**Design, Synthesis, and In Vitro and In Vivo Antifungal Activity of Novel Triazoles Containing Phenylethynyl Pyrazole Side Chains**

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Abstract: A series of triazole derivatives containing phenylethynyl pyrazole moiety as side chain were designed, synthesized, and most of them exhibited good in vitro antifungal activities. Especially, compounds 5k and 6c showed excellent in vitro activities against *C. albicans* (MIC = 0.125, 0.0625 µg/mL), *C. neoformans* (MIC = 0.125, 0.0625 µg/mL), and *A. fumigatus* (MIC = 8.0, 4.0 µg/mL). Compound 6c also exerted superior activity to compound 5k and fluconazole in inhibiting hyphae growth of *C. albicans* and inhibiting drug-resistant strains of *C. albicans*, and it could reduce fungal burdens in mice kidney at a dosage of 1.0 mg/kg. An in vivo efficacy evaluation indicated that 6c could effectively protect mice models from *C. albicans* infection at doses of 0.5, 1.0, and 2.0 mg/kg. These results suggested that compound 6c deserves further investigation.

Keywords: triazole; CYP51; antifungal; molecular docking; synthesis

1. Introduction

During the last four decades, the morbidity of invasive fungal infections (IFIs) has been increasing in patients which acquired immunodeficiency, and the mortality keeps high [1,2]. According to the clinic report, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* are the three most common human pathogenic fungi [3,4]. It is estimated that more than 300 million people suffer from serious fungal-related diseases, and fungi collectively kill over 1.6 million people annually, which is more severe than malaria and similar to tuberculosis [5]. Moreover, the rate of emergence of drug resistance is greater than the discovery pace of antifungal research. Therefore, it is urgent to develop novel antifungal agents [6].

Compared with the other few antifungal agents, including amphotericin B, flucytosine and echinocandins, azole antifungals, especially triazoles, were widely applied in treating IFIs and were much more concerned in research due to their higher therapeutic index, broader-spectrum, lower toxicity and superior druggability [7–9]. However, their extensive use has led to the occurrence of resistant fungi, which may cause the failure of antifungal treatment and greatly limit therapeutic options [10]. Hence, new antifungal azoles are therefore highly needed.

Azoles antifungal agents act by inhibition of fungal lanosterol 14α-demethylase (CYP51), which is a necessary enzyme for catalyzing the oxidative removal of the 14α-methyl in sterol biosynthesis of fungi [11]. By summarizing the structures of triazole antifungals...
approved for the treatment of invasive fungal infections, as shown in Figure 1, a clear pharmacophore can be concluded to possess a triazole and a halophenyl ring, which have been proved to bind the heme iron and the hydrophobic pocket of CYP51 [12]. The side chain in the right part of each structure, shown in Figure 1, occupies the substrate access channel of CYP51. Structurally, the difference between newer azole antifungals mainly focuses on the type of side chain attached to the carbinol center of the triazole alcohol scaffold. Accordingly, most of the recent efforts aimed to optimize this part of the molecule correspondingly. Therefore, the study of antifungal azoles has been mainly focused on the structural optimization and SAR research on the side chain for new drugs [13].

Figure 1. Several triazole drugs for IFIs in clinic and the structures of our lead compounds.

Guided by the binding modes of triazole antifungal agents, a number of new triazoles were rationally designed and synthesized by our group [14–18]. Among them, a series of triazoles containing alkynyl side chains showed good antifungal activity with a broad spectrum [18]. They exhibited superior activity to fluconazole and comparable activity to voriconazole, and specially compound A5 (Figure 2) was subjected to evaluate its in vivo efficacy as a promising compound. Unfortunately, it possessed inferior activity in the murine model of disseminated C. albicans infection. Considering the lack of hydrogen bond donors and acceptors as well as the number of rotatable bonds in compound A5, further lead optimization was focused on ameliorating drug-likeness in order to improve the in vivo potency. Herein, a series of novel side chains of triazole antifungals were designed by hybridizing the side chains in lead compound A5 and fluconazole and replacing the 1,2,4-triazole with pyrazole based on the bioisosterism principle [19] to construct a phenylethynyl pyrazole side chain in Figure 2. Herein, we reported in vitro antifungal activity and SAR of all the target compounds 5a–v and 6a–e, and in vivo antifungal potency of 6c.
2. Results and Discussion

2.1. Chemistry

As depicted in Scheme 1, the target compounds were synthesized, starting from 1-((2-(2,4-difluorophenyl)oxiran-2-yl)methyl)-1H-1,2,4-triazole methane sulfonate (1), which the opening-ring reacted with 4-iodo-1H-pyrazole in the presence of K₂CO₃ in DMF. Sonogashira reaction of the key intermediate (2) with substituted (4-ethynylphenoxy)methyls or aryl alkyne was conducted in an NMP solution of Pd(PPh₃)₂Cl₂, CuI and DIEA at 60 °C to afford (3) or 6a–e. In addition, the ester hydrolysis reaction of methyl 4-((1-(2-(2,4-difluorophenyl)-2-hydroxy-3-(1H-1,2,4-triazol-1-yl)propyl)-1H-pyrazol-4-yl)ethynyl)benzoate (4) was reacted with LiOH in a mixed solvent of H₂O and THF, which was then subjected to the amidation reaction conditions with substituted alkyl amine or aromatic amine to give target compounds 5a–5v.

Scheme 1. Synthesis of the target compounds. (i) 4-Iodo-1H-pyrazole, K₂CO₃, DMF, 80 °C, 4 h; (ii) Methyl 4-ethynylbenzoate, PdCl₂(PPh₃)₂, CuI, DIEA, NMP, 60 °C, 6 h; (iii) LiOH, THF/H₂O (V/V= 1:1), 50 °C, 6 h; (iv) Alkyl amines or aromatic amines, DIEA, PyBOP, 50 °C, 4–8 h; (v) substituted (4-ethynylphenoxy)methyls, PdCl₂(PPh₃)₂, CuI, DIEA, NMP, 60 °C, 6 h.

2.2. In Vitro Antifungal Activity

All 29 compounds were evaluated by means of the minimum inhibitory concentration (MIC) according to the regulations recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [20,21]. MIC was defined as the first well with an approximate 80% reduction in growth compared to the growth of the drug-free well. The three most common human pathogenic fungi, including Candida albicans, Cryptococcus neoformans and Aspergillus fumigatus, were tested. Clinic antifungal drugs fluconazole (FCZ) and
posaconazole (POS) were used as the reference drugs to compare with target compounds. All data were the means of three replicate tests performed with each target compound and are summarized in Table 1.

Table 1. In vitro antifungal activity of the target compounds (MIC, μg/mL).

| Compd. | R₁   | R₂   | MIC (μg/mL) |
|--------|------|------|-------------|
|        |      |      | C. alb SC5314 | C. neo 32609 | A. fum 7544 |
| 3      | -    | -    | 0.5 | 8.0 | >64.0 |
| 4      | -    | -    | 0.5 | 1.0 | >64.0 |
| 5a     | -    | -    | 0.0625 | 0.125 | >64.0 |
| 5b     | -    | -    | 0.0625 | 0.125 | >64.0 |
| 5c     | -    | -    | 0.5  | 0.5  | 64.0  |
| 5d     | -    | -    | 0.25 | 0.5  | >64.0 |
| 5e     | -    | -    | 0.5  | 0.5  | >64.0 |
| 5f     | -    | -    | 0.5  | 1.0  | >64.0 |
| 5g     | -    | -    | 0.5  | 0.5  | >64.0 |
| 5h     | -    | -    | 0.5  | 2.0  | >64.0 |
| 5i     | -    | -    | 0.25 | 0.5  | 64.0  |
| 5j     | -    | -    | 0.0625 | 0.125 | >64.0 |
| 5k     | -    | -    | 0.125 | 0.125 | 8.0   |
| 5l     | -    | -    | 0.5  | 0.5  | >64.0 |
| 5m     | -    | -    | 0.5  | 0.5  | >64.0 |
| 5n     | -    | -    | 1.0  | 0.5  | 16.0  |
| 5o     | -    | -    | 0.0625 | 0.25 | >64.0 |
| 5p     | -    | -    | 0.25 | 0.5  | >64.0 |
| 5q     | -    | -    | 0.25 | 0.25 | >64.0 |
| 5r     | -    | -    | 0.5  | 0.25 | >64.0 |
| 5s     | -    | -    | 0.25 | 0.25 | >64.0 |
In this study, the grades of MIC values were regarded as: excellent: <0.25 µg/mL; good: 0.25–1 µg/mL; moderate: 1–64 µg/mL. As shown in Table 1. Most of the target compounds exhibited good to excellent inhibitory activity against C. albicans and C. neoformans with MIC values ranging from 1 to 0.0625 µg/mL. Especially, compounds 5a, 5b, 5j, 5k, 5o, 6a and 6c exhibited MIC values of 0.0625 µg/mL against C. albicans. Compounds 6a and 6c exhibited MIC values of 0.0625 µg/mL against C. neoformans. These compounds showed superior activity to fluconazole (FCZ) and posaconazole (POS). Moreover, compounds 5k, 5t, 5u, 5v, 6c exhibited moderate activity against A. fumigatus (MIC = 8.0 µg/mL). However, fluconazole (FCZ) is inactive against A. fumigatus (MIC > 64.0 µg/mL). Among all the compounds, 5k and 6c are promising leads for the development of new generations of triazole antifungal agents. These results suggested that phenylethynyl pyrazole could be considered a novel privileged structure of side chain that deserves further investigation.

Table 1. Cont.

| Compd. | R₁ | R₂ | MIC (µg/mL) |
|--------|----|----|-------------|
|        |    |    | C. alb SC5314 | C. neo 32609 | A. fum 7544 |
| 5t     | -  | -  | 0.125        | 0.125        | 8.0          |
| 5u     | -  | -  | 0.25         | 0.125        | 8.0          |
| 5v     | -  | -  | 0.25         | 0.25         | 8.0          |
| 6a     | F  | -  | 0.0625       | 0.0625       | 16.0         |
| 6b     | Cl | -  | 0.125        | 0.25         | >64.0        |
| 6c     | CN | -  | 0.0625       | 0.0625       | 4.0          |
| 6d     | CF₃| -  | 0.125        | 0.25         | 16.0         |
| 6e     | OCF₃| - | 0.125       | 0.125        | 64.0         |
| FCZ    | -  | -  | 0.5          | 0.25         | >64.0        |
| POS    | -  | -  | 1.0          |              | 1.0          |

Abbreviations: C. alb: Candida albicans; C. neo: Cryptococcus neoformans; A. fum: Aspergillus fumigatus; FCZ: fluconazole; POS: posaconazole.
activity than other compounds against both tested drug-resistant strains with MIC values of 4.0 µg/mL.

Table 2. In vitro antifungal activity of the target compounds (MIC, µg/mL).

| Compd. | C. alb. Strain 100 | C. alb. Strain 103 |
|--------|-------------------|-------------------|
| 5a     | 16.0              | 2.0               |
| 5b     | 8.0               | 8.0               |
| 5j     | 8.0               | 8.0               |
| 5k     | 16.0              | 4.0               |
| 6a     | 8.0               | 2.0               |
| 6c     | 4.0               | 4.0               |
| FCZ    | >64.0             | >64.0             |

Abbreviations: C. alb: Candida albicans; strain 100, fluconazole-resistant strains of Candida albicans; strain 103, fluconazole-resistant strains of Candida albicans; FCZ: Fluconazole.

2.3. Theoretical Evaluation of ADME/T Properties

Since compounds 5a, 5b, 5j, 5k, 6a and 6c exhibited excellent antifungal activity, their ADMET prediction was performed using the DS-ADMET and DS-TOPKAT modules to evaluate their druggability, and the predicted data are summarized in Figure 3 and Table S1.

Most compounds were positioned in the 95% and 99% confidence ellipses for human intestinal absorption (absorption), and only compound 6c was positioned between the interval of 95% and 99% confidence ellipses (Figure 3). Meanwhile, compounds 5a and 5b were positioned between the interval of 95% and 99% confidence ellipses for blood–brain barrier (BBB) penetration, and compounds 5j, 5k, 6a and 6c were beyond the 99% confidence ellipse of the BBB model. These results indicated that these compounds with proper aqueous solubility and relatively low BBB penetration may enter blood circulation
through intestinal absorption and exert their antifungal effect in vivo without causing nervous system damage.

Next, we analyzed the A logP and PSA of these compounds, and their values were distributed within a reasonable absorption range, suggesting that these compounds can achieve appropriate drug concentrations in vivo (Table S1). In addition, the toxicological properties of target compounds were further predicted according to the TOPKAT calculation module. We found these compounds had the characteristics of non-mutagen, non-irritant, and non-carcinogen, which greatly reduces the drug risk. Although all compounds showed strong skin sensitization, we did not consider this side effect as a concern, as the control drug fluconazole had the same predicted results.

2.4. Anti-Hyphal Activity

Hyphae growth is a significant morphological feature of fungi and is one of the virulent factors [22]. Morphological transitions from yeast to filamentous forms are the major contributor to the in vivo pathogenicity of C. albicans [23–26]. As the basis for studying in vivo potency, we further investigated the activity of compounds 5k and 6c against the yeast-to-hyphal transition of C. albicans with fluconazole as the control drug. As shown in Figure 4, compounds 5k and 6c exhibited mild activity against fungi hyphal formation, with fewer hyphae and more pseudohyphal cells compared with the group without drug treatment at 1.0 µg/mL or higher concentrations. Even at the concentration of 0.0625 µg/mL, compound 6c showed an obvious difference between compound 5k and fluconazole, which grew more hyphaes.

Figure 4. Anti-hyphal effects of different concentrations of compounds 5k and 6c with FCZ (fluconazole) as a positive agent. Exponentially growing C. albicans SC5314 cells were transferred to hypha-inducing Spider liquid medium. The cellular morphology was photographed after incubation at 37 °C for 3 h. Scale bar = 20 µm.

2.5. Fungal Burden Evaluation

C. albicans has a strong tropism in kidney tissue, so the fungal burden is an important indicator for evaluating systemic fungal infection. Compound 6c, which demonstrated significant in vitro and anti-hyphal activity, was tested for the evaluation of the fungal burden of systemic C. albicans SC5314 in ICR mice. Following 3 days of treatment, we determined the changes in the fungal burdens in the kidney by measuring the number of CFU in Figure 5. Significant reductions in fungal burdens were observed with compound 6c in a dose-dependent manner compared with the vehicle control. Meanwhile, there is no
significant difference between compound 6c (at the dosage of 1.0 mg/kg) and FCZ (at the dosage of 0.3 mg/kg). The results highlighted the antifungal potential of compound 6c.

![Figure 5](image_url)

**Figure 5.** Effects of the antifungal treatments on the tissue burden. Each mouse was intravenously infected with *C. albicans* SC5314 $5 \times 10^6$. Saline, FCZ (fluconazole, 0.3 mg/kg), compound 6c (0.3 mg/kg and 1.0 mg/kg) were administered orally once daily for 3 days after infection. Kidneys of mice were harvested on day 4 and colony-forming units were measured. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, determined by ANOVA.

### 2.6. In Vivo Potency

Considering the good evaluation of the fungal burden of compound 6c, an in vivo potency study was evaluated in disseminated *C. albicans* SC5314 models. As summarized in Figure 6, interestingly, compound 6c showed potent in vivo antifungal activity. The survival rate of compound 6c exhibited a dose-dependent manner. At the dose of 0.5 mg/kg, 20% of ICR mice survived at the end of the test, which was moderate to that of the fluconazole group (0.5 mg/kg). Meanwhile, it could effectively protect mice from fungal infection at the dose of 2.0 mg/kg ($p < 0.001$). This result indicated that it possessed potent activity, which could effectively protect mice from *C. albicans* infection.

![Figure 6](image_url)

**Figure 6.** In vivo efficacy of compound 6c (0.5, 1.0 and 2.0 mg/kg) and FCZ (fluconazole, 0.5 mg/kg) in a systemic infection of an ICR mouse model ($n = 10$) with *C. albicans* SC5314. ** $p < 0.01$, *** $p < 0.001$, determined by ANOVA.

### 2.7. Molecular Docking

In order to investigate whether the target compounds could have a well binding with CYP51, a molecular docking study was performed. All compounds were docked into
the active site and scored using the Surfex-Dock program in the SYBYL-X 2.0 software (Table S2). The published crystal structures of C. albicans CYP51 (PDB ID: 5TZ1) served as a useful template for generating binding modes [27]. The most active compound, 6c, will be shown in Figure 7 as a representative. As shown in Figure 7, the iron in the heme group was coordinated by triazole moiety, meanwhile, the 2,4-difluorophenyl could be placed into the hydrophobic pocket formed by Tyr-132, Phe-126, Met-306 and Phe-145. As surmised, the rigid linear phenylethynyl moiety was designed as a bar to anchor the side chain into the hydrophobic channel. The hydroxyl group in compound 6c formed a hydrogen-bonding interaction with Tyr-132 (2.4 Å). The long alkynyl side chain extended into a hydrophobic channel formed by the surrounding residues Tyr-118, Phe-228, Leu-376 and Phe-380 to form van der Waals and hydrophobic interactions. It is worth noting that the π–π stacking interaction was found between 2,4-difluorophenyl and Phe-126 and between pyrazole and Tyr-118, respectively. This may further improve the affinity and specificity of the inhibitors. Due to flexibility, the benzyloxy produced a bend in the channel. Especially, the strong hydrogen bonds existing between terminal nitrile and Lys-90 (1.9 Å) and between benzyloxy and His-377 (3.0 Å) may be a significant factor in the effective antifungal activity.

![Image](image_url)

**Figure 7.** The binding mode of compound 6c in the active site of CYP51. Compound 6c, heme group, residues are shown in brown, yellow or green sticks, respectively. Hydrogen-bonding interaction was shown in red and π–π stacking interaction was shown in forest green. Image depicting the proposed binding mode was generated using PyMOL.

3. Materials and Methods

3.1. Chemistry

$^1$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were reported in DMSO-$d_6$ unless otherwise indicated, by a Bruker AC-300P spectrometer. Tetramethylsilane (TMS) was considered as the internal standard. Chemical shifts ($\delta$ values) and coupling constants (J values) are given in ppm and Hz, respectively. HPLC purity was determined by Agilent Technologies 6120 Quadrupole LC-MS. HRMS analyses were produced on an Agilent Technologies 6538 UHD Accurate-Mass Q-TOF LC/MS. Silica gel plates GF254 (Yantai Huanghai Chemical, Yantai, China) were applied to thin-layer chromatography (TLC) analysis. All the solvents and reagents were purchased from commercial vendors and were used as received or dried prior to use as needed.
3.1.1. Procedure for the Synthesis of 2-(2,4-Difluorophenyl)-1-(4-iodo-1H-pyrazol-1-yl)-3-(1H-1,2,4-triazol-1-yl)propan-2-ol (2)

To a solution of compound 1 (50 mmol) and 4-Iodo-1H-pyrazole (50 mmol) in DMF (150 mL) was added K$_2$CO$_3$ (100 mmol). The mixture was stirred continuously for 4 h and heated at 80 °C. The reaction was monitored by TLC. After the reaction was finished, the mixture was cooled to room temperature, poured into ice water, then stirred for 1 h. The product solid was filtered and then dried at 50 °C to obtain compound 2 (15.5 g, yellow solid, yield 72%).

3.1.2. Procedure for the Synthesis of Methyl 4-((1-(2-(2,4-Difluorophenyl)-2-hydroxy-3-(1H-1,2,4-triazol-1-yl)propyl)-1H-pyrazol-4-yl)ethynyl)benzoate (3)

Under nitrogen atmosphere, compound 2 (40 mmol) and Methyl 4-ethynylbenzoate (40 mmol) were dissolved in NMP (140 mL). To this solution was added CuI (20 mmol%), Pd(PPh$_3$)$_2$Cl$_2$ (5 mmol%) and DIEA (200 mmol). The mixture was degassed under a nitrogen atmosphere prior to heating at 60 °C for 6 h. The reaction was monitored by TLC. After the reaction was finished, the mixture was poured into ice water, and then extracted with ethyl acetate (3 × 250 mL). The organic phases were combined, washed with saturated aqueous sodium chloride solution (2 × 300 mL), dried over anhydrous Na$_2$SO$_4$ and evaporated under reduced pressure. The crude products were purified by chromatography on silica gel (PE:EA = 20:1~5:1) to obtain compound 3 (14.57 g, yellow solid, yield 78%).

3.1.3. Procedure for the Synthesis of 4-((1-(2-(2,4-Difluorophenyl)-2-hydroxy-3-(1H-1,2,4-triazol-1-yl)propyl)-1H-pyrazol-4-yl)ethynyl)benzoic acid (4)

To a solution of compound 3 (31 mmol) in a mixed solution of THF (100 mL) and H$_2$O (100 mL), were added LiOH (200 mmol). The mixture was stirred continuously for 6 h and heated at 50 °C. The reaction was monitored by TLC. After the reaction was finished, THF was evaporated under reduced pressure. Aqueous hydrochloric acid (5 mol/L) was dropped into the remaining liquid to adjust pH to 3–4. After stirring for 1 h, the precipitated solid was filtered and dried to obtain compound 4 (12.1 g, yellow solid, yield 67%).

3.1.4. General Procedure for the Synthesis of Target Compound (5a–5v)

To a solution of compound 4 (1.0 mmol) and amines (1.0 mmol) in DMF (5 mL), were added DIEA (2.0 mmol) and PyBOP (1.1 mmol). The mixture was stirred continuously for 4–8 h and heated at 50 °C. The reaction was monitored by TLC. After the reaction was finished, the mixture was poured into ice water, and then extracted with ethyl acetate (3 × 20 mL). The organic phases were combined, washed with saturated aqueous sodium chloride solution (2 × 30 mL), dried over anhydrous Na$_2$SO$_4$ and evaporated under reduced pressure. The crude products were purified by reverse phase (mobile phase was A: H$_2$O, B: acetonitrile, gradient elution, 0–20 min, 30–90% B) and then lyophilized to obtain the target compounds.

3.1.5. General Procedure for the Synthesis of Target Compound (6a–6e)

Under nitrogen atmosphere, compound 2 (1.0 mmol) and alkynes (1.0 mmol) were dissolved in NMP (10 mL). To this solution was added CuI (20 mmol%), Pd(PPh$_3$)$_2$Cl$_2$ (5 mmol%) and DIEA (5 mmol). The mixture was degassed under nitrogen prior to heating at 60 °C for 6 h. The reaction was monitored by TLC. After the reaction was finished, the mixture was poured into ice water, and then extracted with ethyl acetate (3 × 20 mL). The organic phases were combined, washed with saturated aqueous sodium chloride solution (2 × 30 mL), dried over anhydrous Na$_2$SO$_4$ and evaporated under reduced pressure. The crude products were purified by chromatography on silica gel (PE:EA = 10:1~5:1).
3.2. In Vitro Antifungal Activities Assay

In vitro antifungal activity was measured according to the protocols from the National Committee for Clinical Laboratory Standards (NCCLS) [20,21]. The serial dilution method in a 96-well microtest plate was used to measure the minimum inhibitory concentration (MIC) of the target compounds. For *C. albicans* and *C. neoformans*, the initial concentration of fungal suspension in the RPMI 1640 medium was $10^3$ CFU/mL. For *A. fumigatus*, the initial concentration of fungal suspension in the RPMI 1640 medium was $5 \times 10^3$ CFU/mL. Targeted compounds were dissolved in DMSO and serially diluted in a growth medium. The final concentrations of each well ranged from 0.125 to 64 µg/mL. The yeasts were incubated at 35 °C and the filamentous fungi were incubated at 37 °C. After 48 h incubation, the optical density (OD630 nm) in each well was measured by spectrophotometer. The MICs were defined as the minimum concentration of drugs to inhibit ≥80% growth of fungal cells compared to that of a drug-free control at 30 °C at 48 h incubation.

3.3. Hyphal Formation Assay

Firstly, *C. albicans* SC5314 cells were harvested by centrifugation (3000 rpm, 5 min) and washed with PBS three times. The *C. albicans* suspension was then adjusted to $1 \times 10^6$ cells/mL with Spider medium. The *C. albicans* suspension was divided into every well in 6-well plates with different concentrations of compounds 5k/6c added. Then the 6-well plates were incubated at 37 °C. After 3 h incubation, the cellular morphology was photographed.

3.4. Fungal Burden Evaluation

In the fungal burden study, antifungal treatments began 1 day after fungal inoculation and continued for 3 days. The day after therapy had stopped, mice were humanely euthanized and the kidneys were collected for the quantitative determination of the tissue fungal burden. After the weights of kidneys were determined, tissues were homogenized in 1 mL PBS. Homogenates were serially diluted in 10-fold steps and aliquots (100 µL) of homogenate were plated on Sabouraud dextrose agar (SDA) plates. The plates were incubated at 30 °C for 72 h, and the numbers of CFU were counted. The fungal burdens were indicated as Log_{10}CFU/g.

3.5. In Vivo Antifungal Potency

All animal experiments were done according to institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of Second Military Medical University. Female ICR mice (weighing between 18 to 22 g) were housed and fed to acclimatize for 3 days. All mice were operated on intraperitoneal injection with 0.4 mL of cyclophosphamide (100 mg/kg, 3 days, qd). Then, mice were administered orally with 0.2 mL of a suspension containing $5 \times 10^6$ CFU/mL of *C. albicans* SC5314. In this model, compound 6c and FCZ (as a suspension in 0.5% carboxymethylcellulose in distilled water) were administered orally 2 h after infection fungi (7 days, qd). The control group consisted of mice treated with NS. Mice were monitored and recorded for survival conditions once daily for a total period of 20 days post-infection. At the end of the observation period, the surviving mice were humanely sacrificed.

3.6. Statistics

Survival was plotted by Kaplan–Meier analysis, and a log-rank test was used to assess for significant differences in median survival time. Differences in fungal burdens between groups were assessed for significance by analysis of variance (ANOVA) with Tukey’s posttest for multiple comparisons. A $p$-value of <0.05 was considered statistically significant, a $p$-value of <0.01 was considered statistically highly significant, and a $p$-value of <0.001 was considered statistically extremely significant.
3.7. Computational Methodology

3.7.1. ADME/T Prediction

All target compounds were predicted using DS-ADMET and DS-TOPKAT modules. The operation process was performed as follows: the target compound files were imported into the program, and “ADMET descriptors” and “Toxicity Prediction” modules were selected, respectively. The prediction items (aqueous solubility, blood brain barrier penetration, intestinal absorption, plasma protein binding, FDA rodent carcinogenicity, Ames mutagenicity, Skin_Irritancy, Skin_sensitization) were set as research objects in the parameter browser, respectively. Finally, the program was run to obtain the corresponding result.

3.7.2. Molecular Docking

The structure of compound 6c was drawn by SYBYL-X 2.0 software, optimized using the standard Tripos molecular mechanics force field with a Gasteiger–Hückel charge, and other parameters were set as default values. Import the PDB (5TZ1) file into SYBYL-X 2.0, first remove water molecules and the ligand, then analyze the protein structure, repair the terminal side chains, and add hydrogen to amino acid residues. The protein structure was optimized using the standard Tripos molecular mechanics force field with the AMBER7 FF99 charge. Next, the active pocket was generated, and molecule 6c was docked into the active pocket through the “Surface Dock” module. Finally, this module will score the interaction between 6c and CYP51 protein and retain the 20 highest scoring ligand-protein complex structures for analysis. The docking results were plotted by Pymol software.

4. Conclusions

In summary, a series of novel triazole derivatives containing alkynyl side chains have been designed, synthesized, and their antifungal activities were evaluated for the three most common human pathogenic fungi. Most of the target compounds have strong antifungal activities against the tested fungi, including fluconazole-resistant strains, and especially, compound 6c exhibited potent in vitro antifungal activity against the three tested fungi strains and in vivo efficacy in the mice model of disseminated C. albicans infection. It could effectively protect mice models from C. albicans infection at doses of 0.5, 1.0, and 2.0 mg/kg. A molecular docking study also demonstrated that the long alkynyl side chain could form closely the van der Waals and hydrophobic interactions with CYP51. The strong hydrogen bond existing between 4-cyanobenzyloxy and residues played an important role in the binding affinity and antifungal activity. Further pharmacokinetic evaluation and structure optimization on compound 6c is under investigation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27113370/s1. Table S1: In silico ADME/T prediction of target compounds with fluconazole; Table S2: Molecular docking results.

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