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IN-SILICO AND *IN-VITRO* STUDIES ON FUNGAL CHITINASE AS A TARGET ENZYME FOR ANTIFUNGAL ACTIVITY OF CLOSANTEL

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ABSTRACT

Drug development is a dynamic field which undergoes changes continuously. The past decade or so has witnessed huge strides in the field of screening of the drugs as well as target and ligand identifications but unfortunately this has not led to useful drugs. Many diseases still remain untreatable because we do not have proper drugs against them. In case of fungi the situation is graver due to the limited drug targets peculiar to fungi. Thus, in order to combat the fungal diseases the need of the hour is to develop new antifungals that have fewer side effects and broad spectrum activity.
The current work deals with closantel, a veterinary drug targeting chitinase, which is utilized as a therapeutic option against helminths. The fact that chitin is an important constituent of the cell wall of the fungi also and it undergoes regular degradation and remodelling through an enzyme called fungal chitinase motivated the authors to test the efficacy of closantel against fungal chitinase. In the present study *Cryptococcus neoformans* has been used as a model organism against which the antifungal activity of closantel has been tested.
In-silico studies carried out using PatchDock and FireDock predicted the global energy (binding energy)involved in docking closantel on chitinase as -47.58 Kcal/mol. Further, the results obtained through the *in-silico* studies were validated by the minimum inhibitory concentration assay (MIC). It was observed that 736 (±7.024) µg/ml of closantel can inhibit the cryptococcal growth by 80% (MIC_{80}).

Keywords: Closantel; Antifungal; *In-silico*; Chitinase; Minimum inhibitory concentration

INTRODUCTION

Our environment is the habitat to humongous number of micro-organisms which are responsible for the mortality and morbidity of the human beings (**Chu et al., 1996**). Amongst the large number of micro-organisms are the fungi which are ubiquitous in environment and are saprophytic or parasitic in nature. Some of them are beneficial to mankind as they can be exploited for food, drug and industrial applications whereas others cause health hazards. Fungi cause infections in both immunocompetent and immunocompromised individuals (**Reedy et al., 2007**). The burden of invasive fungal infections (IFIs) has increased, majorly due to the increased number of immunosuppressed patients leading to their morbidity and mortality (**Wade et al., 2013; Enoch et al., 2006; Denning et al., 1990**). Invasive fungal infections represent a continuous and serious threat to human health as they are associated with at least 1.5 million deaths worldwide each year (**Pianalto et al., 2016; Brown et al., 2012; Campoy et al., 2017**).
In order to combat these fungal infections there is a continuous need of new antifungals. This need intensifies as the fungi have got an intrinsic capacity to mutate and undergo genetic modifications making them resistant to the action of fungicidals and fungistatics. Additionally, fungal infections are often confused with the bacterial infections making their diagnosis increasingly difficult. Furthermore, increasing multidrug resistance has made situation more adverse and it has become far more difficult to tackle the infectious diseases caused by microorganisms.
Contrary to the development of new antibacterial drugs, antifungal drug development is more challenging because fungi are eukaryotes and many potential targets for therapy are common to humans with substantial host toxicity risk (**Roemer et al., 2014**). The commonly used antifungal agents are not completely effective due to the development of resistance and host toxicity. More threatening, new resistance patterns have been observed including simultaneous resistance to different antifungal classes (**Wade et al., 2013**). Further, the undesirable side effects limit their use in medical practice.
In the last 5 years, there has been an upsurge in the availability of new antifungal therapies. After a long dry spell following the release of fluconazole, itraconazole and lipid-associated amphotericin B formulations more than a decade ago, voriconazole and caspofungin were approved for the treatment of systemic fungal infections (**Proia, 2006**). Unfortunately, both polyenes and azoles do not work efficiently and have side effects. Conventional amphotericin B deoxycholate (CAB), is associated with adverse effects in electrolyte imbalances, and nephrotoxicity (**Clements Jr. et al., 1990; Enoch et al., 2006**). Voriconazole is generally well tolerated but it also has been reported to cause reversible disturbance of vision (**Purkins et al., 2002; Lazarus et al., 2002**). Voriconazole and fluconazole introduced in 1990s although changed the approach of treating many fungal infections but neither of them was an ideal agent. Itraconazole was plagued by absorption problems whereas fluconazole had a limited spectrum of antifungal activity, and resistance was soon noted in immunosuppressed hosts who received long term treatment (**Saravolatz et al., 2003**).
Fungal chitinase plays an essential role in exogenous chitin decomposition, fungal cell wall degradation and remodelling (**Hartl et al., 2012**). Based on the amino acid sequences of their glycoside hydrolase (GH), 18 modules of fungal chitinases have been divided into three different subgroups, namely, A, B and C .Subgroup A are known to contain chitinases involved in processes during fungal growth and autolysis. Autolysis is the natural process of self-digestion of aged hyphal cultures (**White et al., 2002**). Subgroup B chitinases appear mainly to be involved in nutritional functions. **Hartl et al (2012)** suggested that hyphal interaction and network formation could be relevant for C chitinase gene regulation. Subgroup C chitinases are involved in several aspects of self- and non-self chitin degradation.This was, so far, investigated in the two mycoparasites *Trichoderma atroviride* and *Trichoderma virens* (**Hartl et al., 2012**).
Fungal chitinases provide structural integrity to the fungal cell and when its synthesis is disrupted, the cell wall becomes osmotically unstable. Its absence in humans and any other vertebrates allows it to be used as a drug target. (**Lenardon et al., 2010**).
Closantel (N-(5-chloro-4(a (4-chlorophenyl)~-cyanomethyl)-2-methylphenyl)-2-hydroxy-3,5,diiodobenzamide), is a halogenated salicylanilide which is widely used as veterinary anti-helminthic drug against *Fasciola* and *Haemonchus* species (**Rothwell and Sangser, 1997; Swan, 1999; Rassouli et al.,2013).** Closantel acts as chitinase inhibitor (**Tran et al., 2016**). The inhibitors of chitinase have chemotherapeutic potential against fungi (**Rao et al., 2005**). Closantel is also known for its other mode of action which is uncoupling of oxidative phosphorylation (**Williamson, 1967**).
Here, it has been tested for its antifungal activity against *Cryptococcus neoformans* (MTCC 1431). *Cryptococcus* is a basidiomycetes and is ubiquitous

in the environment, because of its ubiquity, it has been suggested that most people are exposed to *C. neoformans* early in life (**Goldman et al., 2001**). It is the causative agent of cryptococcosis, a life-threatening fungal infection **(Hagen, 2011)**. Its infections can occur in individuals with both normal and impaired immune function, but most cases are found in patients with immune deficiency (**Firacative and Meyer, 2017**). *C.neoformans* has become one of the most prevalent causes of fungal disease leading to the fatal mycotic infection amongst AIDS patients world-wide (**Brandt et al., 2001; Mitchell and Perfect 1995**).
Initially, *in-silico* approach utilizing docking studies was carried out in order to ascertain the effectivity of closantel against chitinase of *C.neoformans*. Positive results obtained from the docking studies lead the authors to perform the minimum inhibitory concentration (MIC) studies in order to confirm the antifungal activity of closantel.

MATERIALS AND METHODS

In-silico studies

Sequence retrieval, analysis and homology modeling

The structure of closantel was obtained from PubChem (PubChem Substance ID 329754809) whereas due to the unavailability of the suitable structure of the chitinase enzyme of *C. neoformans*, its sequence was obtained from National Center for Biotechnology information (NCBI) (Genbank ID: GenBank: OWZ37771.1) and was modelled through the Modeller (ModWeb webserver) webserver (https://modbase.compbio.ucsf.edu/modweb/). Further, the modelled structure of chitinase enzyme was validated through PROVE module RAMPAGE and SAVES webserver.

Structure refinement and Active site identification

Active site prediction was done through an online webserver GHECOM 1.0 (http://strcomp.protein.osaka-u.ac.jp/ghecom/). Further refinement of the structure was performed through the Deep view software by energy minimization.

Docking

PatchDock and FireDock softwares were used in order to dock closantel on to the modelled structure of the chitinase enzyme. Visualizations of the docked structures, interactions between chitinase enzyme and closantel and electrochemical potential diagram were made through the pymol visualization software.

In-vitro studies of closantel against *C. neoformans* (MTCC Ref 1431)

Micro-organism

One reference strain (MTCC Ref 1431) of *C. neoformans* was used to conduct this study.

Antimicrobial agents

Closantel was obtained from Sigma-Aldrich (CAS Number: 57808-65-8) whereas amphotericin B (AMB) (brand name AMPHOTRET) was obtained from Bharat Serums and Vaccines Limited.
A stock solution of closantel was prepared in 1% acetone. Three controls viz. negative, vehicle (acetone) and positive (amphotericin B), were used for this experiment. The concentration range of closantel used in this experiment was 0.5mg/ml to 20 mg/ml.

Preparation of inoculum

Fresh cultures maintained on Sabouraud Dextrose Agar (SDA) for 48 hours at 37^0 C medium were used for the preparation of inoculum. Fungal colonies were gently scraped from the culture plate and dissolved in 0.9% sterile saline forming a fungal suspension. Further, 0.5 McFarland scale of turbidity was set for the fungal suspension with the help of a spectrophotometer. This suspension was further diluted in the ratio of 1:10 with RPMI medium to obtain the final concentration of $1.0\text{-}5*10^6$ cfu/ml.

In vitro susceptibility testing

Minimum inhibitory concentration (MIC) assay of *C. neoformans* against closantel was performed according to the guidelines issued by CLSI for the broth macro-dilution method M27-A3 (CLSI, 2008) with a few modifications **(Neelabh & Singh, 2017).** An incubation time span of 72 hours and 35º C temperature was followed in the experiment. Additionally, all the samples were tested in triplicate. The optical density of all the samples was measured at 420 nm using spectrophotometer (ThermoScientific UV1).

Statistical analysis

One way ANOVA followed by Holm Sidak test was performed for statistical analysis of the readings obtained.

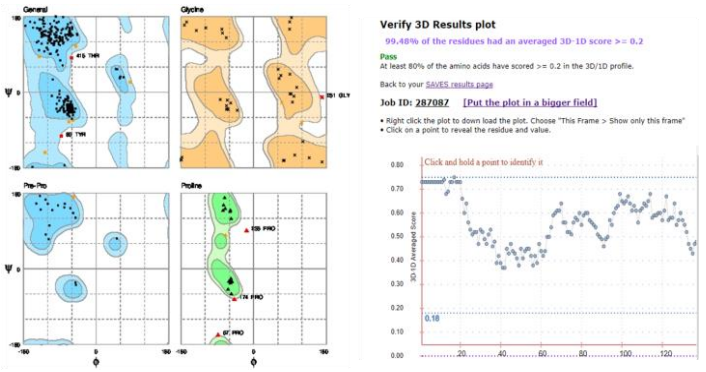
RESULTS

The sequence of chitinase was obtained from NCBI (GenBank: OWZ37771.1) (Table 1).

Table 1 Sequence of the chitinase enzyme used in the present study.

Chitinase (OWZ37771.1) [Cryptococcus neoformans var. grubii e45]	MAHSPKPALYPSSDKKPTSKASLAFLAVLLLAAIVFLFAQ TDFAFPSRPWKAVREPLKGDEIEMNNPKRTVGYFVNWGI YGRKFFPQNIPGQHLTHINYAFGNVKADSGEVVLSDTWA DVEIHYDGDSWDEPGTNLYGCFKAIYLMKKQNRNLKVL LSIGGWSFSPNFAGIVHPKWRSTFVQSAVKLVEDVGLDG LDIDYEYPKTPRDAEAYVDLLRELRQGLEQLAQSKGKP QGQYQLTVAAPCGWEQMQVLRVREMDQVLDFWNLMA YDFAGSWDSVAGHQANLYSDKPDGQSVDRSVRFYLEAG VHPTKLVIGLPVYGRAFANTKGIGSPFSGTGEGSWEAGM WDYKALPQPGAQETNDHRLGASYSYDPAKRLLITYDTQ AIAHQKASYIAYHGLGGAMWWELDSDKPEETGQSLVRT VREALGQLEWRENELDYPGSKYDNLRRRME

Further, due to unavailability of the structure of the cryptococccal chitinase it was modelled utilizing Modeller (ModWeb webserver). Best model was chosen on the basis of the model score and it was further validated through SAVES webserver (VERIFY3D) and RAMPAGE. Through VERIFY3D, 99.48% of the residues were found to have an averaged 3D-1D score >= 0.2 and through RAMPAGE 95.9% of the total residues were found in favoured region, 2.6% in the allowed region and 1.6% in the outlier region (figure 1) proving the authenticity of the model. This model was further submitted to Protein Model Databse.



a b

Figure 1 Validation of the chitinase model. a: Modelled structure as analyzed by RAMPAGE b: Modelled structure as analyzed by SAVES (VERIFY 3D)
Additionally, the energy of this structure was minimized to -16352.138 KJ/mol using molecular simulation based on the DeepView software.The minimized energy structure has been shown in figure 2.

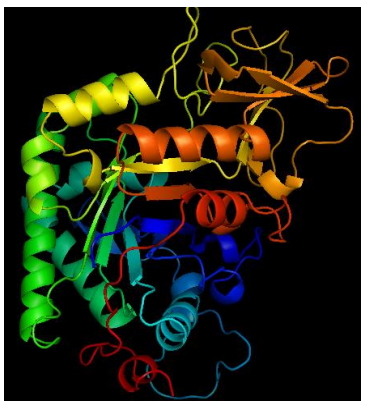


Figure 2 Cartoon structure of the modelled and energy minimized chitinase enzyme

As, *in-silico* modelling was conducted to obtain the structure of chitinase therefore, the active site region of this molecule was unknown. Hence, in order to

identify the active site region of the molecule it was subjected to Ghecom 1.0 webserver (figure 3).

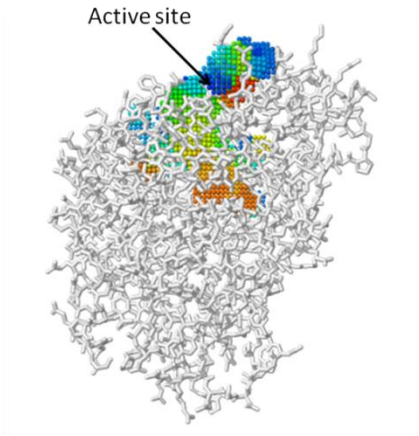


Figure 3 Active site region shown in the modelled chitinase molecule

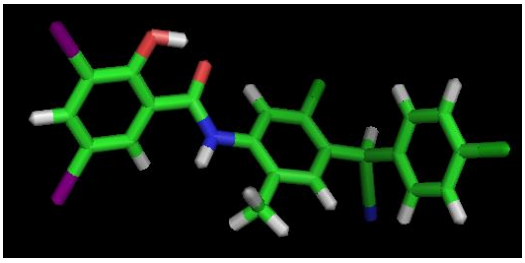


Figure 4 3D structure of closantel

Closantel (figure 4) was docked on the active site of chitinase molecule with the help of PatchDock and further refinement of the results was performed using FireDock. The structure having the best binding energy of closantel docked to the chitinase active site has been shown in figure 5a. Closantel has bound to the electronegative pocket of the chitinase depicting its electropositive nature (figure 5b). The chitinase residues interacting with closantel (distance < 5 A^0) were found to be proline 244, glycine 246, aspartatae 199, glutamine 201, tryptophan 405, tyrosine 270 and methionine 268 (figure 5c).

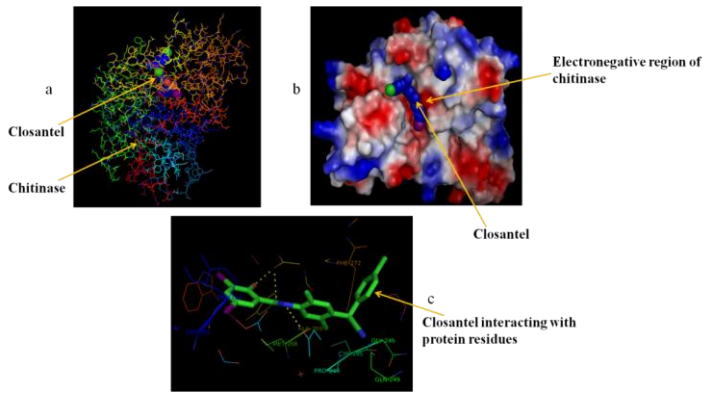


Figure 5 Docked structures of closantel on chitinase molecule. a: depicting closantel docked on the active site of the chitinase molecule, b: depicting the electrostatic potential of the chitinase molecule, c: depicting the interaction between closantel and chitinase.

The global energy value of the closantel bound to the chitinase molecules were found high which motivated the authors to conduct its minimum inhibitory concentration assay. The different parameters of the best structure obtained on docking have been provided in Table 2.

Table 2 Different parameters of the best structure obtained after docking of closantel on chitinase

Global energy	Attractive VdW	Repulsive VdW	ACE
-47.58	-22.23	4.85	-11.59

Global energy or the binding energy is the most important parameter amongst various parameters (table 2). It is well known that only the negative binding energies are energetically favourable. Here, the ΔG_{bind}= -47.58Kcal/mol, which is negative therefore suggesting that the process of binding of closantel to chitinase is thermodynamically favourable and the complex formed is stable. Further, *in-vitro* activity of closantel was carried out through minimum inhibitory concentration to test for its antifungal susceptibility. It was observed that at 736 (±7.024) µg/ml of closantel, 80% of the fungal growth was inhibited (MIC_{80}). Further, for the same isolate amphotericin B was found to have an MIC_{80} of 67.5 (±2.754) µg/ml (figure 6).

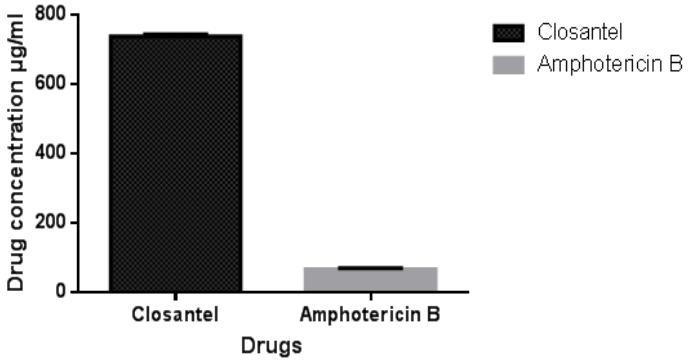


Figure 6 MIC_{80} of both Closantel and Amphotericin B against *C.neoformans*.

DISCUSSION

In the last few years invasive fungal infections have remarkably increased and the number of patients at risk for these infections is increasing as immunomodulatory therapies continue to expand. Though there are numerous available antifungal drugs, they do not meet the expectations for managing these fungal infections. Mortality and morbidity rates are still unacceptably high. The number of antifungal agents is very limited as compared to antibacterial drugs. Fungi are eukaryotic organisms that parasitize eukaryotic hosts and therefore the scarce physiologic differences between both make more difficult to develop safe and broad spectrum antifungal agents. At present the efforts are being made on identifying new fungal specific targets that are critical for fungal growth and have minimal similarity to targets among human proteins. Infections caused by *C. neoformans* are increasing globally and are already a major burden on the public health-care system. Although azoles and polyenes are increasingly used as a last-line therapy against it but the development of new antifungals is the need of the hour (**Neelabh et al., 2016**). In the present manuscript closantel, which is already a known anti-helminthic drug acting against the chitinase enzyme has been tested for its action against *C. neoformans*.

Interestingly, closantel showed antibacterial activity but its mechanism of action is unclear. However, closantel has been reported to exhibit antimicrobial activity against Gram-positive bacteria *in vitro*. MIC for closantel against *B.subtilis* is 3.8 µM (**Hlasta et al.,1998**) and against *Staphylococcus aureus* and *E.faecium* was found to be between 1-2 µg/ml. MIC for closantel against various organisms are *E.coli* (>50 µg/ml) , *B subtilis* (<0.78 µg/ml), multidrug-resistant *Staphylococcus aureus* (< 0.78µg/ml),VRSA (0.78µg/ml) and *E.faecalis* (< 0.78µg/ml), (**Rajamuthiah et al., 2014**). In another study on multidrug-resistant *Acinetobacter baumannii*, it was observed that closantel increased the bacterial killing when administered along polymyxin B (**Tran et al., 2016**). Similar studies have been carried out by Neelabh and Singh in 2017 reporting MIC_{50} of griseofulvin on *C. neoformans* (MTCC Ref 1431) as 128 µg/ml. Most recently, a study aiming at screening the inhibitors against *C.neoformans* and *C.gattii* targeting the TPS1(trehalose-6-phosphate (T6P) synthetase (TPS) identified closantel as a potent inhibitor with a minimum inhibitory concentration of less than 1mg/ml. However, no *in-vivo* effect of closantel was found in the murine model (**Perfect et al., 2017**). Herein, we have conducted both *in-silico* and *in-vitro* tests for testing the activity of closantel against *C.neoformans.* The results obtained here strengthen our speculation that closantel acts on chitinase resulting in fungal death (figure 7).

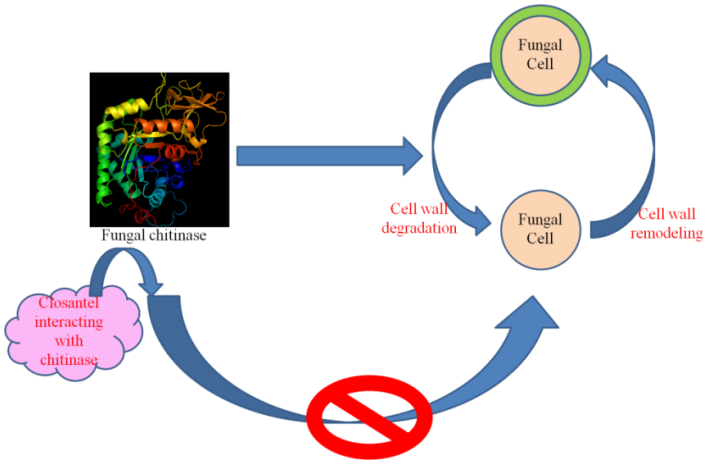


Figure 7 Possible mode of action of closantel against *C.neoformans*

Minimum inhibitor concentration of closantel was determined to be 736 (±7.024) µg/ml which was able to inhibit 80% of the fungal growth (MIC_{80}). This was found to be higher as compared to the standard drug amphotericin B, the MIC_{80} of which was determined to be 67.5 (±2.754) µg/ml against the same isolate. But as already discussed, it has high number of side effects henceforth closantel can be used as an alternative or even as a combinatorial therapy after rigorous *in-vivo* tests.

CONCLUSION

The need for safe and effective antifungal agent is increasing in parallel with the expanding number of immunocompromised patients at risk for invasive fungal infections. The use of antifungal agents, particularly in long-term suppressive regimens, has raised concern about the development of drug resistance in *C. neoformans* .The findings from the present study demonstrate that closantel, an already known anti-helminthic drug having chitinase as its target, has antifungal activity for *C. neoformans*. A two way approach was utilized in the current study in order to determine the antifungal activity of closantel against *C. neoformans*. Primarily, an *in-silico* study was conducted against the chitinase enzyme present in the *C. neoformans* which gave positive results and a global energy (Binding energy) of -47.58 Kcal/mol was observed. Further, it was validated through MIC assay and observed that at 736 (±7.024) µg/ml of closantel caused 80% of the fungal growth to be inhibited (MIC_{80}). This study presents closantel as a therapeutic option against *C.neoformans* infection, although *in-vivo* studies are required to order to strengthen the results obtained in *in-vitro* and *in-silico* studies.

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REFERENCES

Brandt, M. E., Pfaller, M. A., Hajjeh, R. A., Hamill, R. J., Pappas, P. G., Reingold, A. L. & Warnock, D. W. (2001). Trends in antifungal drug susceptibility of Cryptococcus neoformans isolates in the United States: 1992 to 1994 and 1996 to 1998. *Antimicrobial agents and chemotherapy*, *45*(11), 3065-3069.http://dx.doi.org/10.1128/AAC.45.11.3065-3069.2001

Brown, Gordon D., David W. Denning, Neil AR Gow, Stuart M. Levitz, Mihai G. Netea, and Theodore C. White. Hidden killers: human fungal infections. *Science translational medicine* 4, no. 165 (2012): 165rv13-165rv13. http://dx.doi.org/10.1126/scitranslmed.3004404

Campoy, S., & Adrio, J. L. (2017). Antifungals. *Biochemical pharmacology*, *133*, 86-96. http://dx.doi.org/10.1016/j.bcp.2016.11.019

Chu, D. T., Plattner, J. J., & Katz, L. (1996). New directions in antibacterial research. *Journal of medicinal chemistry*, *39*(20), 3853-3874. http://dx.doi.org/10.1021/jm960294s

Clements Jr, J. S., & Peacock Jr, J. E. (1990). Amphotericin B revisited: reassessment of toxicity. *The American journal of medicine*, *88*(5N), 22N-27N. doi not found.

Denning, D. W., & Stevens, D. A. (1990). Antifungal and surgical treatment of invasive aspergillosis: review of 2,121 published cases. *Reviews of infectious diseases*, *12*(6), 1147-1201. http://dx.doi.org/10.1093/clinids/12.6.1147

Enoch, D. A., Ludlam, H. A., & Brown, N. M. (2006). Invasive fungal infections: a review of epidemiology and management options. *Journal of medical microbiology*, *55*(7), 809-818. http://dx.doi.org/10.1099/jmm.0.46548-0

Firacative, C., Trilles, L., & Meyer, W. (2017). Advances in the understanding of the Cryptococcus neoformans and C. gattii species complexes and cryptococcosis. *Microbiology Australia*, *38*(3), 106-111. http://dx.doi.org/10.1071/MA17043

Goldman, D. L., Khine, H., Abadi, J., Lindenberg, D. J., Pirofski, L. A., Niang, R., & Casadevall, A. (2001). Serologic evidence for Cryptococcus neoformansinfection in early childhood. *Pediatrics*, *107*(5), e66-e66. http://dx.doi.org/ 10.1542/peds.107.5.e66

Hagen, F. (2011). *Cryptococcus gattii and Cryptococcus neoformans-Cosmopolitans on the move*. Utrecht University. (Thesis) doi not known

Hartl, L., Zach, S., & Seidl-Seiboth, V. (2012). Fungal chitinases: diversity, mechanistic properties and biotechnological potential. *Applied microbiology and biotechnology*, *93*(2), 533-543. http://dx.doi.org/10.1007/s00253-011-3723-3

Hlasta, D. J., Demers, J. P., Foleno, B. D., Fraga-Spano, S. A., Guan, J., Hilliard, J. J.& Webb, G. C. (1998). Novel inhibitors of bacterial two-component systems with gram positive antibacterial activity: pharmacophore identification based on the screening hit closantel. *Bioorganic & medicinal chemistry letters*, *8*(14), 1923-1928. http://dx.doi.org/10.1016/SO960-894X(98)00326-6

Lazarus, H. M., Blumer, J. L., Yanovich, S., Schlamm, H., & Romero, A. (2002). Safety and pharmacokinetics of oral voriconazole in patients at risk of fungal infection: a dose escalation study. *The Journal of Clinical Pharmacology*, *42*(4), 395-402. http://dx.doi.org/10.1177/00912700222011445

Lenardon, M. D., Munro, C. A., & Gow, N. A. (2010). Chitin synthesis and fungal pathogenesis. *Current opinion in microbiology*, *13*(4), 416-423. http://dx.doi.org/10.1016/j.mib.2010.05.002

Mitchell, T. G., & Perfect, J. R. (1995). Cryptococcosis in the era of AIDS--100 years after the discovery of Cryptococcus neoformans. *Clinical microbiology reviews*, *8*(4), 515-548. doi not known

Neelabh, Singh, K., & Rani, J. (2016). Sequential and Structural Aspects of Antifungal Peptides from Animals, Bacteria and Fungi Based on Bioinformatics Tools. *Probiotics and antimicrobial proteins*, *8*(2), 85-101. http://dx.doi.org/10.1007/s12602-016-9212-3

Neelabh & Singh, K (2017). In silico studies on the effect of griseofulvin on tubulin protein of *Cryptococcus neoformans* and its *in vitro* validation. *Journal of Microbiology, Biotechnology and Food Sciences*, 6(6), 1280-1283. http://dx.doi.org/ http://dx.doi.org/10.15414/jmbfs.2017.6.6.1280-1283

Perfect, J. R., Tenor, J. L., Miao, Y., & Brennan, R. G. (2017). Trehalose pathway as an antifungal target. *Virulence*, *8*(2), 143-149.

Pianalto, K. M., & Alspaugh, J. A. (2016). New Horizons in antifungal therapy. *Journal of Fungi*, *2*(4), 26. http://dx.doi.org/10.3390/jof2040026

Proia, L. A. (2006). New antifungal therapies. *Clinical Microbiology Newsletter*, *28*(22), 169-173. http://dx.doi.org/10.1016/j.clinmicnews.2006.11.001

Purkins, L., Wood, N., Ghahramani, P., Greenhalgh, K., Allen, M. J., & Kleinermans, D. (2002). Pharmacokinetics and safety of voriconazole following intravenous-to oral-dose escalation regimens. *Antimicrobial agents and chemotherapy*, *46*(8), 2546-2553. http://dx.doi.org/10.1128/AAC.46.8.2546-2553.2002

Rajamuthiah, R., Fuchs, B. B., Jayamani, E., Kim, Y., Larkins-Ford, J., Conery, A. & Mylonakis, E. (2014). Whole animal automated platform for drug discovery against multi-drug resistant *Staphylococcus aureus*. PLoS ONE 9, e89189. http://dx.doi.org/10.1371/journal.pone.0089189

Rao, F. V., Houston, D. R., Boot, R. G., Aerts, J. M., Hodkinson, M., Adams, D. J. & van Aalten, D. M. (2005). Specificity and affinity of natural product cyclopentapeptide inhibitors against A. fumigatus, human, and bacterial chitinases. *Chemistry & biology*, *12*(1), 65-76. http://dx.doi.org/10.1016/j.chembiol.2004.10.013

Rassouli, A., Arab, H., Ghezelloo, Y., & Shams, G. R. (2013). A bioequivalence study on two closantel oral suspensions in sheep: an Iranian product (fascinil®) versus flukiver® as a reference product. *Iran J Vet Med*, *7*, 263-9. http://dx.doi.org/ 10.22059/ijvm.2013.36286

Reedy, J. L., Bastidas, R. J., & Heitman, J. (2007). The virulence of human pathogenic fungi: notes from the South of France. *Cell host & microbe*, *2*(2), 77-83. http://dx.doi.org/10.1016/j.chom.2007.07.004

Roemer, T., & Krysan, D. J. (2014). Antifungal drug development: challenges, unmet clinical needs, and new approaches. *Cold Spring Harbor perspectives in medicine*, *4*(5), a019703. http://dx.doi.org/10.1101/cshperspect.a019703

Rothwell, J. & Sangser, N. (1997). Haemonchus contortus: The Uptake and metabolism of Closantel. *International Journal of Parasitology*, 27(3):313-319. http://dx.doi.org/10.1016/0020-7519(93)90162-R

Saravolatz, L. D., Johnson, L. B., & Kauffman, C. A. (2003). Voriconazole: a new triazole antifungal agent. *Clinical Infectious Diseases*, *36*(5), 630-637. http://dx.doi.org/10.1086/367933

Swan, G. E. (1999). The pharmacology of halogenated salicylanilides and their anthelmintic use in animals. *Journal of the South African Veterinary Association*, *70*(2), 61-70. http://dx.doi.org/10.1016/j.jcpa.2006.11.007

Tran, T. B., Cheah, S. E., Heidi, H. Y., Bergen, P. J., Nation, R. L., Creek, D. J.& Velkov, T. (2016). Anthelmintic closantel enhances bacterial killing of polymyxin B against multidrug-resistant Acinetobacter baumannii. *The Journal of antibiotics*, *69*(6), 415. http://dx.doi.org/10.1038/ja.2015.127

Wade, R. L., Chaudhari, P., Natoli, J. L., Taylor, R. J., Nathanson, B. H., & Horn, D. L. (2013). Nephrotoxicity and other adverse events among inpatients receiving liposomal amphotericin B or amphotericin B lipid complex. *Diagnostic microbiology and infectious disease*, *76*(3), 361-367. http://dx.doi.org/10.1016/j.diagmicrobio.2013.04.001

White, S., McIntyre, M., Berry, D. R., & McNeil, B. (2002). The autolysis of industrial filamentous fungi. *Critical reviews in biotechnology*, *22*(1), 1-14. http://dx.doi.org/10.1080/07388550290789432

Williamson, R. L. & Metcalf, R. L. Salicylanilides: a new group of active uncouplers of oxidative phosphorylation (1967). Science 158, 1694–1695. http://dx.doi.org/10.1126/science.158.3809.1694