A semisynthetic borrelidin analogue BN-3b exerts potent antifungal activity against *Candida albicans* through ROS-mediated oxidative damage

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In the process of investigating the antifungal structure-activity relationships (SAR) of borrelidin and discovering antifungal leads, a semisynthetic borrelidin analogue, BN-3b with antifungal activity against *Candida albicans*, was achieved. In this study, we found that oxidative damage induced by endogenous reactive oxygen species (ROS) plays an important role in the antifungal activity of BN-3b. Further investigation indicated that BN-3b stimulated ROS accumulation, increased malondialdehyde (MDA) levels, and decreased reduced/oxidized glutathione (GSH/GSSG) ratio. Moreover, BN-3b decreased mitochondrial membrane potential (MMP) and ATP generation. Ultrastructure analysis revealed that BN-3b severely damaged the cell membrane of *C. albicans*. Quantitative PCR (RT-qPCR) analysis revealed that virulence factors of *C. albicans* SAPs, PLB1, PLB2, HWP1, ALSs, and LIPs were all down-regulated after BN-3b exposure. We also found that BN-3b markedly inhibited the hyphal formation of *C. albicans*. In addition, in vivo studies revealed that BN-3b significantly prolonged survival and decreased fungal burden in mouse model of disseminated candidiasis.

*Candida albicans* is an opportunistic fungal pathogen1, causing skin and mucosal infections in healthy individuals2. Moreover, *C. albicans* can cause fatal systemic disease when immune function is damaged3. Disseminated candidiasis is the main cause of death in immunocompromised patients3,4. Borrelidin (BN), an 18-membered macrolide polyketide5, was isolated from the fermentation broth of *Streptomyces vinaceusdrappus*. In the process of investigating the antifungal SAR of BN and discovering antifungal leads, forty-seven borrelidin derivatives (BNs) were synthesized by our research group5. Among them, a BN ester analogue BN-3b was greatly promising antifungal candidate. The MIC (minimum inhibitory concentration) values of BN-3b against *C. albicans* and *Candida parapsilosis* were 50 μg/mL and 12.5 μg/mL, respectively (Table 1). In this study, we will explore the antifungal mechanism of BN-3b.

Several studies have suggested that endogenous ROS mediated oxidative damage participate in the antifungal activity of amphotericin B (AMB) and fluconazole (FLC)7–11. These findings implied that oxidative stress involved in the antifungal mechanism of antifungal agents. ROS are the byproducts of cellular metabolism and mainly produced in the mitochondria12. However, overproduction of ROS resulted in damage of nucleic acids, proteins, and lipids3.

*C. albicans* has developed an effective battery of virulence factors13 that promote disease establishment and progression14. Among these virulence factors, lipases (LIPs), phospholipases, agglutinin-like sequences (ALSs), secreted aspartyl proteinases (SAPs), and hyphal wall protein (HWP1) are most significant in virulence15–17. Fungal virulence factors are potential targets for drug development15. In this study, we determined the expression of virulence factors (SAPs, PLB1, PLB2, HWP1, ALSs, and LIPs) of *C. albicans* after exposure to BN-3b using RT-qPCR.

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In vitro antifungal activities of BN-3b. The antifungal activities of BN-3b were evaluated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. BN-3b showed strong antifungal activity against C. albicans SC5314, C. albicans CGMCC 2.2086 and C. parapsilosis, with MIC values of 50, 50, and 12.5 μg/mL, respectively (Table 1). In addition, BN-3b displayed antifungal activity against a wide range of plant pathogenic fungi and inhibited mycelial growth of fungus including Fusarium oxysporum, Alternaria alternata, Aspergillus fumigatus, and Botrytis cinerea in a dose-dependent manner (Fig. 1A). The MIC values of BN-3b against F. oxysporum, A. alternata, A. fumigatus, and B. cinerea were 50, 50, 50, and 25 μg/mL, respectively (Table 1). In order to further evaluate the effect of BN-3b on C. albicans, an analysis of fungal viability was carried out by CFU counting (time-kill curves; Fig. 1B). The results indicated treatment with BN-3b at 0.5 × MIC reduced the cell viability of C. albicans SC5314 even after 2 h of treatment compared with the vehicle group; treatment with BN-3b at MIC and 1.5 × MIC exhibited fungicidal activity against C. albicans SC5314 in a dose and time-dependent manner within 8 h; BN-3b at higher concentrations (2 × MIC and 3 × MIC) decreased yeast viability in a time-dependent manner even after 8 h (Fig. 1B).

Effect of BN-3b on ultrastructure of C. albicans. We next utilized transmission electron microscopy to reveal ultrastructure of C. albicans SC5314 cells after treated with BN-3b. As shown in Fig. 2, BN-3b treated cells exhibited obvious alteration in the morphology compared to vehicle. C. albicans SC5314 cells showed normal cellular morphology with a distinct cell wall and an intact cell membrane in vehicle treated group (Fig. 2A). In contrast, the cell membrane of C. albicans was seriously destroyed after treated with BN-3b (Fig. 2D–F). The results indicated that BN-3b killed fungi through destroying the cell membrane.

BN-3b inhibited C. albicans in vivo. To evaluate the antifungal effect of BN-3b against C. albicans in vivo, we used two wild-type strains of C. albicans (C. albicans SC5314 and CGMCC 2.2086) in a model of disseminated candidiasis in mice. Percent survival over time for BN-3b, AMB, and FLC are shown in Fig. 3. All animals in the vehicle treatment group died by day 11 after infection with C. albicans SC5314, or day 13 with strain CGMCC 2.2086. Survival times for all groups of treated mice were significantly prolonged compared to the vehicle group. BN-3b or FLC treatments at dose of 2.0 mg/kg survived through days 15 and 19 for 2.2086. Survival times for all groups of treated mice were significantly prolonged compared to the vehicle group.

The model of disseminated candidiasis has been used extensively to study antifungal drug efficacy. In current study, we also evaluated the efficacy of BN-3b in mouse model of disseminated candidiasis caused by C. albicans.

### Table 1

| Strains                   | BN-3b | FLC | AMB |
|---------------------------|-------|-----|-----|
| MIC (μg/mL)               | MIC<sub>a</sub> (μg/mL) | MIC<sub>b</sub> (μg/mL) | MIC<sub>c</sub> (μg/mL) |
| C. albicans SC5314        | 50    | 12.5 | 2.0  | 2.0  | 0.5  |
| C. albicans CGMCC 2.2086  | 50    | 12.5 | 2.0  | 2.0  | 0.5  |
| C. parapsilosis           | 12.5  | 3.13 | 2.0  | 1.0  | 0.5  |
| C. neoformans             | >100.0| >100.0| >4.0 | 2.0  | 0.25 |
| A. niger                  | >100.0| >100.0| >8.0 | 2.0  | 0.5  |
| R. solani                 | >100.0| >100.0| >8.0 | 8.0  | 2.0  |
| E. oxysporum              | 50    | 12.5 | >8.0 | 2.0  | 0.5  |
| A. alternata              | 50    | 12.5 | >8.0 | 2.0  | 0.5  |
| A. fumigatus              | 50    | 12.5 | >8.0 | 2.0  | 0.5  |
| B. cinerea                | 25    | 6.25 | >8.0 | 2.0  | 0.25 |

The mic value was defined as the minimal inhibitory concentration of a compound. Identical values were obtained for each compound in three replicates by visual investigation as stated in the experimental section. The drug MIC<sub>a</sub> was defined as the first well with an approximate 80% reduction in growth compared to the growth of the drug-free well. Fluconazole (FLC) and amphotericin B (AMB) were served as the positive controls.
BN-3b enhanced the ROS production. Intracellular ROS production was detected by using the oxidant-sensitive probe 2',7'-dichlorofluorescin diacetate (DCFH-DA). Generation of ROS was monitored by incubation of BN-3b at 25.0, 50.0, and 75.0 μg/mL with the C. albicans SC5314 cells for 8 h, respectively. As shown in Fig. 5A, the ROS production induced by BN-3b increased significantly in a dose-dependent manner (p < 0.01), which was in turn attenuated by the addition of antioxidant N-acetylcysteine (NAC). The data indicated that BN-3b promoted ROS production in C. albicans.

Phospholipid peroxidation of C. albicans induced by BN-3b. Overproduction of ROS lead to phospholipid peroxidation of membrane. MDA was one of the final products of phospholipids peroxidation and could directly reflect the level of cell membrane damage. To further determine the involvement of oxidative damage induced by ROS in BN-3b antifungal activity, we examined phospholipid peroxidation levels of
C. albicans SC5314 cells treated with or without BN-3b by measuring the content of MDA. As shown in Fig. 5B, the content of MDA were significantly increased in a dose-dependent manner after treated with BN-3b compared with the control ($p < 0.01$), which was reduced by the addition of NAC. These findings indicated that BN-3b could stimulate ROS accumulation and thus resulted in C. albicans membrane phospholipid peroxidation.

Figure 2. Ultrastructure of C. albicans SC5314 cell. C. albicans SC5314 were treated with BN-3b or vehicle and were observed by transmission electron microscopy. (A) vehicle treatment; (B) treated with 25.0μg/mL of FLC; (C) treated with 2.0μg/mL of AMB; (D) treated with 25.0μg/mL of BN-3b; (E) treated with 50.0μg/mL of BN-3b; (F) treated with 75.0μg/mL of BN-3b; (G) structures of BN-3b, FLC, and AMB. The cell membrane of C. albicans was seriously destroyed by BN-3b, FLC or AMB. The white bar represents a length of 1 μm.

Figure 3. Survival and body weight of immunocompromised mice infected intravenously with C. albicans SC5314 (A) and CGMCC 2.2086 (B). Animals were immunocompromised by intraperitoneal injection of cyclophosphamide (100.0 mg/kg) at 3 days before and 1 day after infection. Fungal suspension (C. albicans: $2 \times 10^5$ CFU/mouse) was inoculated into the lateral tail vein of mice, 3 days (72 h) after the intraperitoneal injection of cyclophosphamide. Animals were treated for 5 days with BN-3b (2.0 mg/kg or 4.0 mg/kg), FLC (2.0 mg/kg), AMB (2.0 mg/kg), or vehicle, starting at 24 h after infection. There were 14 animals in each treatment group.
Effect of BN-3b on GSH. GSH plays a vital role in the protection of yeast cells against damage induced by oxidative stress\(^\text{20}\). As overproduction of ROS may consume GSH, we therefore examined the GSH levels of *C. albicans* SC5314 cells after treated with BN-3b. As shown in Fig. 5C, the content of GSH were significantly reduced in a dose-dependent manner after treated with BN-3b compared with the control \((p < 0.01)\). Moreover, we also

**Figure 4.** Fungal burden in liver tissues, kidney tissues, spleen tissues and lung tissues of immunocompromised mice infected intravenously with *C. albicans* SC5314 (A) and CGMCC 2.2086 (B). Intraperitoneal BN-3b (2.0 mg/kg or 4.0 mg/kg), FLC (2.0 mg/kg) and AMB (2.0 mg/kg) daily treatment was started 24 h after the *Candida* infection and lasted 5 days. Data represent the mean ± SD of 4 mice. \(*P < 0.05, **P < 0.01\) vs. vehicle group; \(#P < 0.05\) vs. FLC group. CFU, Colony-forming units.
found that the ratio of GSH/GSSG were significantly decreased in the BN-3b treatment groups (Fig. 5D). These results further confirmed that oxidative damage induced by ROS was involved in the antifungal activity of BN-3b.

Effect of BN-3b on MMP and ATP synthesis. In general, excessive ROS production triggered the mitochondria dysfunction. To investigate whether BN-3b affected the function of mitochondria, we determined the intracellular MMP level and ATP production of C. albicans SC5314 treated with or without BN-3b. The MMP was measured using a JC-1 fluorescent probe, and the JC-1 red/green fluorescence intensity ratio was used to represent MMP. (F) The intracellular ATP content. The results were represented as the mean ± SD from three independent experiments.

*P < 0.05, **P < 0.01 vs. control group; §p < 0.05 vs. FLC group; #p < 0.05 vs. 75.0 μg/mL BN-3b group.

Figure 5. Oxidative damage induced by endogenous ROS involved in the antifungal activity of BN-3b. C. albicans SC5314 cells were treated with 0, 25.0, 50.0, 75.0 μg/mL BN-3b, 5 mM NAC, 5 mM NAC + 75.0 μg/mL BN-3b, respectively. (A) BN-3b induced ROS generation. (B) BN-3b increased lipid peroxides MDA. (C) The content of GSH. (D) The ratio of GSH/GSSG. (E) The intracellular MMP levels. The MMP was measured using a JC-1 fluorescent probe, and the JC-1 red/green fluorescence intensity ratio was used to represent MMP. (F) The intracellular ATP content. The results were represented as the mean ± SD from three independent experiments.

*P < 0.05, **P < 0.01 vs. control group; §p < 0.05 vs. FLC group; #p < 0.05 vs. 75.0 μg/mL BN-3b group.
In addition, ATP content is one of the important indexes of mitochondrial activity. As shown in Fig. 5F, the content of intracellular ATP decreased significantly in a dose-dependent manner after treatment with BN-3b. The content of intracellular ATP decreased to 86.7 ± 4.2% (p < 0.05), 59.3 ± 2.5% (p < 0.01), and 48.7 ± 3.1% (p < 0.01), respectively, when the C. albicans SC5314 cells were exposed to 25.0, 50.0, and 75.0 μg/mL BN-3b. At the same time, the decrease of intracellular ATP content caused by BN-3b could be in turn attenuated by the addition of antioxidant NAC. The above data suggested that the mitochondria function was impaired in BN-3b treated cells.

**Effect of BN-3b on the hyphal formation in C. albicans.** The ability to switch from yeast to hypha was important for virulence of C. albicans. C. albicans SC5314 cells incubated with vehicle or different concentrations of BN-3b (25.0, 50.0, and 75.0 μg/mL) for 8 h, and then observed by microscopy. In the vehicle treated group, large numbers of hyphae were observed in C. albicans. In contrast, BN-3b markedly inhibited the hyphal formation of C. albicans in a dose-dependent manner (Fig. 6). Especially, BN-3b completely inhibited the hyphal formation of C. albicans at the concentration of 75.0 μg/mL (Fig. 6F).

**Effect of BN-3b on expression of virulence-related genes.** The effect of BN-3b on the virulence-related genes (Supplementary Table S1) was examined in C. albicans SC5314 using RT-qPCR. RT-qPCR analyses revealed that the expressions of virulence factors of C. albicans SC5314 were significantly down-regulated in a dose-dependent manner after treated with BN-3b compared with the control (p < 0.01) (Fig. 7). These data indicated that BN-3b could exert additional antifungal activity by inhibiting the expression of virulence factors (SAPs, PLB1, PLB2, HWP1, ALS, and LIPs) in C. albicans.

**Discussion**

The kill curves study indicated that BN-3b significantly reduced the cell viability of C. albicans in a dose-dependent manner (Fig. 1B). Following, we evaluated the in vivo antifungal activity of BN-3b by establishing the systemic mouse model of disseminated candidiasis. The results revealed that BN-3b significantly prolonged survival and decreased fungal burdens in mouse model of disseminated candidiasis. Based on the good antifungal activity of BN-3b in vivo and in vitro, we further discussed its action mechanism.

Increasing evidence demonstrated that oxidative damage induced by endogenous ROS was involved in the antifungal activity of antifungal agents. Kobayashi et al. reported that the ROS production of C. albicans were significantly increased in a dose-dependent manner after treated with 0.125 (MIC), 1.25, and 12.5 μg/mL miconazole, respectively. FLC treatment also enhanced ROS production, especially in the 5 and 50 μg/mL groups. In another study, Li et al. showed that treatment with 8 μg/mL of AMB significantly increased the ROS production in C. albicans. According to the literatures and our experiment results, we used 25 μg/mL FLC or (and) 2 μg/mL AMB as positive controls for the antifungal mechanism research in the current study.

Excessive ROS may lead to oxidative damage of nucleic acids, proteins, and lipids, and ultimately lead to cell death. In this study, we found that BN-3b stimulated ROS production in C. albicans cells (Fig. 5A). To further confirm the ROS production, we examined the intracellular MDA production and GSH concentration. The excess ROS produced will react with cell membranes, produce lipid peroxide radical, and eventually form MDA. Our
current study showed that the production of MDA were significantly increased in a dose-dependent manner in the BN-3b treated cells (Fig. 5B). GSH protects the cells from oxidative damage by scavenging ROS. Thus, the generation of excess ROS may consume GSH. In fact, our results indicated that the content of GSH were significantly reduced in a dose-dependent manner in the BN-3b treated cells (Fig. 5C). Besides, BN-3b treatment could markedly decrease the ratio of GSH/GSSG (Fig. 5D). Conversely, antioxidant NAC could significantly attenuate BN-3b induced oxidative stress. These results indicated that BN-3b treatment impaired the balance of antioxidant system in C. albicans cells. In general, the overproduction of ROS might damage the membranes. Consistent with this, the cell membrane of C. albicans appeared obvious shrinkage and breakage (Fig. 2D–F) after exposure to BN-3b. Collectively, these data strongly suggested that BN-3b induced the endogenous ROS-mediated oxidative damage and destroyed the cell membrane, ultimately resulting in cell death.

The above results confirmed that BN-3b stimulated ROS accumulation in C. albicans. The excessive ROS production can lead to mitochondrial dysfunction, mainly characterized by the loss of MMP and decrease of ATP generation. Our current study showed that the MMP levels of C. albicans cells were significantly reduced in a dose-dependent manner after treated with BN-3b. Furthermore, the results also showed that the intracellular ATP content was significantly decreased after treated with BN-3b compared with the control. These results indicated that the mitochondria function was impaired in BN-3b treated cells.

BN-3b belongs to the BN ester analogue and was synthesized by our research group. In comparison with BN, the cytotoxicity of BN-3b was significantly decreased. More significantly, BN-3b displayed potent antifungal activity against C. albicans, while BN was inactive. It is generally accepted that the multiple bioactivities of BN were associated with the inhibition of threonyl-tRNA synthetase (ThrRS). The molecular docking study indicated that the side chain of BN-3b was projected deeper into the bottom of binding pocket in ThrRS than BN, which indicated the action mechanisms of BN-3b was different from BN. In the present study, we found that oxidative damage induced by endogenous ROS plays an important role in the antifungal activity of BN-3b. Besides, we speculated that the unique interaction of BN-3b with ThrRS might also play a vital role in its antifungal activity, but it needs to be further verified.

Many virulence factors of C. albicans are involved in the infective process, such as extracellular hydrolases production, adhesion to host tissue, and hyphal formation. In this study, we also examined the mRNA level of the virulence-related genes after exposure to BN-3b using RT-qPCR in C. albicans. The three most significant extracellular hydrolases produced by C. albicans are the SAPs, LIPs, and phospholipase B (PLBs). C. albicans SAPs, encoded by a multigene family, contribute to pathogenesis by digestion of host cell membranes and molecules of the host immune system. C. albicans LIPs, encoded by at least ten members, contribute to the provision of nutrients and promote fungal penetration of host barriers. Phospholipase B encoded by at least two genes (PLB1 and PLB2) also contributes to the pathogenicity of C. albicans by abetting the fungus in damaging and traversing host cell membranes. The results of our study showed that all of these genes were significantly down-regulated after BN-3b treatment (p < 0.01). The pathogenic potential of C. albicans was positively correlated with its adhesive capacity of the organism. Currently, most studies focus on two well-characterized adhesins, Hwp1 and the ALS family. The C. albicans ALS family has eight members (ALS1 to ALS7, and ALS9), each encodes a large glycoprotein whose function is adhesion to host. Furthermore, it has been suggested that some ALS proteins were involved in growth-related functions. Our current study showed that these genes were significantly down-regulated after BN-3b treatment (p < 0.01). Therefore, down-regulation of these genes not only affect the infection ability of C. albicans but also affect the cell proliferation of C. albicans. HWP1, a hypha-specific gene, which encodes a cell-surface adhesin that promotes interactions between C. albicans and host cells. It is interesting to note that the mRNA level of HWP1 decreased significantly after treatment with BN-3b. Hyphae formation plays a key role in C. albicans pathogenicity. In this study, we found that BN-3b strongly inhibited the hyphal formation of C. albicans. Thus we suggested that BN-3b could inhibit the yeast-to-hypha transition and down-regulate the expressions of virulence factors to weaken the pathogenicity of C. albicans.

Our results indicated that BN-3b exerts antifungal effect through increasing the generation of ROS, decreasing the MMP, reducing the intracellular ATP level, and destroying the cell membrane. In addition, BN-3b exerts added antifungal activity by inhibiting the yeast-to-hypha transition and down-regulating the expressions of virulence factors. These findings suggested that BN-3b may be a promising lead for the development of antifungal agent.
Materials and Methods

Drugs. BN-3b was synthesized according to our previous report\(^\text{7}\). BN-3b, AMB (Sigma-Aldrich) and FLC (Solarbio Science & Technology Co., Ltd., Beijing, China) were dissolved in dimethyl sulphoxide (DMSO) to prepare 150.0 mg/mL, 10.0 mg/mL and 10.0 mg/mL of stock solutions and stored at \(-20^\circ\text{C}\). Working solutions of BN-3b, AMB, and FLC were prepared by diluting the stock solution with DMSO prior to use\(^\text{8}\). N-acetylcysteine (Beyotime Biotechnology Co., Shanghai, China) was dissolved in phosphate-buffered saline (PBS) to prepare 0.5 M of stock solution and stored at \(-20^\circ\text{C}\). Cyclophosphamide (CY, J&K Scientific Ltd., Beijing, China) was dissolved in 0.9% NaCl sterile solution to prepare 50.0 mg/mL of stock solution and stored in the dark at 4 °C.

Organism and culture conditions. C. albicans SC5314 (ATCC MYA-2876), C. albicans CGMCC 2.2086, C. parapsilosis (ATCC 22019), Cryptococcus neoformans (ATCC 208821), and Rhizoctonia solani (CGMCC 3.7376) were obtained from China General Microbiological Culture Collection Center. Aspergillus niger (CCTCC AF 93021), A. fumigatus (CCTCC AF 93048), A. alternata (CCTCC AF 93103), F. oxysporum (CCTCC AF 93247), and B. cinerea (CCTCC AF 93110) were obtained from China Center for Type Culture Collection. All strains were maintained on YPD or YPDA\(^\text{9}\). Hyphal development was induced using the yeast carbon base/fetal bovine serum (YCB/FBS) liquid media, containing 1.17% (w/v) yeast carbon base (BD Biosciences), 1% (w/v) glucose, and 10% (v/v) fetal bovine serum (Gibco BRL, USA).

In vitro assay for antifungal activities. The MICs of BN-3b were determined using the method described by CLSI guidelines\(^\text{34}\). FLC and AMB were used as positive controls. The tested compounds were dissolved in DMSO and 2-fold serially diluted to eight different concentrations \(10^{-6}-10^{-8}\) mg/mL for BN-3b, 0.8-0.00625 mg/mL for FLC and AMB. The above samples (1 μL) and 100 μL of prepared fungal suspensions (in RPMI-1640 medium) containing 2 × 10⁴ CFU/mL of fungus were added to each well of 96-well microtiter plates\(^\text{3}^\). The culture treated wells were used as control. The plates were incubated for 48 h at 37 °C, and the absorbance was recorded spectrophotometrically at 620 nm using a microplate reader (BioTek Synergy H1, BioTek Instruments, Inc., Vermont, USA). The MICs of the BN-3b and AMB were defined as the lowest concentrations that completely inhibited visual growth of an organism\(^\text{35}\). The concentration of FLC which caused a 80% reduction in the absorbance compared to the control was considered as the MIC\(^\text{36}\).

The viability assay of C. albicans. Fungal suspensions at 6 × 10⁵ CFU/mL in YPD liquid medium were exposed to BN-3b (0, 0.5 × MIC, MIC, 1.5 × MIC, 2 × MIC, and 3 × MIC), and incubated for 2, 4, 6, 8, 10 or 12 h at 37 °C. Yeasts were washed twice with PBS, diluted serially (1:10) and spread on Sabouraud dextrose agar (SDA) plates. After 48 h of incubation at 37 °C, the viability of C. albicans was determined by colony counting\(^\text{12}\). Each concentration was performed with three biological replicates.

Ultrastructure analysis. C. albicans SC5314 cells treated with BN-3b (0, 25.0, 50.0, or 75.0 μg/mL) for 8 h. The samples were treated according to our previous report\(^\text{37}\). The ultrastructure of C. albicans was observed under a Hitachi H-7650 transmission electron microscope (Tokyo, Japan). The treatment of AMB (2.0 μg/mL) and FLC (25.0 μg/mL) served as positive controls.

In vivo assay for antifungal activity. Male BALB/c mice (weight, 20–25 g) were purchased from Changsheng biotechnology Co., Ltd. (Liaoning, China). All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006)\(^\text{38}\) and approved by the Laboratory Ethics Committees of College of Life and Health Sciences of Northeastern University\(^\text{39}\).

In order to quickly establish mouse model of disseminated candidiasis, all mice used in this study were received CY at 100 mg/kg body weight administered intraperitoneally 3 days before and 1 day after infection\(^\text{38}\). Fungal suspension (C. albicans: 2 × 10⁵ CFU/mouse in volume of 0.1 mL) was inoculated into the lateral tail vein of mice, 3 days (72 h) after the intraperitoneal injection of CY. Mice were randomly separated into five groups \((n = 14\) per group). BN-3b therapy with a dose of 2.0 mg/kg or 4.0 mg/kg of body weight daily, AMB or FLC therapy with a dose of 2.0 mg/kg of body weight daily by intraperitoneal injection were initiated at 24 h after infection, and continuous administration for 5 days. Control group were treated with vehicle (60% 1,2-propanediol) in the same way. Twenty-four hours after the last dose of antifungal agent, four mice of each group were sacrificed to monitor daily until 15 days after the end of therapy (21 days after infection).

Measurement of ROS production. Followed the methods as previously described, C. albicans SC5314 (10⁷ CFU/mL) incubated with 40 μM DCFH-DA at 37 °C for 60 min in the dark\(^\text{40}\), the cells were collected, washed twice and then diluted to 6 × 10⁵ CFU/mL with YPD\(^\text{12}\). After that, a series of BN-3b were added and incubated at 37 °C for 8 h. And then washed and re-suspended in 100 μL of PBS. The fluorescence intensity of a cell suspension (100 μL) containing 10⁷ cells was measured using a microplate reader with excitation at 480 nm and emission at 530 nm\(^\text{4}\). The NAC (5 mM) treated cells were used as negative control, and FLC (25.0 μg/mL) as positive control.

Measurement of phospholipid peroxidation. We examined phospholipid peroxidation of membranes by measuring the levels of MDA\(^\text{3}\). Briefly, C. albicans SC5314 (6 × 10⁷ CFU/mL) incubated with different concentrations of BN-3b for 8 h. And cells (3 × 10⁷) were collected and washed three times with PBS. The pellet was...
resuspended in 200μL PBS and freeze overnight at −80°C, then samples were heated in a boiling bath for 5 min and, cooled to room temperature and added the reagents of MDA Assay Kit (Jiancheng Institute of Biotechnology, Nanjing, China). Finally, the absorbance of the supernatant was measured at 532 nm using a microplate reader27. The FLC (25.0μg/mL) treated cells were used as positive control.

**GSH/GSSG assay.** *C. albicans* SC5314 (6 × 10^5 CFU/mL) incubated with different concentrations of BN-3b (0, 25.0, 50.0, and 75.0μg/mL) for 8 h. And cells (3 × 10^7) were collected and washed three times with PBS. The pellet was resuspended in 200μL PBS and freeze overnight at −80°C, then samples were heated in a boiling bath for 5 min and, cooled to room temperature, then the supernatants were collected by centrifugation. GSH and GSSG levels were quantified using GSH/GSSG Assay Kit (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer’s protocol. The absorbance was measured at 405 nm. The FLC (25.0μg/mL) treated cells were used as positive control.

**Measurement of MMP.** The MMP was measured with a Mitochondrial Membrane Potential Assay Kit with JC-1 (Beyotime Biotechnology Co., Shanghai, China)28. *C. albicans* SC5314 (6 × 10^5 CFU/mL) incubated with vehicle or different concentrations of BN-3b (25.0, 50.0, and 75.0μg/mL) for 8 h. And then cells (3 × 10^7) were incubated with a JC-1 staining solution at 37 °C for 20 min in the dark, washed twice with PBS, and resuspended in the buffer28. Green (excitation/emission wavelength: 514/529 nm) and red (excitation/emission wavelength: 585/590 nm) fluorescence were detected on a microplate reader. The ratio of red/green fluorescence intensity represents MMP22.

**Synthesis of ATP assay.** The cellular ATP level was detected using an ATP Bioluminescence Assay Kit (Beyotime Biotechnology Co., Shanghai, China)28. *C. albicans* SC5314 (6 × 10^5 CFU/mL) incubated with vehicle or different concentrations of BN-3b (25.0, 50.0, and 75.0μg/mL) for 8 h. After that, cells (5 × 10^6) from each culture were lysed and centrifuged, and then 100μL of ATP detection working solution as well as 50μL supernatant were added to 96-well plate, and then luminescence was measured on a microplate reader28.

**Hyphal formation assay.** The hyphal formation of *C. albicans* induced by YCB/FBS medium40,41. *C. albicans* SC5314 (6 × 10^5 CFU/mL) incubated with different concentrations of BN-3b (25.0, 50.0, and 75.0μg/mL) for 8 h at 37°C. The hyphal formation of *C. albicans* was recorded with a microscope with the magnification of 400×. The treatment of AMB (2.0μg/mL) and FLC (25.0μg/mL) severed as positive controls.

**RNA extraction and RT-qPCR.** *C. albicans* SC5314 cells were treated with vehicle and BN-3b (25.0 or 50.0μg/mL) in YCB/FBS medium as the same method above. Total RNA was extracted with AxyPrep Multisource Total RNA Miniprep Kit (Axygen, China) and reverse transcribed with GoScriptTM reverse transcription system (Promega, USA) by following the manufacturer’s instructions. RT-qPCR was performed with the primer sets listed in Supplementary Table S1. ACT1 gene was used as the internal control. Fold changes were calculated using the 2^−ΔΔCt method30.

**Statistical analysis.** All data were represented as the mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was determined by using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, p ≤ 0.05. The SPSS 17.0 statistical software package was used for data analysis.

**Data availability**

The datasets that were generated and/or analysed during the current study are freely available from the corresponding author on a request.

Received: 25 February 2019; Accepted: 2 March 2020; Published online: 19 March 2020

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Acknowledgements
This work was funded by National Natural Science Foundation of China (No. 81573327), and the Fundamental Research Funds for the Central Universities, China (Nos. N172004004 and N172008008).

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L.H. and X.H. conceived and designed the research; H.S., C.H., B.C., X.Q., P.G. and Y.M. performed the evaluations of bioactivity and the action mechanism investigations. H.S., L.H. and X.H. wrote and edited the manuscript. All co-authors contributed to the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-61681-0.

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