

IN SILICO STUDIES ON THE EFFECT OF GRISEOFULVIN ON TUBULIN PROTEIN OF *CRYPTOCOCCUS NEOFORMANS* AND ITS *IN VITRO* VALIDATION

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doi: [10.15414/jmbfs.2017.6.6.1280-1283](https://doi.org/10.15414/jmbfs.2017.6.6.1280-1283)

ARTICLE INFO

Received 16. 11. 2016
Revised 28. 3. 2017
Accepted 19. 4. 2017
Published 1. 6. 2017

Regular article



ABSTRACT

Griseofulvin is a well known drug against dermatophytes. It is particularly prescribed for an infection called scrap ringworm or tinea capitis. In general, griseofulvin inhibits the tubulin protein that is responsible for the cell division. Not much is known about the effect of griseofulvin on *Cryptococcus neoformans*. Therefore, the authors made an effort to check the activity of griseofulvin against it.

The webservers (T-Coffee, Blues simulation and CASTp) and software (Autodock 4.0) have been used in order to determine the activity of griseofulvin against the beta subunit of tubulin protein of *C. neoformans*. The results obtained from the *in silico* studies show a high affinity of griseofulvin towards the beta chain of tubulin protein. The negative value of binding energy (-9.02 kcal/mol) also shows that the complex is thermodynamically favourable and stable. These *in silico* results were further validated by MIC assay showing 74.1% inhibition of *C. neoformans* (isolate no. 5) against griseofulvin.

The present study reports that apart from dermatophytes, the drug has significant effect on *C. neoformans* and it may be used in the combinatorial therapy against the same.

Keywords: *Cryptococcus neoformans*; Griseofulvin; *In silico*; *In vitro* validation

INTRODUCTION

Cryptococcus neoformans is commonly known for causing cryptococcosis and cryptococcal meningitis. Most commonly the infection of *C. neoformans* starts with lungs but patients having advanced immune suppression end up with meningitis (Centers for disease control and Prevention, 2014). The general treatment for cryptococcosis in patients suffering from asymptomatic or mild to moderate pulmonary infections is fluconazole. This is slightly different from the treatment plan of those who have severe lung infections in which amphotericin B is prescribed in combination with flucytosine (Centers for disease control and Prevention, 2014). But in the recent past, some isolates of *C. neoformans* have shown resistance against these drugs and patients have witnessed relapse or failure on treatment with flucytosine, fluconazole and amphotericin B (Lopez-Jodra et al., 2000).

Griseofulvin is an antifungal drug known to be produced by 3 species of *Penicillium* viz. *P. patulum*, *P. griseofulvum* and *P. janczewskii* (Drugs.com, 2015). It has been used for the treatment of human and animal dermatophytic infections. The reason for its success against dermatophytes is due to its peculiar pharmacological property that after oral administration it is localized in the keratinized cells of skin, hairs and nails. The growth of the dermatophytes parasitizing these cells is thus inhibited and removed by desquamation (Drugs.com, 2015). Many sensitivity studies have been done on the activity of griseofulvin against superficial fungal infections (Millikan, 2016). But anticryptococcal activity of griseofulvin has not been reported yet.

Many studies advocate the *in vitro* endorsement of the *in silico* results, owing to the technical limitations of bioinformatics approach. For instance, Nyarady et al (2005) validated the results of *in silico* prediction of epitopes by a multipin ELISA. Similarly, the mechanism of serine hydroxymethyltransferase (SHMT) inhibition by pemetrexed was validated by *in vitro* assays and the calculated interaction energy of pemetrexed in the active site of SHMT and the corresponding predicted binding energy were found to be in good agreement with the values of K_d and K_i obtained in Isothermal Titration Calorimetry (Daidone et al., 2011).

The general mode of action of griseofulvin is to interact with tubulin molecule in the fungal cells (Ronnest et al., 2012). Hence, in the current manuscript the activity of griseofulvin against the tubulin protein of *C. neoformans* has been tested *in silico* and validation of the same has been done through the minimum inhibitory concentration assay (MIC) following the Clinical and Laboratory Standards Institute (CLSI, 2008) guidelines.

MATERIALS AND METHODS

Retrieval of the structure of griseofulvin and tubulin protein

The structure of griseofulvin (Drug data bank ID: DB00400) was obtained from drug data bank (<http://www.drugbank.ca/>) whereas reference structure of tubulin of *Sus scrofa* (wild boar) was obtained from the Protein data bank (PDB) (PDB ID: 1TUB). The sequence of beta chain of tubulin of *C. neoformans* was obtained from National Center for Biotechnology Information (NCBI) (Accession number: XP_568244.1). Modelling was done by Phyre² webserver with default parameters.

Determination of the active site region of the tubulin protein and docking studies

CASTp server, an online webserver which determines the structural pockets and cavities with the help of Delaunay triangulation and the alpha complex for shape measurements was used in order to determine the active site regions on the tubulin protein. The docking studies were performed through Autodock 4.0 (<http://autodock.scripps.edu/>). Further, Blues simulation software (<http://protein.bio.unipd.it/blues/>) was used to analyse the structural stability of the model. It calculates the total energy of the structure on the basis of generalized Born atom radii.

In vitro studies of griseofulvin against isolates of *C. neoformans*

Drug griseofulvin under the brand name of GRISOVIN-FP was obtained from GlaxoSmithKline and amphotericin B (AMB) (brand name AMPHOTRET) was obtained from Bharat Serums and Vaccines Limited.

Micro-organisms

One reference strain (Ref 1431), two environmental isolates (NCBI accession no. KJ175192 and KJ175193) and five clinical isolates (4CI2, 49, CSF2, CNS and CNS45) of *C. neoformans* were used in the study.

Antimicrobial agent

A stock solution of griseofulvin in dimethyl sulfoxide (DMSO) at a concentration of 100% was prepared (Brilhante et al., 2014). Three controls viz. negative, vehicle (DMSO) and positive (amphotericin B), were used in the current experimental plan. The concentration range of griseofulvin against which the growth of the fungi has been tested is from 2 µg/ml to 1.024 mg/ml.

Preparation of inoculum

The inocula of *C. neoformans* were prepared from the fresh cultures maintained in Sabouraud Dextrose Agar (SDA) medium for 48 hours at 37°C. Each fungal culture was added to 0.9% sterile saline solution, ensuring gentle scrapping of the fungal colonies with the aid of the inoculation loop to form a fungal suspension. The resulting fungal suspension was adjusted to 0.5 McFarland scale of turbidity. This suspension was further diluted in the ratio of 1:10 with RPMI medium to obtain the final concentration of 1.0-5*10⁶ CFU/ml.

In vitro susceptibility testing

The susceptibility testing of *C. neoformans* against griseofulvin was performed according to the guidelines issued by CLSI for the broth macro-dilution method M27-A3 (CLSI, 2008) with a few modifications. An incubation time span of 72 hours and 35° C temperature was followed in the experiment. Additionally, all the samples were tested in triplicate.

Determination of MIC

MIC of all isolates of *C. neoformans* was determined using broth macro-dilution method (CLSI, 2008). The optical density of all the samples was measured at 420 nm using spectrophotometer (ThermoScientific UV1).

Statistical analysis

The OD readings obtained at different concentrations of griseofulvin for each isolate were statistically analysed by F test followed by Holm Sidak test.

RESULTS

Griseofulvin is known to interact with tubulin protein which plays an important role during the cell division. The structure of the alpha beta tubulin of *C. neoformans* was unavailable, therefore, a multiple sequence alignment was performed between the eukaryotic tubulin protein sequences having known structures with *C. neoformans* tubulin sequence. T-Coffee software was used to carry out the multiple sequence alignment process (Notredame et al., 2000). A very high sequence similarity (99%) was found between the tubulin protein of *C. neoformans* and *Sus scrofa* (Fig 1).

Henceforth, 1 TUB, the respective tubulin protein of *S. scrofa* was taken as reference in the current study. Top 10 active sites were determined on 1TUB. These active sites were docked with griseofulvin. It was observed that the best binding energy was obtained at pocket ID: 167 having an area of 381.6 and a volume of 590.9 (Fig 2). The docked structure of griseofulvin on this pocket has been shown in fig 3 and the docking parameters have been provided in table 1.

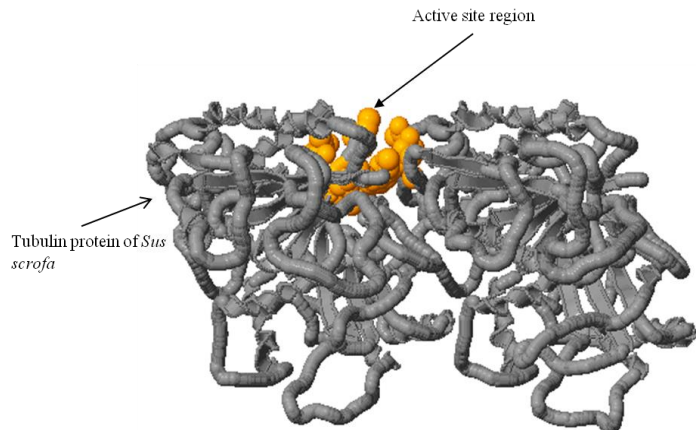


Figure 2 Prediction of active site of tubulin protein of *S. scrofa*

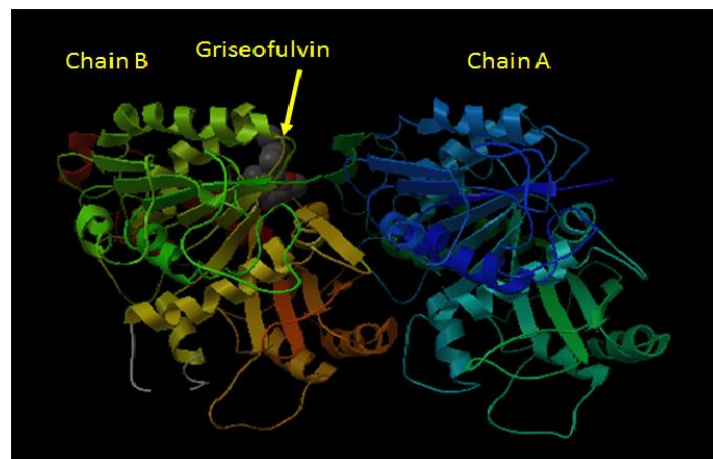


Figure 3 Best structure obtained after docking griseofulvin on tubulin protein of *S. scrofa*



Figure 1 MSA of beta tubulin of *S. scrofa* and *C. Neoformans*

Table 1 Parameters of the best docked structure of griseofulvin on tubulin protein (1TUB) of *S. scrofa*

Parameters	Values
Estimated Free Energy of Binding	-9.02 kcal/mol
Estimated Inhibition Constant (Ki)	243.84 nM (nanomolar)
Final Intermolecular Energy	-9.92 kcal/mol
vdW + Hbond + desolv Energy	-9.84 kcal/mol
Electrostatic Energy	-0.08 kcal/mol
Final Total Internal Energy	-0.45 kcal/mol
Torsional Free Energy	+0.89 kcal/mol
Unbound System's Energy	-0.45 kcal/mol

Estimated Free Energy of Binding or ΔG_{bind} is the most important parameter amongst various parameters shown in table 1. It is well known that only the negative ΔG_{bind} are energetically favourable. Here, the $\Delta G_{bind} = -9.02$, hence this is energetically favourable and the resultant complex formed is thermodynamically stable. Docking result of the reference protein suggested that griseofulvin had a higher affinity to bind the beta chain of tubulin in comparison to its alpha chain therefore, suitable sequence of tubulin beta chain of *C. neoformans* was searched for modelling it.. The modelled structure has been shown in fig 4.

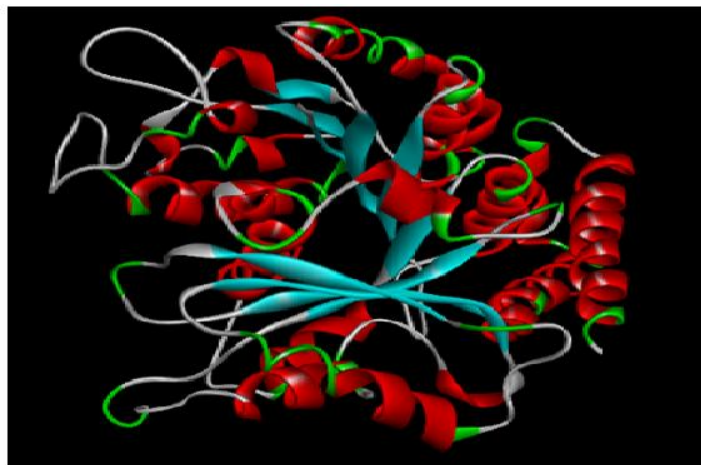


Figure 4 Modelled structure of beta tubulin of *C. neoformans* obtained by Phyre² webserver

The obtained negative total energy assured that the model could be safely used for further docking analysis. The different parameters obtained through the Blues software have been provided in table 2.

Table 2 Protein stability parameters predicted by Blues software

Parameters	Values
Born self energy:	-16169.476521 (kJ)
Coulomb energy:	-109416.947909 (kJ)
Electrostatic solvation energy:	-4719.453945 (kJ/mol)
Total energy:	-112139.794202 (kJ/mol)

Again the best10 active sites for the modelled structure of beta tubulin were determined and griseofulvin was docked on all these 10 sites. It was observed that pocket ID 103 (active site region of beta tubulin of *C. neoformans*) having an area of 219.5 and a volume of 258.6 showed the highest binding energy when docked with griseofulvin (Fig 5 and Fig 6). The parameters related to the best docking structure obtained from Autodock 4.0 have been provided in table 3.

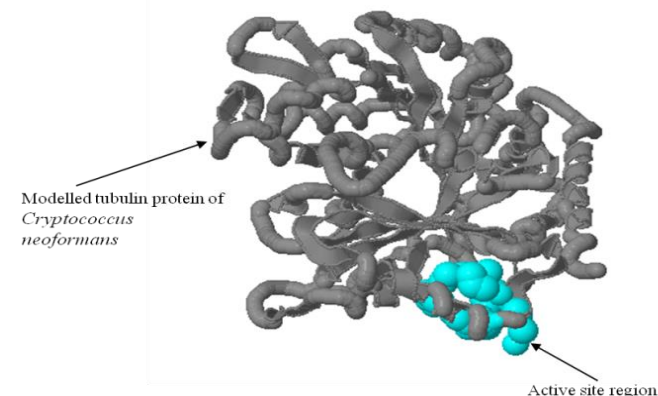


Figure 5 Prediction of active site on modelled tubulin protein of *C. neoformans*

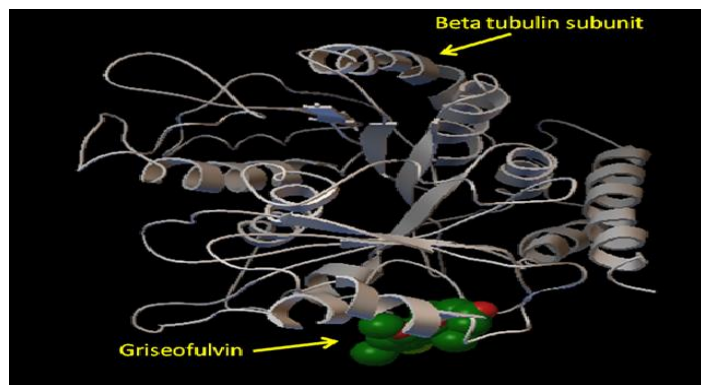


Figure 6 Best structure obtained of docking griseofulvin on beta tubulin subunit *C. neoformans*

Table 3 Parameters of the best docked structure

Parameters	Values
Estimated Free Energy of Binding	-7.05 Kcal/mol
Estimated Inhibition Constant (Ki)	6.75 uM (micromolar)
Final Intermolecular Energy	-7.95 kcal/mol
vdW + Hbond + desolv Energy	-7.94 kcal/mol
Electrostatic Energy	-0.01 kcal/mol
Final Total Internal Energy	-0.52 kcal/mol
Torsional Free Energy	+0.89 kcal/mol
Unbound System's Energy	-0.52 kcal/mol

Table 3 shows that $\Delta G_{bind} = -7.05$ which is energetically favourable hence providing evidence that complex formed would be structurally favourable.

Further, MIC of griseofulvin against eight isolates of *C. neoformans* (one reference, two environmental and five clinical) was found to inhibit the growth of the strain 4CI2, CNS, Ref 1431, 9, 49, CSF2, 5 and CNS45 by 35%, 43%, 45%, 39.5%, 54%, 66.6%, 74.1% and 49% respectively on comparison with their respective controls (Table 4).

Table 4 MIC of griseofulvin against eight isolates of *C. neoformans*

Drug	Isolate	Range	MIC	% inhibition at this MIC
Griseofulvin	<i>C. neoformans</i> Ref 1431	64 µg/ml - 1.024 mg/ml	128 µg/ml	45%
	<i>C. neoformans</i> 4CI2	64 µg/ml - 1.024 mg/ml	1.024 µg/ml	35%
	<i>C. neoformans</i> CNS	64 µg/ml - 1.024 mg/ml	128 µg/ml	43%
	<i>C. neoformans</i> 9	64 µg/ml - 1.024 mg/ml	64 µg/ml	39.5 %
	<i>C. neoformans</i> 49	2-64 µg/ml	16 µg/ml	54%
	<i>C. neoformans</i> CSF2	64 µg/ml - 1.024 mg/ml	256 µg/ml	66.6%
	<i>C. neoformans</i> 5	64 µg/ml - 1.024 mg/ml	512 µg/ml	74.1%
	<i>C. neoformans</i> CNS45	64 µg/ml - 1.024 mg/ml	128 µg/ml	49%

DISCUSSION

Griseofulvin was the only drug available for treatment of *Tinea capitis*, a fungal infection caused by dermatophytes until the approval of terbinafine in 2007 (Gupta and Summerbell, 2000; Seebacher et al., 2007). It is known to inhibit microtubule assembly and the growth of the cells by inducing abnormal mitosis and blocking the cells at G₂/M phase of cell cycle (Panda et al., 2005; Rebazc et al., 2007). Tubulin is a major structural component of microtubule and consist of α and β subunits (Ludueno et al., 1977). It has already been reported that griseofulvin does not disrupt the microtubules, (Watson, 2004) instead it interacts with tubulin or with one or more associated proteins of microtubules (Sloboda et al., 1982; Roobol et al., 1977; Chaudhari and Ludueno, 1996).

In the present study, anticryptococcal activity of griseofulvin was performed against eight isolates of *C. neoformans* and significant (74.1%) inhibition was observed against isolate number 5. However, MIC of griseofulvin was found to be very high than MIC of amphotericin B but low toxicity and pharmacokinetic parameters like high elimination rate of griseofulvin than amphotericin B suggests that it could be a better drug.

In the present study, due to unavailability of structure of tubulin protein of *C. neoformans*, structure of tubulin alpha beta dimer of *S. scrofa* was taken as reference. Griseofulvin was found to efficiently bind with beta domain of tubulin. Ten griseofulvin binding sites on tubulin were also predicted. Unlike, Rathinasamy et al. (2010), the binding site at Pocket ID:167 gave the best binding results. However, in case of modelled structure of beta tubulin of *C. neoformans* the best docking was obtained at Pocket ID:103.

Keeping in view, the ability of griseofulvin to inhibit mitosis in fungal cells, to stabilize microtubule dynamics and its high elimination rates, it can be suggested that the drug may be used in combination therapy for the treatment of cryptococcosis. Further, its low toxicity and weak binding to mammalian brain tubulin (Wehland, 1977; Panda et al., 2005) makes it safer for human use.

Henceforth, this study will certainly be helpful in designing more potent and specific analogues of griseofulvin against the tubulin domains of *C. neoformans* with least side effects to the host and will provide understanding about the uniqueness of binding site of griseofulvin.

CONCLUSION

Griseofulvin is a well-known drug against dermatophytes but its activity against *C. neoformans* is not known. The activity of griseofulvin has been tested against eight isolates of the same. *In silico* docking results have been validated by

minimum inhibitory concentration assay. The high inhibition in growth gives an understanding that griseofulvin is effective against *C. neoformans*. The results obtained in this study can be extended and griseofulvin can further be investigated for its effect in *in vivo* condition. Its use as combinatorial therapeutic is also suggested.

Acknowledgements: One of the authors (Neelabh) gratefully acknowledges ICMR, New Delhi, India for providing the Junior Research fellowship. The authors are also thankful to The Principal, MMV, BHU for providing infrastructure facilities.

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