

**Inhibition of *Aspergillus fumigatus* conidia binding to extracellular matrix proteins by sialic acids: A pH effect?**

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Abbreviations: DMB, 1,2-Diamino-4,5-methylenedioxybenzene; ECM, extracellular matrix; ManNAc, *N*-acetylmannosamine; Neu5Ac, *N*-acetylneuraminic acid; Neu5,9Ac<sub>2</sub>, 5-*N*-acetyl-9-*O*-acetylneuraminic acid; Neu5,7Ac<sub>2</sub>, 5-*N*-acetyl-7-*O*-acetylneuraminic acid; Sia, sialic acid

## Summary

Infection by *Aspergillus fumigatus*, which causes the life threatening disease invasive aspergillosis, begins with the inhalation of conidia that adhere and germinate in the lung. Previous reports have shown that *A. fumigatus* conidia express high levels of the negatively charged 9-carbon sugar sialic acid, and that sialic acid appears to mediate the binding of *A. fumigatus* conidia to basal lamina proteins. However, despite the ability of sialic acid to inhibit *A. fumigatus* conidia adherence, the exact mechanism by which this binding event occurs remains unresolved.

Utilising various free sialic acids and other carbohydrates, sialic acid derivatives, sialoglycoconjugates, glycoproteins,  $\alpha$ -keto acid related compounds and amino acids we have found that the binding of *A. fumigatus* conidia to type IV collagen and fibrinogen was inhibited by (i) glycoproteins (in a sialic acid-independent manner), and (ii) free sialic acids, glucuronic acid and  $\alpha$ -keto acid related compounds. However, inhibition by the latter was found to be the result of a shift in pH from neutral (pH 7.4) to acidic (less than pH 4.6) induced by the relatively high concentrations of free sialic acids, glucuronic acid and  $\alpha$ -keto acid related compounds used in the binding assays. This suggests that previous reports describing inhibition of *A. fumigatus* conidia binding by free sialic acid may actually be due to a pH shift similar to that shown here.

As previously reported, we found that *A. fumigatus* conidia only express *N*-acetylneuraminic acid, the most common sialic acid found in nature. However, *A. fumigatus* appears to do so by an alternative mechanism to that seen in other organisms. We report here that *A. fumigatus* (i) does not incorporate sialic acid obtained from the environment, (ii) does not synthesise and incorporate sialic acid from exogenous *N*-acetylmannosamine, and (iii) lacks homologues of known sialic acid biosynthesising enzymes.

## INTRODUCTION

*Aspergillus fumigatus* is the most prevalent airborne fungal pathogen in developed countries, and in immuno-compromised patients causes the usually fatal disease, invasive aspergillosis (IA) (Latge, 2001). There are a number of anti-fungal drugs recommended for the treatment of IA; however, their success rate is limited (Denning, 1998; Stevens *et al.*, 2000) due mainly to varying response rates, and severe adverse effects. In addition, troubling reports of multi-drug resistance against one of the major classes of anti-fungal drug (Manavathu *et al.*, 2001; Mosquera & Denning, 2002; Warris *et al.*, 2002), makes research aimed at better understanding fungal pathogenesis an imperative.

Sialic acids (Sia) are a family of 9-carbon  $\alpha$ -keto acids found predominantly at distal positions of oligosaccharide chains of glycoproteins and glycolipids. They are glycosidically linked to either the 3- or 6-hydroxyl group of galactose (Gal) residues or to the 6-hydroxyl group of *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) residues, and can form oligo- to polysialic acid chains through either their 8-hydroxyl or 9-hydroxyl groups. The complexity of sialylated glycans is further enhanced through various substitutions of Sia - approximately 50 naturally occurring Sia-derivatives have been identified (Angata & Varki, 2002; Schauer & Kamerling, 1997). Because of their terminal position and unique physicochemical properties, sialylated oligosaccharide sequences are considered key determinants in a variety of complex biological regulatory and signaling events. Not only do Sia act as ligands in their own right, modulating a variety of recognition processes between cells and molecules mediated through Sia-specific lectins, but they also mask underlying structures, thus preventing binding of other lectins (Crocker, 2002; Lehmann *et al.*, 2006; Varki & Angata, 2006).

The predominant species of Sia identified in pathogenic fungi, including *A. fumigatus*, is *N*-acetylneuraminic acid (Neu5Ac) (Alviano *et al.*, 1999; Warwas *et al.*, 2007), however 5-*N*-

acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac<sub>2</sub>) has also been found in *Cryptococcus neoformans* (Rodrigues *et al.*, 1997). In *C. neoformans*, *Paracoccidioides brasiliensis* and *Candida albicans* Sia are mainly linked *via* an  $\alpha$ 2,6-linkage forming terminal  $\alpha$ 2,6-sialyl-galactose structures with much fewer  $\alpha$ 2,3-sialyl-galactose structures present (Rodrigues *et al.*, 1997; Soares *et al.*, 1993; Soares *et al.*, 2000). The same has now also been observed in *A. fumigatus* (Warwas *et al.*, 2007). Even though Sia is expressed by fungi, the function and biosynthesis of Sia by these organisms remains poorly understood, a situation accentuated by the fact that putative enzymes involved in Sia biosynthesis have not been identified in the genomes *S. cerevisiae*, *C. albicans*, or *C. neoformans* (Angata & Varki, 2002).

*A. fumigatus* infection begins with the inhalation of conidia, the infectious particle, which adhere to and germinate in the lung (Latge, 1999). Several groups have investigated the adhesion of *A. fumigatus* conidia to purified ECM proteins, including type IV collagen (Bromley & Donaldson, 1996; Gil *et al.*, 1996), laminin (Bouchara *et al.*, 1997; Bromley & Donaldson, 1996; Gil *et al.*, 1996; Tronchin *et al.*, 1997; Wasylnka & Moore, 2000), fibrinogen (Bouchara *et al.*, 1997; Bromley & Donaldson, 1996) and fibronectin (Bromley & Donaldson, 1996; Gil *et al.*, 1996; Penalver *et al.*, 1996; Wasylnka & Moore, 2000; Wasylnka *et al.*, 2001). These studies suggest that binding of *A. fumigatus* conidia to ECM proteins is mediated *via* Sia; however, the exact mechanism by which this occurs remains unresolved. There are two schools of thought, for both of which supporting data have been reported. Firstly, Sia on *A. fumigatus* conidia interact with ECM proteins of the lung basal lamina (Wasylnka & Moore, 2000; Wasylnka *et al.*, 2001). The second, opposing theory suggests that a Sia-recognising lectin on *A. fumigatus* conidia binds Sia on ECM proteins of the lung basal lamina (Bouchara *et al.*, 1997; Tronchin *et al.*, 2002). Even though the mechanism by which Sia is involved is still open to debate, the common denominator in all studies was the

apparent ability of adhesion to be inhibited by Sia and Sia-containing glycoconjugates (Bouchara *et al.*, 1997; Wasylnka & Moore, 2000; Wasylnka *et al.*, 2001).

In order to better understand the role of Sia in *A. fumigatus* conidia binding to ECM proteins, as well as the biosynthesis of Sia by *A. fumigatus*, we: (i) determined the effect of Sia and other carbohydrates, Sia-derivatives, sialoglycoconjugates, glycoproteins,  $\alpha$ -keto acid related compounds and amino acids on *A. fumigatus* conidia binding to ECM proteins; (ii) performed homology searches of the *A. fumigatus* genome; and (iii) evaluated the impact on Sia expression of supplementing minimal growth media with *N*-acetylmannosamine (ManNAc, a key Sia precursor) and Neu5Ac.

## **METHODS**

**Materials, *Aspergillus* species, media and cultivation.** All reagents were purchased from (Sigma-Aldrich, St Louis, MA, USA) unless otherwise stated, and were of the highest possible quality. *A. fumigatus* ATCC 13073 was obtained from the American Type Culture Collection and was grown on complex media (YM) and chemically-defined minimal (MM) agar as described previously (Wasylnka *et al.*, 2001) in 250 ml vented tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany), and MM agar supplemented with 5 mM Neu5Ac and 5 mM ManNAc. Conidia were harvested with glass beads in the presence of 20 mM PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 (PBST) by vigorous shaking. Subsequently, the dislodged conidia were filtered through sterile glass wool, collected by centrifugation (1000 x g, 5 min), resuspended in 20 mM PBS and counted.

**Extravidin-peroxidase labelling of *A. fumigatus* conidia and 96 well-based binding inhibition assays.** Isolated *A. fumigatus* conidia were labelled with Extravidin-peroxidase and adherence assays were performed essentially as previously described (Penalver *et al.*, 1996). Briefly, SpectraPlate-96 HB plates (PerkinElmer, Australia) were coated with type IV collagen, fibrinogen and fibronectin at a concentration of 25 g/mL, 50 g/mL and 50 g/mL, respectively, and subsequently blocked with 0.1% BSA in 20 mM PBS. Extravidin-peroxidase labelled conidia (100 L) in 20 mM PBS ( $10^8$  conidia/mL) were then added to individual wells and incubated at 37°C for 1 hr. As negative control 100 L Extravidin-peroxidase labelled conidia were incubated at 37°C for 1 hr in wells coated with 0.1% BSA. Non-adherent conidia were removed by washing twice with PBST and absorbance at 490nm determined using a VICTOR3 multilabel plate reader (PerkinElmer) following the addition *o*-phenylenediamine (Sigma-Aldrich) and 3 M H<sub>2</sub>SO<sub>4</sub>. Identical assays were performed at pH

values between 2.6 and 8.0, and in the presence and absence of free Sia, Sia-derivatives, sialoglycoconjugates, glycoproteins, free carbohydrates, free amino acids, and commercially available  $\alpha$ -keto acids,  $\alpha$ -oxy acids, L-ascorbic acid, and D-saccharic-1,4-lactone at low (20 mM PBS) and high (200 mM PBS; 200 mM sodium phosphate, 150 mM NaCl, pH 7.4) buffering capacity. The A490nm of the negative control was subtracted prior to calculating percentage inhibition of each compound tested using the following equation:

$$\% \text{ inhibition} = 100 \times (1 - A490\text{nm (compound present)} / A490\text{nm (compound absent)})$$

**Mammalian cell culture and cell binding inhibition assays.** Human lung epithelial cells A549 (ATCC CCL-185) were seeded in 96-well cell culture plates (Griener) at  $10^5$  cells/well and grown overnight in DMEM/F-12 supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 10% fetal calf serum to confluence in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. Cells were washed with 20 mM PBS and Extravidin-peroxidase labelled conidia in 20 mM PBS added at a concentration of  $10^8$  conidia/mL. Following incubation at 37°C for 1 hr non-adherent conidia were removed by washing twice with PBST and absorbance at 490nm determined using a VICTOR3 multilabel plate reader (PerkinElmer) following the addition *o*-phenylenediamine (Sigma-Aldrich) and 3 M H<sub>2</sub>SO<sub>4</sub>. Identical assays were performed in the presence of Sia-derivatives, sialoglycoconjugates, glycoproteins, free carbohydrates, and commercially available  $\alpha$ -keto acids and  $\alpha$ -oxy acids at low buffering conditions (20 mM PBS).

**Fluorometric HPLC analysis of *A. fumigatus* conidia sialic acids.** Sialic acids were released from isolated conidia with 2 M propionic acid at 80 °C for 4 hours (Mawhinney & Chance, 1994). Following centrifugation (1000 x g, 5 min) the supernatant was applied to an

ultrafiltration centrifugal device (Microsep 3K Omega; Pall Gelman Laboratories, Australia). Sia, which were not retained by the filtration device, were subsequently purified by sequential ion-exchange chromatography essentially as previously described (Reuter & Schauer, 1994). Briefly, acid-released Sia was applied to a column of Dowex Marathon C (3 mL resin, H<sup>+</sup> form) and eluted with 10-15 column volumes of H<sub>2</sub>O. The pH of the eluent was adjusted to 5-6 if required and applied to a column of Dowex 2X8-400 anion exchange resin (1.5 mL resin, formate form). Following washing with H<sub>2</sub>O, Sia was eluted with 10-15 column volumes of 0.8 M formic acid.

Purified Sia were derivatized with 1,2-Diamino-4,5-methylenedioxybenzene (DMB) reagent (Toronto Research Chemicals, Canada) and analysed by fluorometric HPLC using the method described by (Hara *et al.*, 1989) on a Synergy 4u Fusion-RP column (250 x 4.6 mm, Phenomenex, Sydney, Australia). The retention times of Sia separated by HPLC were compared with an authentic Neu5Ac standard. The amount of Sia detected by fluorometric HPLC was calculated *via* a standard curve constructed from known amounts of Neu5Ac (10-1000 ng) against the corresponding area of the integrated peak.

The identification of Sia was afforded through mild periodate oxidation. Purified Sia were incubated prior to fluorometric-HPLC with 1 mM sodium periodate for 20 min at 0 °C. Utilising periodate oxidation, un-substituted Sia such as Neu5Ac could be identified by monitoring for the disappearance of the corresponding peaks by HPLC. For the identification of putative *O*-acetylated Sia, purified Sia samples were treated with 5 % ammonia solution for one hour at 37°C prior to fluorometric HPLC.

**Homology searches for putative enzymes involved in Sia biosynthesis, activation, and transfer in the *A. fumigatus* genome.** All homology searches were performed using the BLASTP program of the GenBank database at the National Center for Biotechnology

Information (NCBI) ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and the *A. fumigatus* database at the Wellcome Trust Sanger Institute ([www.sanger.ac.uk/Projects/A\\_fumigatus/](http://www.sanger.ac.uk/Projects/A_fumigatus/)). The protein sequences used in homology searches were: UDP-GlcNAc epimerase/ManNAc kinase (Mouse, Acc. No. Q91WG8), Sia synthase (*S. agalactiae*, Acc. No. ABV59028; and *E. coli*, Acc. No. AAC43302), Sia-9-P synthase (Mouse, Acc. No. AAH57977); CMP-Sia synthetase (Mouse, Acc. No. NP\_034038; *S. agalactiae*, Acc. No. P0A4V0; *E. coli*, Acc. No. AAD53077 and conserved motifs); sialyltransferase (Mouse  $\alpha$ 2,6; Acc. No. NP\_666045; mouse  $\alpha$ 2,3, Acc. No. P97325 ; mouse  $\alpha$ 2,8, Acc. No. O35696 and sialyl motifs), and trans-sialidase (*T. brucei*, Acc. No. AAZ12746; *T. cruzi*, Acc. No. BAA09333; and *T. rangeli*, Acc. No. AAC95494).

## RESULTS

### **Effect of free sialic acid, sialic acid derivatives, sialoglycoconjugates and $\alpha$ -keto acid-related compounds on the interaction of *A. fumigatus* conidia with type IV collagen and fibrinogen**

Even though some aspects of the mechanism by which Sia is involved in the adherence of *A. fumigatus* to ECM proteins is still open to debate, the common denominator in all studies was the ability of adhesion to be inhibited by Sia and Sia-containing glycoconjugates (Bouchara *et al.*, 1997; Wasylnka & Moore, 2000; Wasylnka *et al.*, 2001). Therefore, Sia, Sia-derivatives and sialoglycoconjugates, including glycoproteins were evaluated for their ability to interfere with the Sia-dependent binding of *A. fumigatus* conidia to type IV collagen and fibrinogen, using a published adherence assay that measures the activity of peroxidase labelled conidia following adhesion to ECM proteins coated onto microtiter plates (Penalver *et al.*, 1996).

Ten millimolar free Neu5Ac, Neu5Gc, KDN and glucuronic acid (GlcA), and to a lesser extent bovine mucin, showed significant inhibition of conidia binding to type IV collagen (Table 1). However, 3'-sialyllactose, 6'-sialyllactose,  $\alpha$ Neu5Ac2Me,  $\beta$ Neu5Ac2Me, Neu5Ac2en, sialylated glycoproteins, and the Na<sup>+</sup> salt of GlcA were unable to inhibit conidia binding to type IV collagen. Interestingly, asialofetuin (which contains less than 0.5% Sia) completely inhibited conidia binding to type IV collagen. Similarly, conidia binding to fibrinogen was only inhibited by free Neu5Ac, KDN, GlcA, and asialofetuin; however, inhibition was also observed for the other glycoproteins tested, including fetuin (Table 1). Taken together this suggests that free Sia and not glycosidically linked Sia is responsible for the inhibitory activity observed.

There are two possible explanations/mechanisms that may account for the observed inhibitory effect of free Sia and GlcA on *A. fumigatus* binding. Firstly, in solution, the open (acyclic) and closed ring form of free Sia, as well as GlcA, exist in equilibrium (Figure 1). Although

the closed ring form is favoured, a small portion (less than 1%) of Sia exists in an open ring  $\alpha$ -keto acid form. Conversely, glycosidically linked Sia on glycoconjugates such as sialyllactose and fetuin, or Sia-derivatives such as  $\alpha$ Neu5Ac2Me,  $\beta$ Neu5Ac2Me and Neu5Ac2en cannot undergo ring opening and are locked in their closed ring form. Therefore, our conidia binding inhibition data raised the possibility that the acyclic form, or the  $\alpha$ -keto acid functionality present in the acyclic form of Sia, rather than the closed ring form of Sia, is responsible for the inhibition of *A. fumigatus* conidia adherence to ECM proteins.

However, an alternative scenario was that the observed inhibition is purely a consequence of a pH shift resulting from the relatively high concentration of free Sia and GlcA used in inhibition assays with 20 mM PBS (low buffering capacity). Direct pH measurement revealed that 10 mM Neu5Ac and GlcA lowered the pH of 20 mM PBS from 7.4 to 4.5 and 4 respectively, whereas Sia-derivatives (in the form of  $\text{Na}^+$  salts) and sialoglycoconjugates had no effect on pH. The observation that GlcA  $\text{Na}^+$  salt was also unable to inhibit conidia binding (Table 1) provides further evidence in support of the pH shift hypothesis.

Therefore, to fully investigate both possibilities the inhibitory effect of free Sia, Sia-derivatives, and sialoglycoconjugates on conidia binding was assayed in 200 mM PBS (high buffering capacity) (Table 1), and the effect of  $\alpha$ -keto acid-related compounds was evaluated in 20 mM and 200 mM PBS (Table 2). Table 1 shows that at high buffering capacity (200 mM PBS) only asialofetuin inhibited conidia binding to type IV collagen, and only fetuin and asialofetuin inhibited binding to fibrinogen; 10 mM Neu5Ac and GlcA showed no inhibitory activity against either ECM protein under these assay conditions. It should be noted that the increase in phosphate concentration from 20 mM to 200 mM had no effect on *A. fumigatus* conidia binding to either type IV collagen or fibrinogen.

Using a series of commercially available structurally related  $\alpha$ -keto acids and amino acids, we observed that, apart from pyruvic acid ( $\text{Na}^+$  salt), phenyl acetic acid and the amino acids, all

organic acids tested showed significant inhibition of conidia binding when performed in 20 mM PBS (Table 2). However, as observed for Neu5Ac and GlcA, this inhibitory effect was negated when assays were performed in 200 mM PBS. Direct pH measurement revealed that all the organic acids, with the exception of pyruvic acid, phenyl acetic acid and the amino acids, lowered the pH of 20 mM PBS to less than 4.5. However, no pH shift was measured for all  $\alpha$ -keto acid related compounds and amino acids listed in table 2 when prepared in 200 mM PBS. To confirm the effect of pH on *A. fumigatus* conidia binding to ECM proteins, additional binding assays were performed at pH values between 2.6 and 8.0. Figure 2 shows that conidia binding to type IV collagen, fibrinogen and fibronectin was significantly reduced at pH values less than 4.6, but quickly plateaued at pH 5.6 where maximum binding was observed for all ECM proteins evaluated.

The inhibition data obtained for *A. fumigatus* conidia binding to type IV collagen and fibrinogen at low buffering capacity were mirrored in cell-based assays. Figure 3 shows that conidia adherence to human lung epithelial cells in 20 mM PBS was not affected by the presence of  $\alpha$ Neu5Ac2Me ( $\text{Na}^+$  salt, pH 7) and pyruvic acid ( $\text{Na}^+$  salt, pH 7); however, significant inhibition was observed for oxalacetic acid (pH 4), phenylpyruvic acid (pH 4) and benzoylformic acid (pH 4). Taken together our data strongly suggests that the inhibition of *A. fumigatus* conidia binding to type IV collagen and fibrinogen by Neu5Ac, GlcA and  $\alpha$ -keto acid-related compounds is the result of a pH shift.

#### **Conidia surface sialic acid analysis of *A. fumigatus*.**

High levels of Sia are present on *A. fumigatus* conidia (Wasylnka *et al.*, 2001), and they appear to exist  $\alpha$ 2,6-linked to galactose (Gal) residues (Warwas *et al.*, 2007). This suggests that *A. fumigatus* should express a Gal-specific  $\alpha$ 2,6-sialyltransferase, and therefore may utilise activated Sia (CMP-Sia) as the donor substrate. Rodrigues *et al.*, (2003) also reported

preliminary evidence for a  $\alpha$ 2,6-sialyltransferase in *C. neoformans*. However, extensive homology searches of the *A. fumigatus* genome available through the Wellcome Trust Sanger Institute and the NCBI, failed to identify any enzymes involved in Sia biosynthesis, activation or transfer, including a putative Gal-specific  $\alpha$ 2,6-sialyltransferase. Similar searches of the *S. cerevisiae*, *C. albicans*, and *C. neoformans* genomes performed by others also failed to identify putative enzymes involved in Sia biosynthesis (Angata & Varki, 2002). This suggests that either *A. fumigatus* and other fungi obtain Sia from their environment, or Sia biosynthesis, activation and transfer occurs via an alternative pathway to that seen in other organisms. Trypanosomes, such as *Trypanosoma cruzi* and *T. brucei* express a unique glycosyltransferase called *trans*-sialidase (Pereira-Chioccola & Schenkman, 1999). This enzyme is used by trypanosomes to transfer Sia from the environment onto their own surface molecules. To investigate the possibility of *A. fumigatus* possessing a putative *trans*-sialidase, homology searches of the *A. fumigatus* genome were also performed using the *trans*-sialidase protein sequence from *T. brucei* (Acc. No. AAZ12746), *T. cruzi* (Acc. No. BAA09333) and *T. rangeli* (Acc. No. AAC95494). However, no putative *trans*-sialidases were identified within the *A. fumigatus* genome.

In order to further explore Sia expression on *A. fumigatus* conidia, *A. fumigatus* conidia were cultivated on YM and MM agar, as well as MM agar supplemented with Neu5Ac and ManNAc, and Sia subsequently released by mild acid hydrolysis analysed by fluorometric-HPLC. Figure 4 shows that the predominant Sia released following mild acid hydrolysis of *A. fumigatus* conidia grown on YM media was Neu5Ac (Panel B, peak 1). This was confirmed by the observed reduction in the peak intensity corresponding to Neu5Ac following treatment with sodium periodate (Panel C, peak 1). A number of other DMB-reactive compounds, which were found to be resistant to sodium periodate, were also observed following fluorometric-HPLC analysis (Figure 4, Panel B, Peaks 2-5). Similar DMB-reactive

compounds released following acid hydrolysis of *A. fumigatus* conidia have also been reported by others (Warwas *et al.*, 2007). However, the observation of peak 2 was of particular interest given that its relative retention time corresponds to 5-*N*-acetyl-7-*O*-acetylneuraminic acid (Neu5,7Ac<sub>2</sub>) (Shen *et al.*, 2004), and that it was not observed by Warwas *et al.*, (2007). Therefore, in order to identify possible *O*-acetylated Sia, compounds released by acid hydrolysis were treated with 5 % ammonia solution prior to HPLC analysis. However, this treatment had no effect on the intensity of Peak 2, or any other of the DMB-reactive compounds (Peaks 3-5) (data not shown). Taken together this supports that reported by Warwas *et al.*, (2007), that Neu5Ac is the only Sia found on *A. fumigatus* conidia.

Sia analyses were also performed on conidia harvested from *A. fumigatus* grown on MM agar. Figure 4 (Panel D) shows that although a reduction in the amount of Neu5Ac present on conidia grown on MM agar in comparison to YM agar was observed, this difference was not statistically significant ( $P > 0.05$ ). This observation is consistent with previous studies (Wasylnka *et al.*, 2001). In order to evaluate whether *A. fumigatus* takes up and incorporates Sia from either exogenous Neu5Ac or ManNAc, *A. fumigatus* was cultivated on MM agar supplemented with both precursors. Even though a small increase in the density of Neu5Ac present on conidia was observed upon addition of ManNAc and Neu5Ac to the media, this difference was found not to be statistically significant ( $P > 0.05$ ), with approximately 20 g of Neu5Ac detected per  $10^{10}$  conidia observed regardless of growth media (Figure 4, panel D).

## DISCUSSION

Utilising various free Sia and other carbohydrates, Sia-derivatives, sialoglycoconjugates, glycoproteins,  $\alpha$ -keto acid related compounds and amino acids we found that binding of *A. fumigatus* conidia to type IV collagen and fibrinogen was inhibited by (i) free Sia, GlcA and  $\alpha$ -keto acid related compounds as a result of pH shift and (ii) glycoproteins in a Sia-independent manner.

Although sialyllactose has previously been reported to inhibit conidia binding to ECM proteins (Bouchara *et al.*, 1997), closer inspection of the literature shows that this only occurred at an extremely high concentration (100 mM). The ability of mucin to inhibit conidia binding has also been reported (Bouchara *et al.*, 1997; Tronchin *et al.*, 2002; Wasylka *et al.*, 2001), and our studies confirm this finding. However, inhibition by other highly sialylated glycoproteins (fetuin and  $\alpha$ 1-acid glycoprotein) was only observed for conidia binding to fibrinogen. Significantly, asialofetuin which contains less than 0.5% Sia, was the only glycoprotein tested that completely inhibited conidia binding to both type IV collagen and fibrinogen. Taken together our data suggest that *A. fumigatus* conidia interaction with type IV collagen and fibrinogen is not mediated through glycosidically linked Sia, and that any inhibition attributed to glycoproteins is unrelated to the presence of Sia.

The adhesion of microorganisms to host cells generally occurs by two mechanisms. The first is typically governed by generic physical and chemical forces, the second by more specific interactions. The electrolytic environment, which includes pH, can influence both of these mechanisms. The possibility that the inhibition of *A. fumigatus* conidia binding to ECM proteins by free Sia was in fact due to pH shift was first suggested by Bouchara *et al.*, 1997. Here we provide conclusive evidence showing that *A. fumigatus* conidia adherence is significantly inhibited at acidic pH (less than 4.6) (Figure 2).

The fungal pathogen *Penicillium marneffeii* has also been suggested to interact with ECM proteins in a Sia-dependent manner (Hamilton *et al.*, 1998; Hamilton *et al.*, 1999). However, this was based predominantly on the ability of free Sia to inhibit conidia binding to immobilised ECM proteins. Interestingly, mucin and asialomucin had no statistically significant effect on conidia binding (Hamilton *et al.*, 1998; Hamilton *et al.*, 1999). Based on the data presented here, it is highly probable that the inhibition of *P. marneffeii* conidia binding to ECM proteins by free Sia is also due to pH shift, particularly given that the effect of 200 mM Neu5Ac was assayed in 10 mM PBS (Hamilton *et al.*, 1998; Hamilton *et al.*, 1999).

The inhibitory effect of acidic pH on *A. fumigatus* conidia binding to fibronectin was also established in our study (Figure 2), with adhesion being significantly reduced at pH values less than 4.6. Previous studies have suggested that ionic bond formation, probably between negatively charged Sia on the surface of *A. fumigatus* conidia and the positively charged heparin-binding domain of fibronectin, are important for conidia adhesion (Wasylnka & Moore, 2000; Wasylnka *et al.*, 2001). Given that Sia are relatively strong acids ( $pK_a$  2.2-3.0) (Schauer & Kamerling, 1997), the pH shift observed in our experiments would not significantly impact on the degree of Sia ionisation (at pH 4.5 approximately 99% of Sia will be ionised), and therefore would not significantly alter the electrostatic interaction between Sia and positively charged binding domains. Therefore, our results suggest that additional interaction/s between *A. fumigatus* conidia and ECM proteins, possibly protein-protein interactions that are sensitive to acidic pH, may also be important.

Integrin-like proteins that bind peptide sequences in a similar way to mammalian integrins are also present in *C. albicans* (Hostetter, 1999), and it has been suggested that integrin-like proteins may also be found in *A. fumigatus* (Bromley & Donaldson, 1996; Gil *et al.*, 1996). However, only certain synthetic peptides have been found to inhibit *A. fumigatus* conidia

binding to particular ECM proteins, and even when inhibition was observed the level varied (Bouchara *et al.*, 1997; Bromley & Donaldson, 1996; Gil *et al.*, 1996). This has led various authors to suggest that *A. fumigatus* conidia may have different adherence systems or mechanisms, where either separate *A. fumigatus* receptors exist for each ECM protein (which may or may not be integrin-like); or more than one receptor exists for a single ECM protein; or that a single receptor recognises multiple domains on different ECM proteins (Gil *et al.*, 1996; Wasylnka & Moore, 2000). Clearly, these interactions may be susceptible to changes in pH, either through pH induced protein denaturation, changes in solubility, or subtle changes in the ionisation state of amino acids, specifically exposed Asp and Glu residues that are known to be important for recognition by eukaryotic integrins (Arnaout *et al.*, 2002).

Our study has confirmed previous observations (Warwas *et al.*, 2007; Wasylnka *et al.*, 2001) showing that *A. fumigatus* conidia express significant levels of Sia, specifically Neu5Ac, when cultivated on YM (complex), as well as MM (minimal) growth media. Furthermore, our HPLC analyses have verified the absence of *O*-acetylated Sia on *A. fumigatus* conidia, despite the retention times of many of the DMB-reactive compounds released by mild acid hydrolysis being similar to that expected for Neu5,7Ac<sub>2</sub> (Peak 2) and Neu5,9Ac<sub>2</sub> (Peak 4). Significantly, our data suggests that *A. fumigatus* does not scavenge and incorporate exogenous Neu5Ac into cell surface components, or synthesise and incorporate Sia from exogenous ManNAc.

In *H. influenzae*, *H. ducreyi* and *E. coli* sialylated glycoconjugates can be generated from free Neu5Ac that has been scavenged from the extracellular environment, activated to CMP-Neu5Ac by the action of a CMP-Sia synthetase, and transferred onto cell surface components by a sialyltransferase (Schilling *et al.*, 2001; Vimr *et al.*, 2004). *E. coli* is additionally able to synthesise Neu5Ac from exogenous ManNAc in a multi-step pathway; however, unlike the utilisation of free Neu5Ac, CMP-Neu5Ac is not produced. Instead exogenous ManNAc appears to enter the reversible Neu5Ac degradation pathway (Ringenberg *et al.*, 2001).

Cultured mammalian cells are also able to metabolise exogenous ManNAc, and this has been utilised to incorporate *N*-acyl modified Neu5Ac onto cell surface glycoconjugates (Keppler *et al.*, 2001). However, in mammalian cells ManNAc is converted to ManNAc-6-P by the bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase, a critical enzyme in Sia biosynthesis (CMP-Neu5Ac levels regulate the epimerase activity). Dephosphorylation of Neu5Ac-9-P, resulting from the condensation of ManNAc-6-P with PEP, yields free Neu5Ac, which is then activated to CMP-Neu5Ac (Tanner, 2005). There are two main differences between the way mammalian and bacterial cells biosynthesis Sia. Firstly, the bacterial ManNAc kinase is functionally separate from the UDP-GlcNAc 2-epimerase and appears to be involved predominantly in Sia degradation; and secondly ManNAc instead of ManNAc-6-P is used by the bacterial Neu5Ac synthase to generate free Neu5Ac that is subsequently converted to CMP-Neu5Ac (Ringenberg *et al.*, 2001; Tanner, 2005; Vimr *et al.*, 2004). The inability of *A. fumigatus* to utilise and incorporate exogenous Neu5Ac or ManNAc, and the lack of significant homology with known mammalian and bacterial enzymes involved in Sia biosynthesis, suggests that *A. fumigatus* may possess an alternative pathway/mechanism for synthesizing Sia and generating sialoglycoconjugates.

It is clear from the data presented here that *A. fumigatus* conidia does not bind glycosidically linked Sia associated with type IV collagen, fibrinogen and lung epithelial cells, and therefore does not possess a Sia-specific lectin as previously reported (Tronchin *et al.*, 2002). Further evidence for this is provided by the fact that *A. fumigatus* conidia bind desialylated fibronectin equally as well as native, fully sialylated fibronectin (Wasylnka & Moore, 2000). *A. fumigatus* conidia, however do express Neu5Ac on their surface. Previous studies have shown that *A. fumigatus* conidia can be taken up by lung epithelial cells and mouse macrophages in a Sia-dependent manner (Warwas *et al.*, 2007; Wasylnka & Moore, 2002). The latter binding event probably results from interactions between conidial surface Sia and

sialoadhesin (or Siglec-1), a member of the Sia-recognizing Ig-like lectin (Siglec) family, found exclusively on the cell surface of macrophages (Lehmann *et al.*, 2006). However, we could not verify the apparent Sia-dependent nature of the interaction with A549 lung epithelial cells, and suggest that an alternative process mediates this interaction. The fact that Sia-specific lectins have not been reported in epithelial cells, and that the removal of Sia from A549 lung epithelial cells actually increased *A. fumigatus* conidia binding (Warwas *et al.*, 2007), further supports this notion.

The development of *A. fumigatus* infection is dependent on the ability of conidia to adhere to host lung cells. Molecules capable of interfering with these interactions would be of particular interest, possibly providing the basis for the development of novel anti-fungals. With this in mind, we have shown that the binding of *A. fumigatus* conidia to type IV collagen, fibrinogen, fibronectin and lung epithelial cells is susceptible to pH shift, and this pH shift may account for the previously observed ability of free Sia to inhibit conidia binding to ECM proteins.

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## FIGURE LEGENDS

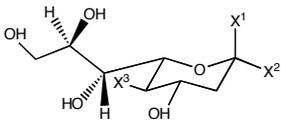
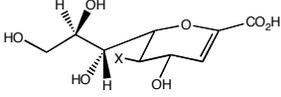
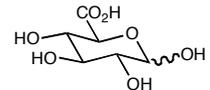
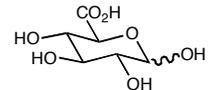
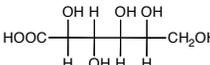
**Fig. 1.** In solution, the acyclic (open ring) and cyclic (closed ring) form of free Sia (top scheme), as well as glucuronic acid (bottom scheme), exists in equilibrium. Although the cyclic form is favoured, a small portion (<1%) of Sia exists in an acyclic  $\alpha$ -keto acid form.

**Fig. 2.** The effect of pH on *A. fumigatus* conidia binding to type IV collagen (■), fibronectin (▲) and fibrinogen (◆) was evaluated using two buffer systems: 20 mM citrate-phosphate buffer (pH 2.6 - 5.6) and 20 mM phosphate buffer (pH 5.9 - 8.0).

**Fig. 3.** The inhibition of *A. fumigatus* conidia adherence to human lung epithelial cells by  $\alpha$ -Neu5Ac2Me and various  $\alpha$ -keto acid-related compounds. Data is presented as mean $\pm$ SD.

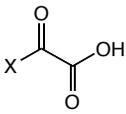
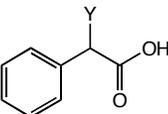
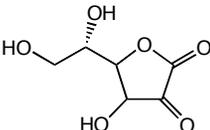
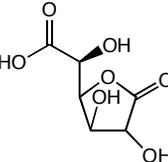
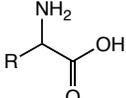
**Fig. 4.** Fluorometric-HPLC analysis of mild acid hydrolysate of *A. fumigatus* conidia (A) Standard DMB-derivatized Neu5Ac. (B) Five DMB-derivatized compounds were observed following mild acid hydrolysis of *A. fumigatus* conidia grown on complex (YM) media. Peak 1, peak 2 and peak 4 possessed retention times similar to Neu5Ac, Neu5,7Ac<sub>2</sub> and Neu5,9Ac<sub>2</sub>, respectively. (C) Only peak 1 (Neu5Ac) was susceptible to sodium periodate treatment, suggesting that peaks 2 and 4 maybe represent *O*-acetylated Sia. However, treatment with 5% ammonia prior to HPLC analysis had no effect on the intensity of peaks 2 and 4 (data not shown). (D) The density of Neu5Ac ( g Neu5Ac/10<sup>10</sup> conidia) present on *A. fumigatus* conidia was not significant (P>0.05) effected by the growth media (YM versus minimal (MM) agar) or the addition of ManNAc or Neu5Ac to MM agar.

**Table 1.** Inhibition of *A. fumigatus* conidia binding to type IV collagen and fibrinogen by Sia, Sia-derivatives, sialoglycoconjugates, glycoproteins and other carbohydrates at low (20 mM PBS) and high (200 mM PBS) buffering capacity.

Structure	Compound (10 mM)	% Inhibition <sup>#</sup>				
		Type IV Collagen		Fibrinogen		
		20 mM PBS	200 mM PBS	20 mM PBS	200 mM PBS	
	X <sup>1</sup> : COOH, X <sup>2</sup> : OH, X <sup>3</sup> : NHAc	Neu5Ac	100	0	85	0
	X <sup>1</sup> : COOH, X <sup>2</sup> : OH, X <sup>3</sup> : NHGc	Neu5Gc	100	ND	ND	ND
	X <sup>1</sup> : COOH, X <sup>2</sup> : OH, X <sup>3</sup> : OH	KDN	100	ND	80	ND
	X <sup>1</sup> : COOH, X <sup>2</sup> : 3Galβ1,4Glc, X <sup>3</sup> : NHAc	3'-Sialyllactose	0	ND	ND	ND
	X <sup>1</sup> : COOH, X <sup>2</sup> : 6Galβ1,4Glc, X <sup>3</sup> : NHAc	6'-Sialyllactose	0	ND	0	ND
	X <sup>1</sup> : COOH, X <sup>2</sup> : OMe, X <sup>3</sup> : NHAc	αNeu5Ac2Me	0	0	0	0
	X <sup>1</sup> : OMe, X <sup>2</sup> : COOH, X <sup>3</sup> : NHAc	βNeu5Ac2Me	0	0	ND	0
		X: NHAc	Neu5Ac2en	0	ND	0
Glycoproteins		Sia-containing glycoprotein	Fetuin*	0	0	75
	Contains < 0.5% Sia	Asialofetuin*	100	100	100	60
	Sia-containing glycoprotein	α1-acid glycoprotein*	0	ND	40	ND
	Sia-containing glycoprotein	Bovine mucin*	35	ND	40	ND
	Sucrose		0	ND	0	ND
	Fucose		0	0	0	0
	Xylose		0	ND	0	ND
	L-Arabinose		0	ND	0	ND
		Glucuronic acid H <sup>+</sup>		100	0	80
Glucuronic acid Na <sup>+</sup> salt			0	0	0	0
	D-Gluconic acid Na <sup>+</sup> salt		20	0	0	0
	D-Gluconic acid lactone		0	0	0	0

<sup>#</sup> The A490nm of the negative control (absence of ECM protein) was subtracted prior to calculating percentage inhibition of each compound tested. Typically, A490nm values for 0% and 100% inhibition were 0.7-0.8 and 0.0-0.05, respectively; \* 250 g/mL; ND, Not Determined.

**Table 2.** Inhibition of *A. fumigatus* conidia binding to type IV collagen and fibrinogen by a number of organic acids, including  $\alpha$ -keto acids,  $\alpha$ -oxy acids, a  $\alpha$ -keto cyclic ester, and a  $\alpha$ -hydroxy acid, and various free amino at low (20 mM PBS) and high (200 mM PBS) buffering capacity.

Structure	Compound (10 mM)	% Inhibition <sup>#</sup>				
		Type IV Collagen		Fibrinogen		
		20 mM PBS	200 mM PBS	20 mM PBS	200 mM PBS	
	X = CH <sub>3</sub>	Pyruvic acid	20	0	0	0
	X = CH <sub>2</sub> COOH	Oxalacetic acid	100	0	70	0
	X = CH <sub>2</sub> C <sub>6</sub> H <sub>5</sub>	Phenylpyruvic acid	100	0	100	0
	X = C <sub>6</sub> H <sub>5</sub>	Benzoylformic acid	100	0	70	0
	Y = OH	$\alpha$ -Hydroxyphenyl acetic acid	100	0	80	0
	Y = OCH <sub>3</sub>	$\alpha$ -Methylphenyl acetic acid	100	0	70	0
	Y = H	Phenyl acetic acid	0	0	0	0
	L-Ascorbic acid	80	0	40	0	
	D-Saccharic-1,4-lactone	100	0	ND	0	
	R = various polar, non-polar and charged groups	Leu, Trp, His, Arg, Asp, Asn,	0	ND	0	ND

<sup>#</sup> The A<sub>490nm</sub> of the negative control (absence of ECM protein) was subtracted prior to calculating percentage inhibition of each compound tested. Typically, A<sub>490nm</sub> values for 0% and 100% inhibition were 0.7-0.8 and 0.0-0.05, respectively; ND, Not determined.

