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Targeting *Candida albicans* Biofilms: Antibiofilm Efficacy of Hemibastadin Alkaloids Analogues

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Abstract

Antibiofilm activity of four hemibastadin analogues was evaluated against a clinical *Candida albicans* strain. Three of four analogues demonstrated significant antibiofilm effects, with EC_{50} values of 34.4 ± 9.6 μM for the most efficient. This most active compounds notably reduced both the biovolume and thickness of the biofilm. Importantly, antifungal susceptibility testing confirmed that the observed effects were specific to biofilm inhibition and not due to general antifungal activity. These results, comparable to those obtained with biofilms of marine Gram-negative bacteria, highlight the potential of hemibastadin analogues as promising lead compounds for the development of broad-spectrum antibiofilm including *Candida albicans* without exerting selective pressure for antimicrobial resistance.

Keywords

Hemibastadin, 1,2,3-Triazole, *Candida albicans*, Biofilm, Antibiofilm

1. Introduction

Biofilms represent the primary mode of life and development for bacteria [1]. The adoption of this lifestyle by bacteria results in the persistence of microorganisms and a greater tolerance to anti-infective agents [2]. However, this mode of life is

not exclusive to bacteria; pathogenic fungi can also adopt it. This is the case for *Candida albicans* [3], a yeast that can cause cutaneous, mucocutaneous, and even deep, severe, or potentially fatal infections [4] [5]. It is important to note that biofilm formation is a significant virulence factor. Indeed, as with bacteria, the formation of a biofilm leads to increased tolerance to antifungal agents and to the immune system [6] [7]. The development of biofilms on both living tissues and medical devices is thus responsible for chronic and/or recurrent fungal infections, as well as nosocomial infections. There is therefore a need for strategies to combat these fungal biofilms.

One approach to control biofilms is the use of strict antibiofilm molecules, particularly those inspired by natural products [8] [9]. Marine organisms (such as sponges and corals), which are constantly exposed to microbial colonization, are a well-known source of such molecules [10]. This is notably the case for bromotyrosins in general, and specifically for hemibastadins [11] [12]. Previous studies have highlighted the interest of hemibastadin analogues in which the oxime group is replaced by a 1,2,3-triazole 1,4-disubstituted core [13]. This core, a bioisostere of many chemical functions (including the oxime group), also has the advantage of being easily accessible via click chemistry reactions, notably by cycloaddition using water combined with various co-solvents [14] [15]. These hemibastadine analogues with a 1,2,3-triazole 1,4-disubstituted core have demonstrated their potential as inhibitors of biofilm formation by marine Gram-negative bacteria, even in multispecies biofilms [13] [16]. The present study aims to evaluate the ability of some of these analogues (**Figure 1**) to also inhibit biofilm formation by *Candida albicans*.

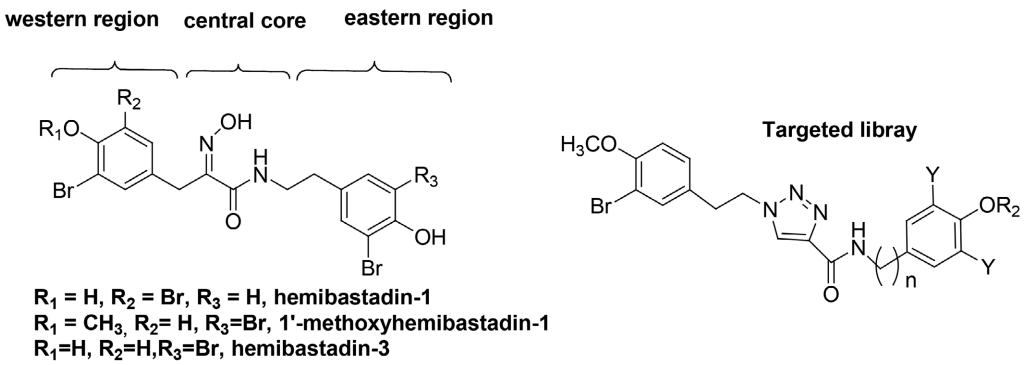


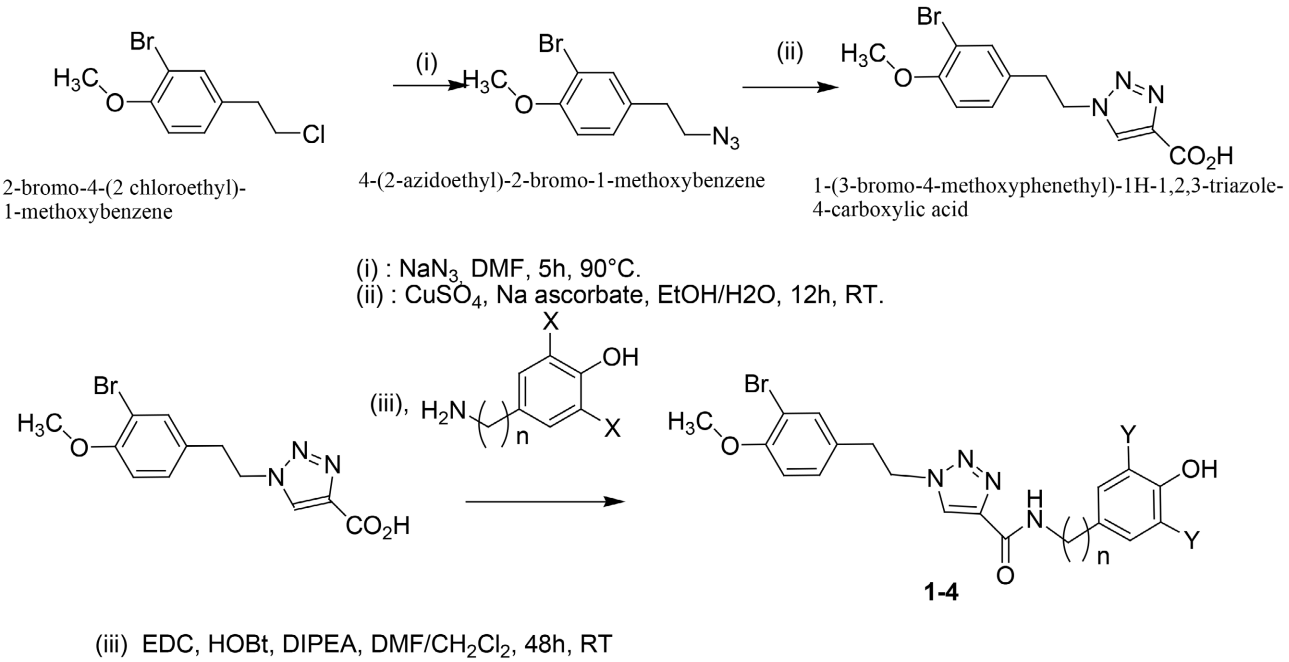
Figure 1. General structure of hemibastadins and evaluated analogue compounds.

2. Experimental Section

2.1. Chemistry

The hemibastadin analogues evaluated in this study were obtained using previously described methods [13] [16]. In practical terms, these compounds were prepared in excellent yield in two steps (**Scheme 1**). 1-(2-Chloroethyl)-4-methoxybenzene was subjected to N-bromosuccinimide to afford 2-bromo-4-(2-chloroethyl)-1-methoxybenzene. Treatment of this halogeno compound by sodium az-

ide in dimethylformamide afforded the 4-(2-azidoethyl)-2-bromo-1-methoxybenzene. Synthesis of the 1-(3-bromo-4-methoxyphenethyl)-1H-1,2,3-triazole-4-carboxylic acid was then achieved by performing the copper(I)-catalyzed 1,3-dipolar cycloaddition of the organic azides with propargylic acid resulting in the formation of 1,2,3-triazoles. In practice, propargylic acid was added at room temperature to a solution of azide, $CuSO_4$/sodium ascorbate in water/ethanol mixture (50/50) and the reaction time was optimized at 12 hours at room temperature. Access to the different hemibastadin analogues was then allowed by a peptide coupling step using EDC/HOBt methodology (22). All amides were obtained in good yields.



Scheme 1. Synthesis of hemibastadin analogues.

2.2. Biology

Fungal strains

Strain selection was performed using three isolates provided by the Institut Pasteur de Côte d'Ivoire (IPCI). These were clinical strains collected from vaginal samples during a 2017 survey among female sex workers. The patients were HIV-seronegative and presented with clinical signs of vaginal candidiasis. Strains from this anatomical site were selected because they are frequently implicated in chronic and/or recurrent infections associated with biofilm formation. Additionally, these strains were chosen for their susceptibility to commonly used antifungal agents, particularly azole antifungals.

Biofilm formation

The methods employed in this study were inspired by those of Pierce and Ramage [17]-[19]. Starting from a colony of a *C. albicans* strain cultured on Yeast Peptone Dextrose (YPD; Sigma-Aldrich, St Louis, USA) agar, 10 ml of liquid YPD were inoculated under sterile conditions and incubated overnight at 30˚C (12 - 14

hours). The resulting suspension was centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged again to remove any residual YPD. After washing, a cell suspension at a density of 10^6 cells/ml in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Gibco, GB) was prepared and distributed into flat-bottomed, transparent 96-well microplates. Following 48 hours of incubation at 37˚C without agitation, the wells were rinsed three times with PBS. Biofilm detection was performed using crystal violet at a concentration of 1%. The dye was added to the wells, and the plate was incubated for 30 minutes. The plates were then washed again, and the dye fixed by the fungus was resolubilized by adding acetic acid at 30 volumes for 15 minutes. Optical density was measured at 590 nm using an ELISA plate reader, allowing determination of the optical density (OD).

Antibiofilm Activity

Biofilm inhibition tests were conducted by exposing the selected strain to a solution of the compound under evaluation. Compound concentrations ranged from 5 to 200 µM. Results were expressed as the percentage of adhesion at each selected concentration, calculated using the following formula. Fluconazole was used as the reference substance. For data analysis, the percentage of adhesion for each well was calculated as follows:

$$\% \text{ adhesion} = (ODT - ODPdt)/(ODTB - ODBlk) \times 100$$

where:

- ODT = Mean optical density (OD) of triplicates for each tested concentration (compound + yeast).
- ODPdt = OD of the compound alone in the presence of the fluorochrome, without yeast.
- ODTB = Mean OD of six replicates for the bacterial adhesion control (yeast).
- ODBlk = Mean OD of blanks (RPMI1640 + fluorochrome).

Based on the adhesion percentages, the EC_{50} values for each compound were calculated using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, Calif.).

Visualisation of Biofilms by Confocal Microscopy

Biofilm visualisation was performed using confocal microscopy, following staining with SYTO® RED 9, both in the presence and absence of the most active compound. The culture was carried out under the same conditions as previously described. However, for SYTO RED 9 staining, 24-well microplates were used, into which coverslips previously sterilised by ultraviolet light were immersed. Yeast suspensions were prepared by incubating them with 200 µM of the compound, compared to a suspension without the molecule. After washing with PBS, the coverslips were mounted on microscope slides using ProLong Diamond Antifade. The preparations were then examined under a confocal microscope at 528 nm.

Data Extraction

Biofilm biovolume and average thickness data were extracted using the COMSTAT plugin in ImageJ [20] [21].

Evaluation of Antifungal Activity

To determine whether the observed antibiofilm activity was specific and not due to an antifungal effect, a sensitivity test was performed with the most active compound. The method used was the standard CLSI protocol, specifically the microdilution method in liquid medium [22]. This approach allows for the determination of the minimum inhibitory concentration (MIC) of the molecules. The procedure involved culturing yeast for 24 hours on a YPD agar plate. Serial dilutions of both the test molecules and the yeast were prepared to carry out the assay. The molecules to be tested, as well as fluconazole as a reference, were weighed to prepare stock solutions according to the following formula:

Mass (mg) = volume (ml) × concentration (µg/ml)/potency (µg/mg)

The specific activity of fluconazole, according to the technical data sheet, is 99.64%, while that of the test molecule was considered to be 100%. From a stock solution of the molecule, the concentrations tested ranged from 0.125 to 64 µg/mL, diluted in RPMI 1640. The yeast stock solution was prepared at 10^6 cells/ml. This inoculum was diluted 1:1000 in RPMI. Then, 100 µl were distributed into each well of the plate except for column 11 (sterility control). The plates were incubated at 37˚C for 72 hours.

3. Results and Discussion

3.1. Chemistry

The characterization of the chemical structures of the various synthesized derivatives has been described in previous articles [13] [16].

1-(3-bromo-4-methoxyphenethyl)-N-(4-hydroxyphenyl)-1H-1,2,3-triazole-4-carboxamide (1).

1H NMR (400 MHz, DMSO) δ 10.17 (s, 1H), 9.26 (s, 1H), 8.62 (s, 1H), 7.57 (d, J = 8.9 Hz, 1H), 7.48 (s, 1H), 7.14 (dd, J = 8.4, 2.0 Hz, 1H), 7.01 (d, J = 8.5 Hz, 1H), 6.72 (d, J = 8.9 Hz, 2H), 4.68 (t, J = 7.1 Hz, 2H), 3.81 (s, 3H), 3.16 (t, J = 7.1 Hz, 2H). 13C NMR (101 MHz, DMSO) δ 158.3, 154.5, 154.2, 143.4, 133.5, 131.5, 130.5, 129.7 (2C), 127.4, 122.6, 115.4 (2C), 112.9, 110.9, 56.6, 51.2, 34.1.

1-(3-bromo-4-methoxyphenethyl)-N-(4-hydroxybenzyl)-1H-1.2,3-triazole-4-carboxamide (2).

1H NMR (400 MHz, DMSO): δ 9.27 (s, 1H, OH), 8.91 (t, J = 6.3 Hz, 1H, NHCO), 8.50 (s, 1H), 7.45 (d, J = 2.1 Hz, 1H), 7.13 (d, J = 2.1 Hz, 1H), 7.10 (d, J = 8.3 Hz, 2H), 7.00 (d, J = 8.5, 1H), 6.68 (d, J = 8.4 Hz, 2H), 4.64 (t, J = 7.1 Hz, 2H), 4.30 (d, J = 6.2 Hz, 2H), 3.79 (s, 3H), 3.13 (t, J = 7.1 Hz, 2H); 13C NMR (101 MHz, DMSO) δ 159.9, 156.6, 154.5, 143.1, 133.5, 131.7, 130.3, 129.7, 129.2 (2C), 126.8, 115.4 (2C), 112.9, 110.9, 56.6, 51.1, 41.8, 34.5; (ESI, m/z) 383.20 $[M + H]^+$, 431.03.

1-(3-bromo-4-methoxyphenethyl)-N-(3.5-dibromo-4-hydroxybenzyl)-1H-1.2,3-triazole-4-carboxamide (3).

1H NMR (400 MHz, DMSO): δ 9.87 (s, 1H, OH), 9.11 (t, J = 6.2 Hz, 1H, NHCO), 8.50 (s, 1H), 7.47 (s, 1H), 7.41 (s, 1H), 7.13 (d, J = 8.4 Hz, 2H), 6.99 (d, J = 8.5 Hz, 1H), 4.64 (t, J = 7.1 Hz, 2H), 4.30 (d, J = 6.2 Hz, 2H), 3.79 (s, 3H), 3.13

(t, J = 7.1 Hz, 4H); 13C NMR (101 MHz, DMSO) δ 160.3, 154.6, 150.3, 142.9, 134.2, 133.5, 131.8, 131.6, 129.8(2C), 127.1, 113.0 (2C), 112.3, 110.9, 56.6, 51.3, 40.5, 34.6; (ESI, m/z) 383.20 $[M + H]^+$, 385.17.

1-(3-bromo-4-methoxyphenethyl)-N-(4-hydroxyphenethyl)-1H-1.2,3-triazole-4-carboxamide (4).

1H NMR (400 MHz, MeOD) δ8.09 (s, 1H), 7.30 (d, J = 2.0 Hz, 1H), 7.05 (m, 2H), 7.02 (dd, J = 8.4, 2.0 Hz, 1H), 6.89 (d, J = 8.4 Hz, 1H), 6.71 (m, 2H), 4.63 (t, J = 7.0 Hz, 2H), 3.81 (s, 3H), 3.55 (t, J = 7.3 Hz, 2H), 3.14 (t, J = 7.0 Hz, 2H), 2.80 (t, J = 7.3 Hz, 2H).13C NMR (101 MHz, MeOD) δ 162.4, 157.0, 156.6, 143.9, 134.5, 132.2, 131.2, 130.7 (2C), 130.1, 127.3, 116.4 (2C), 113.7, 112.8, 56.9, 52.7, 42.0, 36.0, 35.8.

3.2. Selection of the Model Strain

In order to select a strain to serve as a model for antibiofilm assays, the three strains of *Candida albicans* were subjected to established biofilm formation protocols and compared using optical density (O.D.) as the primary criterion. The results of this comparative analysis are presented in Figure 2.

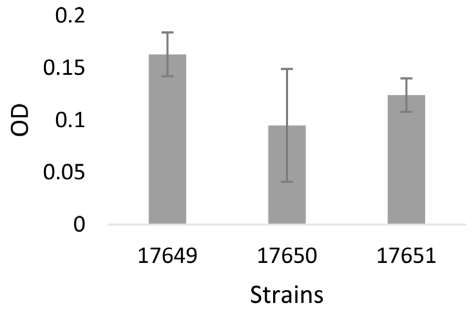


Figure 2. Optical densities of biofilms formed by the three candida strains used to validate the method.

Differences in the thickness of the biofilms formed were observed. It has previously been demonstrated that biofilm thickness varies significantly according to parameters such as HIV serological status or the anatomical origin of the strain (vaginal or oral) [23].

When observed under phase-contrast optical microscopy as well as confocal microscopy (Figure 3), the biofilm formed by this strain exhibits a high cell density and the presence of cells in the form of hyphae, pseudohyphae, and yeast, which are characteristic features of a Candida albicans biofilm.

Under the selected experimental conditions, this strain forms a biofilm with an average biovolume of 0.14 ± 0.04 $\mu m^3/\mu m^2$ and a biomass exhibiting a mean thickness of 7.15 ± 0.84 µm. These properties enable its use for the evaluation of antibiofilm activity.

3.3. Antibiofilm Activity

The selected compounds have been evaluated against the biofilm of Candida albicans 17649. The results are reported in Table 1.

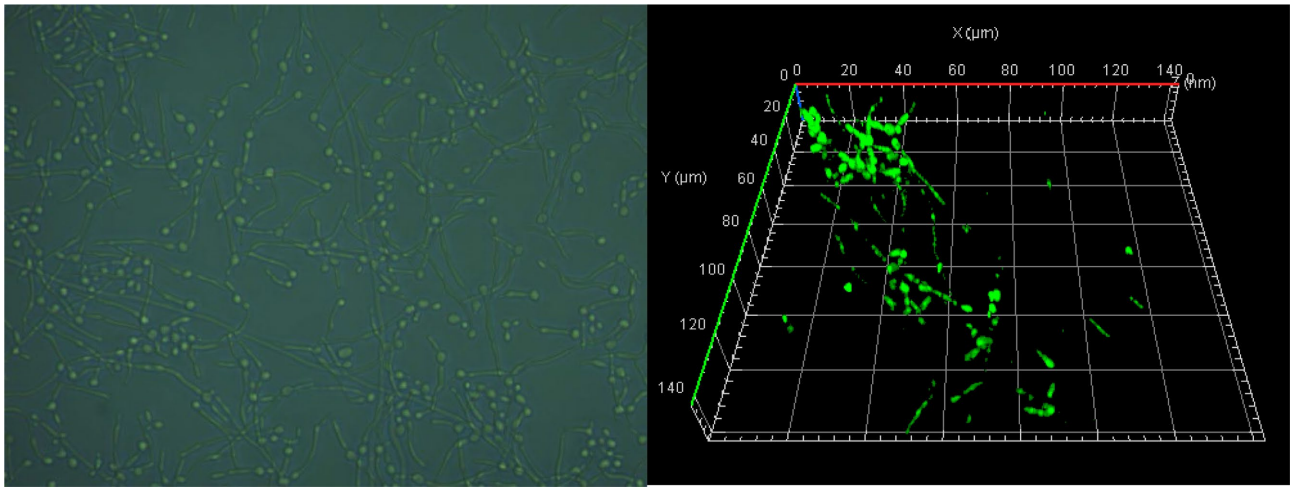
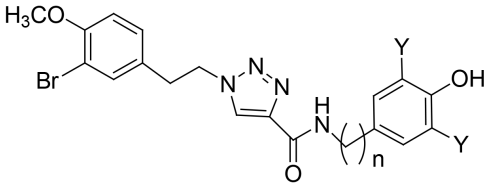


Figure 3. Strain 17649 observed under phase-contrast optical microscopy (left) and confocal microscopy (right).

Table 1. Chemical structure and biological activity of hemibastadin analogues against the biofilm of Candida albicans, IPCI 17649 strain. Results are expressed as effective concentration to inhibit 50% of biofilm formation (EC50) in micromoles/L (µM). Data represent means ± standard deviations values from three independent experiments.



Compound	n	Y	EC50 (µM)
1	0	H	40.9 ± 10.3
2	1	H	34.4 ± 9.6
3	1	Br	>200
4	2	H	71 ± 19.3

These results show that three of the four evaluated molecules exhibit antibiofilm activity; only compound 4 lacks this activity. These findings are consistent with those previously obtained for the same molecules against marine bacteria [13] [16]. Indeed, compound 3 did not inhibit biofilm formation by marine bacteria. The SAR trends parallel those observed in marine bacteria: maximal activity is achieved with a non-brominated single-carbon eastern region. Hyperbromination, typically beneficial, becomes detrimental on this scaffold and abolishes activity, likely due to steric or electronic disruption. In marine bacteria, replacing the western-region methoxyl group with a phenolic hydroxyl restores the positive effect of hyperbromination [24], a modification that should also be evaluated against *C. albicans*. Given this reversal and the persistence of a single-carbon, non-brominated east region as the optimal motif, QSAR modelling and molecular docking studies will be essential to elucidate the mechanistic basis of this unfavorable hyperbromination effect**.** The inhibitory effect of this compound is shown by confocal microscopy (**Figure 4**).

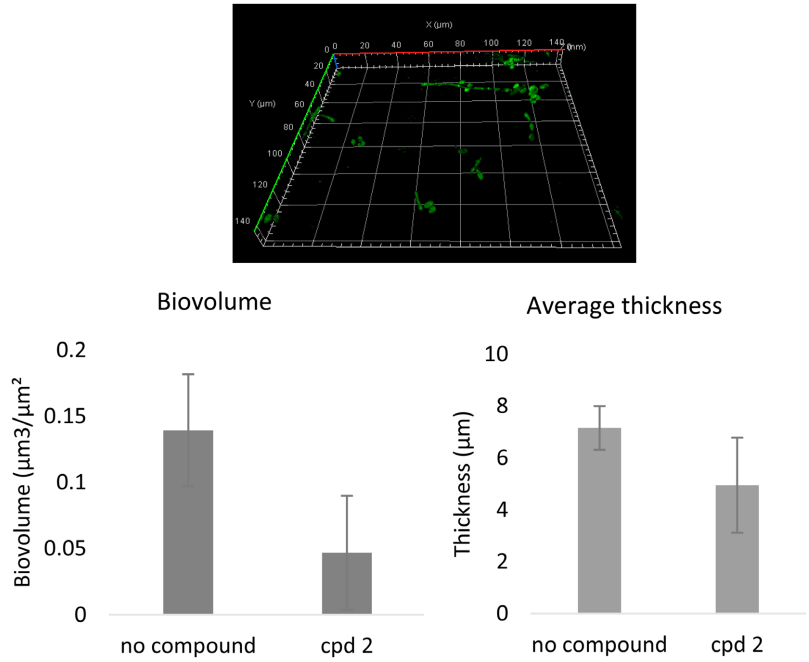


Figure 4. Confocal laser scanning microscopy images for the Candida albicans 17649 strain with compound 2 (above). Effect on the compound on the biovolume (down left) and the average thickness (down right).

Determination of the minimum inhibitory concentration (MIC) according to CLSI standards showed a value greater than 64 $\mu g \cdot mL^{-1}$, compared with fluconazole, which has an MIC of 4.06 $\mu g \cdot mL^{-1}$. Therefore, this compound does not possess intrinsic antifungal activity. Thus, as observed with marine bacteria, the effect on *C. albicans* biofilm appears to be a strictly antibiofilm activity, without antifungal properties. In *C. albicans* biofilms, the hemibastadin analogues may also modulate the early adhesion phase. Although their precise mode of action in fungal systems remains to be fully elucidated, this hypothesis is consistent with observations reported for marine bacteria, where these molecules interfere with initial surface attachment and the establishment of nascent biofilms. Further investigations will be required to determine whether similar molecular targets or signaling pathways are involved in *C. albicans*, and to clarify how these compounds influence adhesion dynamics, morphogenesis, and early matrix deposition.

4. Conclusion

This study, aimed at evaluating the activity of 1,2,3-triazole 1,4-disubstituted hemibastadine analogues on *Candida albicans* biofilms, complements previous work that demonstrated the activity of these same molecules on marine bacterial biofilms. These molecules exhibit antibiofilm activity against *Candida albicans* biofilm that appears comparable to that observed on marine bacteria, without exhibiting antifungal activity. These findings further support their potential as lead compounds for the development of future broad-spectrum antibiofilm agents, without exerting antimicrobial selection pressure. Nevertheless, additional studies

will be required to substantiate and broaden these observations, particularly through the assessment of a more extensive panel of molecules previously investigated in bacterial models, their evaluation across a wider range of fungal strains, and the implementation of QSAR analyses to elucidate the structural determinants underlying their antibiofilm activity.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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