VORICONAZOLE INHIBITS MELANIZATION IN Cryptococcus neoformans

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ABSTRACT

Voriconazole is a triazole antifungal drug that inhibits ergosterol synthesis and has broad activity against yeast and moulds. While studying the interaction of voriconazole and *Cryptococcus neoformans*, we noted that cells grown in the presence of sub-inhibitory concentrations of voriconazole reduced melanin pigmentation. We investigated this effect systematically by assessing melanin production in the presence of voriconazole, amphotericin B, caspofungin, itraconazole, and fluconazole. Only voriconazole impeded the formation of melanin at sub-inhibitory concentrations. Voriconazole did not affect the autopolymerization of L-Dopa and 0.5 MIC of voriconazole did affect gene expression of *C. neoformans*. However, voriconazole inhibited the capacity of laccase to catalyze the formation of melanin. Hence, voriconazole affects melanization in *C. neoformans* by interacting directly with laccase, which may increase the efficacy of this potent antifungal against certain pigmented fungi.
INTRODUCTION

Voriconazole, a synthetic derivative of fluconazole, is a broad-spectrum triazole antifungal that inhibits cytochrome P450-dependent 14α-lanosterol demethylation, which is a critical step in fungal cell membrane ergosterol synthesis. We have previously shown that voriconazole is highly active against melanized and non-melanized Cryptococcus neoformans, an important human pathogenic fungus, in vitro (12) and during experimental infection (4).

Melanins are negatively charged, hydrophobic pigments of high molecular weight that are formed by the oxidative polymerization of phenolic and/or indolic compounds (15) and the pigments are found in all biological kingdoms (2). Melanin synthesis occurs in C. neoformans, dimorphic fungi, and diverse moulds and has been associated with virulence for the human pathogenic fungi Cryptococcus neoformans, Aspergillus species, Exophiala [Wangiella] dermatitidis and Sporothrix schenckii [reviewed in (7)]. In C. neoformans, pigment production protects the fungus against diverse insults, including oxidants, elevated temperature, amphotericin B, caspofungin, microbicidal peptides, enzymatic degradation, and macrophages in vitro [reviewed in (7)]. In our studies with voriconazole on C. neoformans, we noted that the drug appeared to affect C. neoformans melanization and we therefore investigated this phenomenon by assessing the impact of voriconazole, fluconazole, itraconazole, caspofungin, and amphotericin B on melanin production. Additionally, we analyzed the effect of sub-inhibitory voriconazole on gene expression.

MATERIALS AND METHODS

Antifungal drugs, C. neoformans and melanization. Voriconazole and fluconazole were provided by Pfizer (Sandwich, England). We purchased Amphotericin B from Gibco (Invitrogen Corp., Carlsbad, CA), itraconazole from Janssen (Spring House, PA), and caspofungin from
Merck (Whitehouse Station, NJ). Although caspofungin has limited clinical efficacy in cryptococcosis, it has activity against *C. neoformans* in vitro (1) and was used in these experiments to establish proof of principle for the effect of this drug class on melanin production.

*C. neoformans* serotype D strain 24067 from the American Type Culture Collection (Rockville, MD) was selected for these studies since it was used in our prior melanin and cellular morphology studies (8, 10, 12). Cultures inoculated with $5 \times 10^4$ cells *C. neoformans* yeast cells were grown either in 50 mL of a chemically defined minimal medium [15 mM glucose, 10 mM MgSO$_4$, 29.4 mM KH$_2$PO$_4$, 13 mM glycine and 3.0 µM vitamin B$_1$] or on minimal medium agar [minimal medium plus 2% agar] with 1 mM L-Dopa (Sigma, St. Louis, MO) as substrate for melanization at 30°C. Liquid cultures were shaken at 150 rpm. MICs for *C. neoformans* were determined by us previously (11, 12): 0.015 µg/ml for voriconazole, 0.125 µg/ml for amphotericin B, 1 µg/ml for fluconazole, < 0.625 µg/ml for itraconazole, and 8 µg/ml for caspofungin. To determine whether these antifungal drugs could impact melanization of *C. neoformans* at sub-inhibitory concentrations, these compounds were added at various concentrations to a maximum concentration of 0.5 MIC to the minimal medium with L-Dopa. The cultures were wrapped in foil to avert autopolymerization of L-Dopa and examined daily for growth and melanin production.

**Growth studies.** *C. neoformans* strain 24067 was grown in L-Dopa minimal media in the absence and presence of voriconazole or fluconazole at 30°C. Both antifungal drugs were added at various concentrations to a maximum concentration of 0.5 MIC. The initial inoculum was $5 \times 10^4$ cells in 50 ml media for each concentration.

**Isolation of melanin from *C. neoformans* after incubation with antifungal drugs.** A density of $5 \times 10^4$ *C. neoformans* 24067 yeast cells were grown in 50 mL of minimal medium supplemented with 1 mM L-Dopa without or with voriconazole (0.25 or 0.5 MIC) or fluconazole
(0.25 or 0.5 MIC) at 30°C for 7 days. Liquid cultures were shaken at 150 rpm. On day 7, melanized *C. neoformans* cells were treated with enzymes, denaturant and hot acid results in the isolation of purified melanin in the shape and size of the parental melanized cryptococcal cell, and these particles are referred to as melanin 'ghosts' (15). Briefly, *C. neoformans* from the subcultures of cells grown for 10 days and transferred to fresh medium with or without L-dopa for 36 h were collected by centrifugation at 2010 g for 30 min, washed with PBS and suspended in 1·0 M sorbitol/0·1 M sodium citrate (pH 5·5). Cell-wall-lysing enzymes (from *Trichoderma harzianum*; Sigma) were added at 10 mg ml⁻¹ and the suspensions were incubated at 30 °C overnight. The resulting protoplasts were collected by centrifugation, washed with PBS and treated with 1 mg proteinase K ml⁻¹ (Roche Laboratories) made up in a reaction buffer (10 mM Tris, 1 mM CaCl₂ and 0·5 % SDS; pH 7·8) at 37 °C overnight. The debris was collected, washed with PBS and then boiled in 6 M HCl for 1 h. If particles remained, they were collected, washed in PBS and lyophilized. Finally, the amount of melanin produced by yeast cells after incubation with drugs was quantitated by dry weight measurement.

**Autopolymerization.** To determine whether voriconazole directly interacted with L-Dopa to impede melanization, this drug was incubated with L-Dopa in minimal medium and exposed to ambient light to catalyze the autopolymerization of the phenolic compound to melanin. Voriconazole at concentrations 0.0075, 0.015, or 0.03 µg/ml was incubated in Erlenmeyer flasks with 25 ml of minimal media supplemented with 1mM L-Dopa at 30°C with shaking at 150 rpm. A flask without drug was utilized as a control.

**Gene expression.** *Cryptococcus neoformans* yeasts were grown in minimal medium with L-Dopa in triplicates alone or with 0.0625 µg/ml of voriconazole for 3 days. Approximately 2 × 10⁹ to 6 ×10⁹ cells were suspended in 5 ml of PBS and then homogenized with 0.5-mm-diameter
zirconium-silica glass beads (Biospec, Bartlesville, OK) by using a glass bead beater (Biospec) for 4 min to ensure complete lysis. Cell debris was removed by centrifugation at 3,900 x g for 10 min at room temperature. Isolation of high quality *Cryptococcus neoformans* RNA was performed using the Ambion Kit (Ambion, Austin, TX) according to the manufacturer's instructions. At the Microarray Facility at the Genome Sequencing Center of the Washington University in St. Louis, the RNA was hybridized to a microarray containing all the currently predicted genes in serotype D *C. neoformans* [http://genome.wustl.edu/activity/ma/cneoformans/]. The slides were scanned immediately after hybridization on a ScanArray Express HT Scanner (Perkin Elmer, Wellesley, MA) to detect Cy3 and Cy5 fluorescence. The laser power was kept constant and photomultiplier tube values were set for optimal intensity with minimal background. Gridding and analysis of images were performed with ScanArray software Express V2.0 (Perkin Elmer) and the intensity values were imported into GeneSpring 7.3 software (Agilent, Redwood city, CA). A Lowess curve was fit to the log-intensity versus log-ratio plot and 20.0% of the data was used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement and mean signal to Lowess adjusted controlled ratios are calculated. Cross-chip averages were derived from the antilog of the mean of the natural log ratios across the 2 microarrays.

**Real-time RT-PCR for LAC1 gene expression.** *C. neoformans* 24067 yeast cells were grown in minimal medium agar plates supplemented with 1 mM L-Dopa without or with voriconazole (0.25 or 0.5 MIC) or fluconazole (0.25 or 0.5 MIC) at 30°C for 7 days. Plates were covered with aluminum foil to prevent autopolymerization. After incubation, *LAC1* gene expression was analyzed by qRT-PCR. Briefly, cells were collected and washed, then, RNA was isolated according to the RNeasy kit protocol (Qiagen). For real time RT-PCR detection of *LAC1**
transcripts, 10 µg of total RNA was treated with DNase at 37°C for 1 h, precipitated with ethanol, and suspended in 100 µl of nuclease-free water. cDNA synthesis was carried out from equal amounts of RNA in a cyclic BioRad MyCycler (BioRad) using reagents from Invitrogen according to the manufacturers instructions. The expression of the *LAC1* gene was examined via RT-PCR with the primers LAC1a (CCAGCGAGGAGCCTTTGTGAATGT) and LAC1b (GCCGTGCAGGTGGTAAGGATGG). For an internal mRNA control, we used primers specific for the *ACT1* gene of *C. neoformans* ACT1a (GCCCTTGCTCCTTCTTCTAT) and ACT1b (GACGATTGAGGGACCAGACT). To confirm that similar concentrations of cDNA were achieved, signals of *ACT1* PCR were compared. *LAC1* transcript levels were determined and quantitatively assessed using a Bio-Rad iQ iCycler and the Cycler iQ software, respectively. The cycling conditions used were 95°C for 5 min, and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Next, the samples were cooled to 55°C, and a melting curve for temperatures between 55 and 95°C with 0.5°C increments was recorded. Real-time expression measurements were normalized against expression of the reference gene *ACT1*. Relative RNA levels were calculated using the ΔΔCt method; all primers resulted in amplification efficiencies of at least 95%.

**Laccase assays.** A quantitative laccase assay using the oxidation of 2,2′-Azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS, Sigma) as substrate was performed with *C. neoformans* yeast cells, *C. neoformans* cytoplasmic extractions, and a commercially available recombinant laccase from *Rhus vernicfera* (Sigma). For intact cells, yeasts were grown in asparagine medium [1 g/liter asparagine, 10 mM sodium phosphate (pH 6.5), 0.25 g/liter MgSO4, 10 µM CuSO4] with glucose (1.5 g/liter) for 72 h at 30°C. The cells were collected by centrifugation, washed with with phosphate-buffered saline (PBS), and transferred into
asparagine medium without glucose for 36h at 30°C. The strains were collected by centrifugation, washed, and diluted to $1 \times 10^8$ cells/ml in PBS with or without voriconazole. A final concentration of 1 mM ABTS was achieved by adding 100 µl of 10 mM ABTS to 900 µl of a yeast cell suspension. After incubation at 30°C for 2 h, the cells were removed with centrifugation and the absorbance readings of the solutions were measured at 420 nm. A yeast cell suspension without ABTS was used as a baseline. Commercially produced laccase from *Rhus vernicifera* [activity, 50 U per mg of solid] was used as a positive control at 1 unit in 1 mL of PBS. For cytoplasmic extracts, yeast cells were collected, suspended in 0.1 M Na$_2$HPO$_4$ with protease inhibitor, and treated for 6 min in a bead beater at 2-min intervals alternating with 5 min on ice. Supernatants were separated from cellular debris by centrifugation and used in place of the yeast cell suspensions in the ABTS assay. This assay was also used with commercial laccase incubated with voriconazole using various concentrations of either compound.

**Phagocytosis assays.** J774.16 is a well-characterized murine macrophage-like cell line that has been extensively used to study *C. neoformans*-macrophage interactions. The J774.16 cells were maintained at –80°C prior to use and were prepared for the phagocytosis assays as described previously (12). A density of $5 \times 10^4$ *C. neoformans* 24067 yeast cells were grown in 25 mL of minimal medium supplemented with 1 mM L-Dopa without or with voriconazole (0.25 or 0.5 MIC) or fluconazole (0.25 or 0.5 MIC) at 30°C for 7 days. Liquid cultures were shaken at 150 rpm. On days 3, 5 and 7, an aliquot was collected, and washed three times in PBS. Cells were added to the J774.16 monolayer in a macrophage/yeast ratio of 1:1. The plates were incubated for 2 h at 37°C with 10 µg of monoclonal antibody (MAb) 18B7/ml. MAb 18B7 binds to cryptococcal glucuronoxylomannan, the major component of the fungal capsule. The monolayer was washed three times with PBS to remove non-adherent cells, fixed with cold methanol, and stained with
Giemsa (Sigma). The phagocytic index is the number of internalized yeast cells per number of macrophages per field. Internalized cells were differentiated from attached cells by their presence in a well-defined phagocytic vacuole. These measurements were determined by light microscopy using an Axiovert 200 M inverted microscope (Carl Zeiss MicroImaging, NY) at a magnification of 400X. For each experiment, three wells were examined, and the numbers of ingested cryptococcal cells and macrophages in three fields were counted with approximately 100 macrophages per field.

**Statistical analysis.** All data were subjected to statistical analysis using Origin 7.0 (Origin Lab Corp., Northampton, MA). P values were calculated by Student’s t test or analysis of variance depending on the data. P values of <0.05 were considered significant.

**RESULTS**

**Voriconazole inhibits melanization at sub-inhibitory concentrations.** *C. neoformans* melanization was significantly reduced and visibly delayed at ≥ 0.125 MIC of voriconazole (Fig. 1). In contrast, the addition of ≤ 0.5 MIC of amphotericin B, caspofungin, fluconazole, or itraconazole to *C. neoformans* cultures did not visibly affect melanization. Inhibition of melanization occurred in a similar manner in both liquid and solid medium. The growth rate of *C. neoformans* was not affected by the incubation in sub-inhibitory concentrations of voriconazole or fluconazole (Fig. 2). L-Dopa polymerization was not impeded by the presence of voriconazole at drug concentrations of up to 2X MIC for *C. neoformans*. By the third day of incubation, small black particles were visible in the flasks with and without antifungal drug and the particle density increased similarly in all the flasks over a two week period.
To confirm the results obtained by the plating assay, the percent inhibition in melanization of cryptococcal cells was determined by dry weight (Table 1). The amount of melanin produced by fungal cells co-incubated in minimal medium with L-Dopa and voriconazole or fluconazole was isolated, quantified and compared relative to yeast cells grown in minimal medium with L-Dopa. *C. neoformans* melanization was significantly inhibited by voriconazole. Melanin production was reduced 82.1 and 94.5% after co-incubation with 0.25 and 0.5 MIC of voriconazole, respectively. However, fluconazole did not prevent melanization at sub-inhibitory concentrations.

**Voriconazole does not affect global gene regulation at sub-inhibitory concentrations.** Three independent replicate experiments were carried out to generate the data set. After normalization of the data using lowess smoothing, there were no genes identified with at least a two-fold upward or downward change, which is considered to be the cutoff for significance with the microarray system used. Hence, although the concentration of voriconazole was sufficient to inhibit melanization, there was no evidence that the phenotype was transcriptionally based.

**Voriconazole affects the melanin regulator gene LAC1 expression at sub-inhibitory concentrations.** To explore changes in the expression of *LAC1* gene of *C. neoformans*, that is required for melanin production and full virulence, qRT-PCR was performed on RNA extracted from cryptococcal cells grown in the absence or in the presence of sub-MIC of voriconazole and fluconazole (Fig. 3). Our results showed that co-incubation with voriconazole significantly reduced *LAC1* gene expression. Voriconazole decreased *LAC1* gene expression by approximately 40% when compared with control cells. However, *LAC1* was not affected by similar sub-MIC of fluconazole.
Voriconazole directly inhibits laccase activity. Voriconazole reduces the capacity of laccase to oxidize ABTS (Fig. 4). When 0.5 MIC of voriconazole was added to suspensions of cryptococci or to cytoplasmic extracts of the fungus, voriconazole dramatically inhibited the activity of the fungal laccases. In order to reproducibly examine these interactions, a commercial standardized recombinant laccase from *Rhus vernicifera* was used to demonstrate that voriconazole inhibited the activity in a dose dependent fashion (Fig. 5A). Similarly, reducing laccase in the presence of a constant concentration of voriconazole resulted in a dose dependent reduction in activity (Fig. 5B).

Reduction in *C. neoformans* cells melanization by voriconazole alters phagocytosis by J774.16 cells. We investigated whether co-incubation of voriconazole with cryptococci in minimal media supplemented with L-dopa altered the phagocytic activity by macrophage-like cells. Significantly more cryptococcal cells grown in the presence of the sub-MIC of voriconazole were phagocytosed by J774.16 cells compared with cells grown in the absence of antifungal drug or in the presence of sub-inhibitory concentrations of fluconazole (Fig. 6). Voriconazole reduced melanization by fungal cells in culture and increased the phagocytosis of yeast cells by J774.16 cells. As found in previous studies in our laboratory, exposure of *C. neoformans* to sub-inhibitory concentrations of fluconazole also enhanced phagocytosis by macrophages.

**DISCUSSION**

Voriconazole significantly impacts the ability of *C. neoformans* to produce melanin, which makes the yeast cells more susceptible to host effector cells. There are various mechanisms by which voriconazole could interfere with melanization. First, voriconazole could interfere with
protein trafficking as a side effect of its action on fungal sterols with consequent disruption of secretion pathways. In this regard, melanin synthesizing enzymes are often located in the cell wall (5, 17) and the proper expression involves transport to the outside of the cell—possibly in secretory vesicles. Voriconazole may function in a manner analogous to glyphosate, which has been shown to inhibit melanin formation, probably through interference with polymerization of laccase-oxidized precursors (9). The structure of voriconazole differs from fluconazole, its parent structure, by the addition of a methyl group and fluorine, which appears to be sufficient to alter the compounds ability to engage laccase. Interestingly, miconazole, a related imidazole, has been reported to interfere with the production of melanin in melanoma cells by inhibiting tyrosinase in a dose dependent fashion, beginning at a concentration of 30 µM (6). Although the cryptococcal microarray failed to show any global differences upon challenge with sub-inhibitory amounts of voriconazole, the more sensitive qRT-PCR revealed that voriconazole reduced the expression of the \textit{LAC1} gene. In addition to its role in \textit{C. neoformans} melanization, laccase protects the fungus from toxic metabolites produced by macrophage via its iron oxidase activity (3). Hence, if voriconazole binds laccase then the capacity of the enzyme to regulate iron oxidation could be impacted as well. Previous to this study, there was no evidence of any antifungal drug directly inhibiting fungal melanin synthesizing enzymes. However, voriconazole has been shown to be effective against fungi at sub-therapeutic concentrations by inhibiting conidiation in \textit{Aspergillus} species (14).

Demonstrating that voriconazole interferes with melanization suggests the exciting possibility that this drug may retain antimicrobial activity even if the targeted microbe develops resistance by mutations of the sterol synthetic pathway and/or by selection of enhanced efflux mechanism. This attribute would not be detected by in vitro susceptibility tests since these are
standardized in conditions where fungi are not ordinarily melanized. Hence, it is conceivable that voriconazole would be active in vivo against fungi for which it has minimal or no in vitro activity since reduction in melanin production would translate into reduced virulence that in turn would allow increased clearance by host immune mechanisms. In this regard, Serena et al. recently demonstrated that voriconazole reduces fungal burden and enhances survival in a murine model of cryptococcal Central Nervous System (CNS) infection (10). Human brain contains various phenolic compounds, such as norepinephrine, 3, 4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindolacetic acid, serotonin, and dopamine, all of which can serve as substrates for the *C neoformans* laccase. In addition to interfering with the production of ergosterol, our study shows that voriconazole can suppress laccase production, which could reduce the ability of *C. neoformans* yeast cells to utilize phenolic compounds as substrates for melanin production in the brain and other tissues further crippling the organism’s capacity to cause disease.

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FIGURE LEGENDS

Figure 1. C. neoformans yeast were grown on minimal medium 1 mM L-Dopa agar plates for 10 days at 30°C with or without the addition of antifungal drugs. (A) Growth of C. neoformans in the absence of antifungal drugs demonstrating dark pigmentation of the colonies and polymerization of the L-Dopa in the agar surrounding the colonies due to the secretion of laccase into the medium. C. neoformans grown with voriconazole at (B) 0.125, (C) 0.25 and (D) 0.5 MIC showing increasingly lower amounts of melanin formation in the colonies or within the agar. There was no significant reduction of melanin production with 0.5 MIC of (E) amphotericin B, (F) caspofungin, (G) fluconazole, or (H) itraconazole. Plates were done in triplicate. This experiment was done twice, with similar results each time.

Figure 2. Growth curve of C. neoformans with subinhibitory concentrations of voriconazole or fluconazole used in this study.

Figure 3. Voriconazole affects C. neoformans LAC1 gene expression at sub-inhibitory concentrations. * denotes $P < 0.05$ in comparison with control or fluconazole groups. This experiment was done twice, with similar results.

Figure 4. Oxidation of ABTS by C. neoformans laccase from intact cells or from supernatants of yeast cell extracts is suppressed by voriconazole. Recombinant laccase from Rhus vernicifera incubated with ABTS represents the positive control and the negative control is ABTS alone. The experiment was done twice and similar results were obtained.

Figure 5. The oxidation of ABTS by laccase is reduced by the presence of voriconazole. (A) Increasing concentrations of voriconazole in the presence of a constant amount of laccase. # represents $P < 0.05$ * indicates $P < 0.001$ in comparison to the activity of laccase in the absence of voriconazole. (B) Decreasing concentrations of laccase result in the presence of a constant
amount of voriconazole. ** indicates $P < 0.001$ in comparison to the activity of the highest concentration of laccase in the absence of voriconazole. The experiments were done twice with similar results.

**Figure 6.** Voriconazole reduces *C. neoformans* 24067 melanization and alters yeasts phagocytosis by J774.16 cells. Bars are the averages of three wells, and brackets denote standard deviations. * denotes $P < 0.001$ in comparison with control group. # represents $P < 0.05$ in comparison of voriconazole group with fluconazole group. This experiment was done twice, with similar results.

**Table 1.** Inhibition of *C. neoformans* 24067 melanin production by voriconazole.

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC</th>
<th>Dry Weight (g)</th>
<th>% melanin inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voriconazole</td>
<td>0.25</td>
<td>0.0302</td>
<td>82.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.0092</td>
<td>94.5</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>0.25</td>
<td>0.1641</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.1394</td>
<td>17.3</td>
</tr>
</tbody>
</table>

† The percentage of melanin inhibition was determined by comparing dry weights obtained from 50 ml cultures of *C. neoformans* cells grown in the presence of L-Dopa and drugs relative to yeast cells grown in the presence of L-Dopa only.

**REFERENCES**


Figure 2

The graph shows the growth of C. neoformans 24067 per ml over time (h) in different conditions. The y-axis represents the number of organisms per ml, ranging from $10^3$ to $10^9$. The x-axis represents time in hours, ranging from 0 to 100.

- **Control**
- **0.25 MIC Voriconazole**
- **0.5 MIC Voriconazole**
- **0.25 MIC Fluconazole**
- **0.5 MIC Fluconazole**

The data indicates that Voriconazole has a more pronounced effect on the growth of the fungus compared to Fluconazole.
Figure 3

The figure shows the percentage of LAC1 expression relative to wild-type at different concentrations of Voriconazole (0.25 and 0.5) and Fluconazole (0.25 and 0.5). The y-axis represents the percentage of expression, and the x-axis indicates the concentration of the antifungal agents. The asterisks (*) indicate statistically significant differences.
Figure 5

Absorbance (420 nm)

Voriconazole (µg/ml) 100 0 1 1 10 50 100
Laccase (U/µl) 0 1 1 1 1 1 1

A

B

* Significant difference
** Highly significant difference
# Not significant