# Synthesis, *In Vitro* and *In Vivo* Antifungal Activity of 5-Phenylthio-2,4-Bisbenzyloxypyrimidine: A Novel Nucleobase

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**Amareshwar, *et al.*: Synthesis and Antifungal Activity of a Novel Nucleobase**

**A pyrimidne nucleobase, 5-phenylthio-2,4-bisbenzyloxypyrimidine and its analogs were synthesized and scanned for *in vitro* antifungal activity using cup-plate and macrobroth dilution method against *Candida albicans, Aspergillus niger, Aspergillus flavus* and *Asperglus fumigatus*. In the cup-plate method, 5-phenylthio-2,4-bisbenzyloxypyrimidine showed very good antifungal activity compared to clotrimazole at the concentrations of 100 and 1000 µg/ml and in the macrobroth dilution method, it showed comparable activity with respect to standard drugs fluconazole and itraconaole. *In vivo* antifungal activity of 5-phenylthio-2,4-bisbenzyloxypyrimidine at the dose levels of 10 and 30 mg/kg was carried by causing systemic infection of mice using the same fungi used in *in vitro* testing. The results from *in vivo* studies with 5-phenylthio-2,4-bisbenzyloxypyrimidine and fluconazole indicated that 5-phenylthio-2,4-bisbenzyloxypyrimidine had similar potency as fluconazole at both dose levels.**

**Key words: 5-phenylthio-2,4-bisbenzyloxypyrimidine, *in vitro* antifungal activity, *in vivo* antifungal activity, synthesis**

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There is a continuous need for developing potent and safe antifungal agents against fungal pathogens due to rapid emergence of drug resistant mutant fungi, increasing risk of opportunistic infections in immunocompromised patients such as those on cancer chemotherapy, organ transplantation and/or suffering with human immunodeficiency virus infection[1]. The clinical utility of triazole agents such as fluconazole and itraconazole is quite limited, especially against drug resistant *Candida* species[2], many of which are also have been reported to be resistant to amphotericin B[3], ketoconazole[4], or flucytosine[5]. In view of this, here we present the synthesis, *in vitro* and *in vivo* antifungal study of a 5-phenylthio-2,4-bisbenzyloxypyrimidine (PTBP) as preliminary trails. Synthesis of PTBP (Scheme 1, compound 6) and its analogs were carried out as reported earlier by our group[6].

Sabouraud agar media (SDA) was used for the antifungal screening and was followed as described in the literature[7]. This evaluation was carried out against *Candida albians, Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus fumigatus.* Test solution and standard drug clotrimazole were prepared at the concentration of 1000 and 100 µg/ml in DMF. Diameter of zone of inhibition was measured in mm after 24 h of incubation at $37^0$. Sabouraud dextrose broth was used for the evaluation of the synthesized compound against *A. niger*, *A. flavus*, *A. fumigatus* and RPMI-1640 with glutamine and without bicarbonate broth (pH 7.0) was used for *C. albicans*[8]. Minimum inhibitory concentration (MIC) of the compound was estimated according to literature and compared to that obtained for itraconazole, fluconazole and clotrimazole[2-9,10]. Test solution and all standard drugs were prepared at the concentration of 512 µg/ml in distilled dimethylsulphoxide (DMSO) and treated as stock solutions. MICs were scanned ranging the concentration from 256 µg/ml to 0.031 µg/ml. Haemocytometer was used to quantify the inoculum size or spore load per ml[11]. MICs were determined using Elico SL 171 spectrophotometer after 48 h of incubation at $37^0$. Amount of growth of fungal culture was determined by measuring the turbidity at 630 nm (the more turbid the suspension, the less light will be transmitted through.

**TABLE 1: *IN VITRO* ANTIFUNGAL ACTIVITY USING CUP-PLATE METHOD**

| Compound | Dose (µg/ml) | Diameter of zone of inhibition (mm) | | | |
|---|---|---|---|---|---|
| | | *C. albicans* | *A. niger* | *A. fumigatus* | *A. flavus* |
| PTBP | 1000 | 27.5 | 35.0 | 27.0 | 22.0 |
| PTBP | 100 | 26.5 | 35.0 | 24.0 | 22.0 |
| Clotrimazole | 1000 | 20.5 | 32.0 | 26.0 | 25.0 |
| Clotrimazole | 100 | 20.0 | 29.0 | 24.5 | 16.5 |

*In vitro* antifungal activity of PTBP and clortimazole was carried out against *C. albicans, A. niger, A. fumigatus* and *A. flavus* at the concentration of 1000 µg/ml and 100 µg/ml.

**TABLE 2: MIC OF PTBP AND STANDARD DRUGS (µg/ml)**

| Compound | *C. albicans* | *A. niger* | *A. fumigatus* | *A. flavus* |
|---|---|---|---|---|
| PTBP | 128 | 128 | 128 | 64 |
| Clotrimazole | 128 | 32 | 16 | 32 |
| Fluconazole | 128 | 64 | 128 | 128 |
| Itraconazole | 128 | 128 | 128 | 128 |

MICs of PTBP, clotrimazole, fluconazole and itraconazole were carried using macrobroth dilution method against *C. albicans, A. niger, A. fumigatus* and *A. Flavus* at the concentration ranging from 256 µg/ml to 0.031 µg/ml.

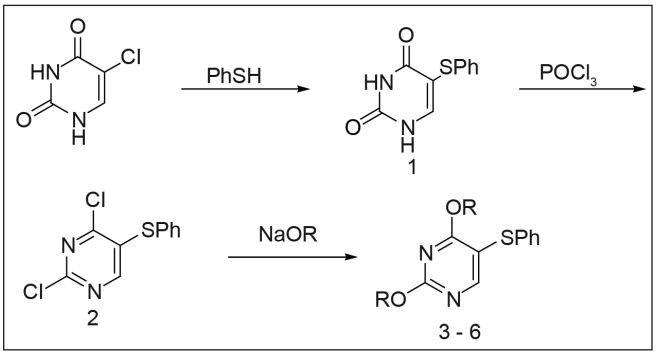
**TABLE 3: RECOVERY OF FUNGAL BURDEN FROM ORGANS OF MICE**

| Groups | | *C. albicans* | | *A. niger* | | *A. fumigatus* | | *A. flavus* | |
|---|---|---|---|---|---|---|---|---|---|
| | | Lung | Liver | Lung | Liver | Lung | Liver | Lung | Liver |
| PTBP (10 mg/kg) | | 38.88±9.62* | 27.63±6.09* | 0.00±0.00 | 0.88±0.74* | 3.33±1.17* | 9.71±5.51* | 2.33±0.76* | 2.0±0.68* |
| Fluconazole (10 mg/kg) | | 37.13±6.92 | 28.63±4.95 | 0.00±0.00 | 0.00±0.00 | 12.83±9.94* | 5.17±3.20* | 2.40±1.17* | 3.33±1.67 |
| PTBP (30 mg/kg) | | 12.75±5.38** | 13.88±4.85** | 2.13±0.79* | 0.88±0.35* | 5.50±4.21* | 6.50±6.10* | 2.33±0.71* | 6.33±3.76* |
| Fluconazole (30 mg/kg) | | 28.75±7.58 | 58.38±4.63 | 0.25±0.25 | 0.38±0.37 | 2.0±0.45 | 1.17±0.06 | 1.17±0.06 | 1.17±0.06 |
| Control | | 85.0±5.83 | 76.88±3.33 | 5.88±1.75 | 4.0±1.93 | 10.67±2.04 | 16.01±2.29 | 6.67±0.33 | 3.33±0.88 |
| One-way ANOVA | F | 13.90 | 28.26 | 8.52 | 2.81 | 0.90 | 0.66 | 4.63 | 0.95 |
| | P | 0.00 | 0.00 | 0.00 | 0.00 | 0.48 | 0.62 | 0.008 | 0.46 |

Values expressed in mean± SE and significant level is set at $P<0.05$. Harmonic mean sample size, as per DMRT post test, n=8 in each group, df=4, 35 for *C. albicans* and *A. niger*, Harmonic mean sample size, as per DMRT post test, n=6 in each group, df=4, 35 for *A. fumigatus* and Harmonic mean sample size, as per DMRT post test, n=4.8, df=4, 21 for lung of *A. flavus* and Harmonic mean sample size, as per DMRT post test, n=5, df=4, 22 for liver of *A. flavus*. *Indicates antifungal activity of PTBP is comparable with fluconazole according to DMRT post test. **Indicates antifungal activity of PTBP is higher than fluconazole according to DMRT post test

Since, turbidity is directly proportional to number of cells). MICs were defined as the concentration of the compound that inhibited the complete growth.

*In vivo* antifungal activity[1,2] was carried using Swiss albino mice of equal sex, weighing between 18-20 g (4-6 week old) were fed standard pellet diet and given tap water *ad libitum*. The experiment was approved by the Institutional Animal Ethics Committee. Mice were divided into five groups of 8 mice each for evaluating against *C. albicans* and *A. niger* and five groups of 6 mice each were used in case of *A. flavus* and *A. fumigatus*. First, second, third, fourth and fifth group were injected with quantified number of fungal spores via tail intravenous route (0.2 ml). Fungal suspension was prepared in sterile saline. Haemocytometer was used to quantify the inoculum size and $6.25\times10^6$ cells of *C. albicans*, $1.13\times10^4$ cells of *A. niger*, $5.23\times10^4$ cells of *A. flavus* and $1.03\times10^5$ cells of *A. fumigatus* were used. PTBP solution and standard drug, fluconazole were prepared in Tween-80 and suspended in distilled water and sterilized before injection. After 24 h of injection with fungal spores, first and second group of animals were treated with PTBP solution of 10 mg/kg and 30 mg/kg, respectively. Third and fourth groups received fluconazole at the dose of 10 mg/kg and 30 mg/kg, respectively. Fifth group received saline and served as control. Injections used for all groups were intraperitoneal for 5 days. Mice were kept under observation for 15 days and deaths occurred during the experiment were recorded during this period. On day 16 survived mice were sacrificed by cervical dislocation and both survived and moribund mice were dissected, liver and lung were removed and triturated in 2 ml of sterile saline and poured on Sabouraud agar media, incubated for 48 h for the growth of fungal burden. Data were analysed by one-way ANOVA followed by Duncan multiple range test (DMRT) and the level of significance was set at $P<0.05$.




**Scheme 1: Synthetic route for the preparation of nucleobases (3-6). 3. R = $CH_3$, 4. R = $CH_2CH_3$, 5. R = $CH(CH)_3$, 6. R = $CH_2C_6H_5$**

In the present study, PTBP showed very good *in vitro* antifungal activity against all the tested fungi, *A. niger*, *A. flavus*, *A. fumigatus* and *C. albicans* compared to standard drug, clotrimazole at both the concentration level of 100 µg/ml and 1000 µg/ml. Hence, we decided to study further *in vivo* antifungal activity of PTBP (Table 1). MIC of PTBP was carried against four fungi, *C. albicans, A. niger, A. flavus and A. fumigatus* and was compared with fluconazole, itraconazole and clotrimazole (Table 2). MIC of PTBP, fluconazole, itraconazole and clotrimazole against *C. albicans* remained same i.e., 128 µg/ml. Clotrimazole has the lowest MIC (32 µg/ml) against *A. niger* followed by fluconazole (64 µg/ml), PTBP (128 µg/ml) and itraconazole (128 µg/ml). Similarly, clotrimazole exhibited lowest MIC against *A. flavus* (16 µg/ml) and *A. fumigatus* (32 µg/ml). However, PTBP has significant less MIC (64 µg/ml) compared to fluconazole (128 µg/ml) and itraconazole (128 µg/ml) against *A. flavus* and has the same MIC of fluconazole and itraconazole against *A. fumigatus* (128 µg/ml). Results of the study demonstrated that, PTBP had comparable MIC against all the four fungi compared to fluconazole and itraconazole except clotrimazole which showed superior activity against *aspergillus* sps.

The *in vivo* antifungal activity of PTBP was compared with standard drug, fluconazole at two dose levels, 10 mg/kg and 30 mg/kg (Table 3). No deaths were occurred during the study period against *C. albicans* and *A. niger* but fungal burden studies showed that there was a significant difference between treated and control groups at the level of $P<0.05$. PTBP showed more efficiency in reducing the *C. albicans* spores in both liver and lung at the dose of 30 mg/kg compared to fluconazole at the same dose level. However, at the dose of 10 mg/kg, PTBP and fluconazole showed similar effect in fungal spore reduction (in lung as well as liver). In case of antifungal activity against *A. niger* there was no significant difference between PTBP and fluconazole treated mice at the dose level of 10 mg/kg and 30 mg/kg. This suggested that PTBP had the same efficiency as that of standard drug. Deaths were observed in all treated (except 30 mg/kg fluconazole group) and control groups of *A. fumigatus* infected mice before the completion of course of experiment. Two female mice and one female mouse were dead in 10 mg/kg fluconazole and 10 mg/kg PTBP groups respectively. One female mouse was dead in PTBP treated at the dose 30 mg/kg and in control group. However, fungal burden study showed that there was no significant difference between PTBP and fluconazole. In control group of *A. flavus*, five of six

mice were dead and only one mouse was alive during the course of experiment. Deaths were also occurred in all the treated groups but numbers of deaths were less compared to control group. Three deaths were occurred in 30 mg/kg fluconazole and 10 mg/kg PTBP groups. Finding of fungal burden of *A. flavus* in control group showed the uncountable number of spores in lungs of three male mice and liver of one male mouse. Similarly, uncountable numbers of *A. flavus* spores were also found in one male mouse of 10 mg/kg fluconazole group. DMRT pos-test showed that PTBP was comparable with fluconazole at both the dose levels at $P<0.05$. However, it should be noted that in control group all the mice were dead except one female mouse. This indicated that fluconazole as well PTBP prolonged the survival rate of the mice. Therefore, *in vivo* antifungal activity of PTBP and fluconazole indicated that PTBP had similar potency as fluconazole at the dose of 10 mg/kg and 30 mg/kg against all the tested fungi.

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