Synthesis of a New Peptide–Coumarin Conjugate: A Potential Agent against Cryptococcosis

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S Supporting Information

ABSTRACT: Antimicrobial peptides (AMPs) are currently being investigated as potential sources of novel therapeutics against an increasing number of microorganisms resistant to conventional antibiotics. The conjugation of an AMP to other bioactive compounds is an interesting approach for the development of new derivatives with increased antimicrobial efficiency and broader spectra of action. In this work, the synthesis of a new peptide–coumarin conjugate via copper(I)-catalyzed azide–alkyne cycloaddition is described. The conjugate was assayed for *in vitro* cytotoxicity and displayed antifungal activity against *Cryptococcus gattii* and *Cryptococcus neoformans*. Additionally, the conjugate exhibited increased antifungal efficacy when compared with the individual peptide, coumarin, or triazole moieties. Treatment of *C. gattii* with the peptide–coumarin conjugate enhanced the production of reactive oxygen species, suggesting that the oxidative burst plays an important role in the mechanism of action of the conjugate.

KEYWORDS: Antimicrobial peptide, copper(I)-catalyzed azide–alkyne cycloaddition, coumarin, cryptococcosis, reactive oxygen species

Antimicrobial peptides (AMPs) are small molecular weight proteins with antimicrobial activity against bacteria, viruses, and fungi.¹ AMPs are currently being investigated as potential sources of novel therapeutic antimicrobial agents because of their broad-spectrum activity and low susceptibility for developing resistance.²

Ubiquitin (UBI) is an antimicrobial peptide, which was first isolated from murine macrophages and then found at low concentrations (as a first line of defense) inside human airway epithelial cells, activated macrophages, and in human colon mucosa. UBI is a cationic peptide consisting of 59 amino acid residues. It is not expected to be immunogenic to humans since it is of human origin.³ UBI 1–59 and some of its fragments have been shown to be microbicidal against a broad spectrum of pathogens. Of particular interest is the fragment UBI 31–38 (Figure 1), which is relatively small, easily synthesized, and exhibits antibacterial activity toward methicillin resistant *Staphylococcus aureus*.⁴

Peptides open up new perspectives in drug design by providing an entire range of highly selective and nontoxic pharmaceuticals. With growing applications of their synthesis and bioactivity, considerable attention has been focused on the research of peptide-based derivatives.⁵

Coumarins form an important class of benzopyrones, which are found in nature. Many coumarins and their derivatives possess antimicrobial, anti-inflammatory, and anticancer properties.⁶ Research on coumarin compounds as antifungal agents has shown that structural modification of the coumarin skeleton (i.e., the benzene ring, lactone ring, or both) results in

Figure 1. Structure of UBI 31–38.

derivatives, which possess potent antifungal activity when compared to clinically used antifungal drugs.⁷ Therefore, numerous efforts have focused on the development of coumarins as potential drugs.⁸

The copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction has previously been employed as a means of orthogonal modification of peptides.^{9,10} CuAAC results in the formation of 1,4-disubstituted 1,2,3-triazoles, which are bioisosteres of the amide bond. This reaction may be used for the synthesis of peptidomimetics with improved medicinal chemistry properties and for the attachment of peptide molecules to other biologically active compounds.¹¹

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Cryptococcus neoformans and *Cryptococcus gattii* are the pathogenic yeasts of cryptococcosis. Immunocompromised patients are more frequently infected with *C. neoformans*, while *C. gattii* has emerged as an important cause of infection in healthy individuals. This disease involves pulmonary and cutaneous sites, but the most severe manifestation occurs in the central nervous system, causing severe meningoencephalitis. A small number of antimycotic drugs are available to treat this disease, with polyenes and azoles being the most commonly employed against cryptococcosis.^{12,13} However, nephrotoxicity has been described as a chronic adverse effect of amphotericin B,¹⁴ and a large number of fluconazole-resistant strains of *C. gattii* and *C. neoformans* have been reported.^{15,16}

Reactive oxygen species (ROS) can damage DNA, RNA, proteins, and lipids, resulting in cell death when the level of ROS exceeds an organism's detoxification and repair capabilities.¹⁷ For instance, some bactericidal antibiotics have been reported to kill bacteria by stimulating the generation of ROS.¹⁸ However, several intracellular mechanisms of action could be involved, and it is yet to be clarified which of these are responsible.¹⁹

Given the current difficulties in treating cryptococcosis and, furthermore, the antimicrobial properties shown by UBI 31–38 and many coumarins, we proposed the synthesis of the novel peptide–coumarin conjugate **4**. The results of this work are reported herein.

The C-terminal amidated peptide UBI 31–38 (RAKRRM-QY) was synthesized by stepwise solid phase peptide synthesis using the Fmoc strategy²⁰ on a Rink amide resin. The alkyne-decorated peptidyl resin **1** was prepared by coupling 4-pentynoic acid to the peptide UBI 31–38 during the solid-phase synthesis. For the purpose of antimicrobial evaluation, compound **2** was obtained from cleavage of the alkyne-decorated peptidyl resin followed by HPLC purification.

The peptide–coumarin conjugate **4** was synthesized via CuAAC,²¹ as represented in Figure 2A. The alkyne-decorated peptidyl resin **1** was directly employed in the CuAAC to avoid undesirable reactions involving the side chains of amino acid residues. The 3-azido-7-diethylaminocoumarin **3** was synthesized as previously reported.²² The first attempt to obtain **4** was unsuccessful; mass spectral data of the product revealed the presence of undesirable copper, possibly forming complexes with amino acids through chelation (data not shown). To overcome this problem, an additional step of resin washing with a solution of 50% EDTA and 25% NH₄OH (1:1) was included in the workup procedure. After cleavage from the resin and purification by HPLC, the product was obtained with high purity, as illustrated by the mass spectrum shown in Figure 2B.

3-Azido-7-diethylaminocoumarin **3** was also conjugated to commercial 4-pentyn-1-ol via classic CuAAC²¹ reaction to yield the novel compound **6** (Figure 3). This coumarin–triazole hybrid was prepared in order to investigate the role of the triazole ring in the antimicrobial activity.

All synthesized compounds were characterized and screened for their antifungal activities against a set of strains of *C. gattii* and *C. neoformans*. The minimum inhibitory concentrations (MICs) of UBI 31–38, the alkyne-decorated peptide **2**, the 3-azido-7-diethylaminocoumarin **3**, the peptide–coumarin conjugate **4**, the coumarin–triazole **6**, and fluconazole (FCZ) were determined by the antifungal microdilution susceptibility standard test proposed by the Clinical and Laboratory Standards Institute M27-A3 method.²³ The results are shown in Table 1.

Figure 2. (A) Synthesis of the alkyne-modified peptide **2** and the peptide–coumarin conjugate **4**. Reagents and conditions: (a) 3.0 equiv of **3**, 0.3 equiv of copper(II) sulfate, 0.6 equiv of sodium ascorbate, 1 mL of DCM, 1 mL of water, rt, 48 h; (b) washing step with 50% EDTA and 25% NH₄OH (1:1); (c) cleavage with TFA/H₂O/TIS/EDT 94.0/2.5/2.5/1.0, 240 rpm, rt, 3 h. (B) MS spectrum of **4** after HPLC purification (m/z calculated for MH⁺: 1445.77).

Figure 3. Synthesis of the coumarin–triazole derivative **6**. Reagents and conditions: 1.0 equiv of **3**, 0.3 equiv of copper(II) sulfate, 0.6 equiv of sodium ascorbate, 1 mL of DCM, 1 mL of water, rt, overnight.

From the results, it is clear that the peptide–coumarin conjugate **4** exhibited moderate to excellent antifungal activities against the tested strains and gave comparable MIC values (0.04–0.18 $\mu\text{mol}\cdot\text{mL}^{-1}$) to the standard drug FCZ (MIC = 0.003–0.15 $\mu\text{mol}\cdot\text{mL}^{-1}$). In addition, the conjugate **4** efficiently inhibited the growth of a fluconazole-resistant strain of *C. gattii* (L27/01_F) at a concentration of 0.09 $\mu\text{mol}\cdot\text{mL}^{-1}$. UBI 31–38 and the derivatives **2**, **3**, and **6** showed low activities against the strains of *C. gattii* and *C. neoformans*, with MIC values of >0.23, >0.22, >0.99, and >0.75 $\mu\text{mol}\cdot\text{mL}^{-1}$, respectively.

Notably, the peptide–coumarin conjugate **4** exhibited better antifungal activities than its precursors **2** and **3**, as well as the peptide UBI 31–38, indicating that the association between the peptide and coumarin using a triazole linker is beneficial to antimicrobial efficacy. Some authors have also reported that a

Table 1. MIC Values ($\mu\text{mol}\cdot\text{mL}^{-1}$) for the Synthesized Compounds against Strains of *C. gattii* and *C. neoformans*

		MIC for the compounds					
Fungi		UBI 31–38	2	3	4	6	FCZ
<i>C. gattii</i> strain	ICB 181	>0.23	>0.22	>0.99	0.09	>0.75	0.05
	L135/03	>0.23	>0.22	>0.99	0.04	>0.75	0.05
	196L/03	>0.23	>0.22	>0.99	0.04	>0.75	0.10
	547/OTT	>0.23	>0.22	>0.99	0.04	>0.75	0.10
	ATCC 32608	>0.23	>0.22	>0.99	0.09	>0.75	0.05
	ATCC 24065	>0.23	>0.22	>0.99	0.18	>0.75	0.01
	1913ER	>0.23	>0.22	>0.99	0.18	>0.75	0.05
	L27/01	>0.23	>0.22	>0.99	0.04	>0.75	0.10
	L28/02	>0.23	>0.22	>0.99	0.09	>0.75	0.10
	LMM 818	>0.23	>0.22	>0.99	0.18	>0.75	0.10
	L24/01	>0.23	>0.22	>0.99	0.09	>0.75	0.05
	23/10993	>0.23	>0.22	>0.99	0.18	>0.75	0.05
	29/10893	>0.23	>0.22	>0.99	0.09	>0.75	0.03
	L27/01 _F ^a	>0.23	>0.22	>0.99	0.09	>0.75	0.42
<i>C. neoformans</i> strain	ATCC 96806	>0.23	>0.22	>0.99	0.09	>0.75	0.15
	ATCC 62066	>0.23	>0.22	>0.99	0.09	>0.75	0.03
	ATCC 24067	>0.23	>0.22	>0.99	0.09	>0.75	0.02
	ATCC 28957	>0.23	>0.22	>0.99	0.09	>0.75	0.003

^aL27/01_F is a fluconazole-resistant strain of *C. gattii*.²⁴

coumarin backbone in combination with various nitrogen-containing heterocycles (e.g., azetidine, thiazolidine, thiazole, etc.) significantly increased the antimicrobial efficacy and broadened the antimicrobial spectrum of activity of these compounds.⁸

Although triazole drugs are extensively used as antifungal agents in clinics,²⁵ the combination of the triazole moiety with other pharmacophores is being investigated in order to develop new types of antifungal drugs, which might exert new mechanisms of action and be effective against multidrug-resistant fungi.²⁶ In the current work, the importance of the peptide in maintaining the antifungal activity of the conjugate **4** has been demonstrated. The absence of UBI 31–38 from the triazole derivative (compound **6**) resulted in a dramatic decrease in inhibition of the growth of the tested strains (MIC >0.75 $\mu\text{mol}\cdot\text{mL}^{-1}$) compared to **4**, suggesting that the peptide, coumarin, and triazole moieties have a synergistic effect.

The cytotoxicity of the peptide–coumarin conjugate **4** was assessed in a noncancerous human cell line (lung fibroblast CCD-Lu ATCC CCL-210) using the methylthiazolotetrazolium (MTT) assay. Briefly, cells were exposed to the conjugate **4** at concentrations ranging from 0.02 to 0.21 $\mu\text{mol}\cdot\text{mL}^{-1}$, and cell survival was determined. The results demonstrated that **4** was nontoxic (cell viability > 95%) up to a concentration of 0.21 $\mu\text{g}\cdot\text{mL}^{-1}$, which is greater than the highest MIC value obtained for this compound, indicating a moderate to high selectivity index for anticryptococcal activity over cytotoxicity.

Since most of the AMPs are cationic and amphiphilic, the mode of action of these molecules is thought to involve the initial electrostatic interaction with a negatively charged surface of microorganisms, followed by insertion into the lipid bilayer by means of hydrophobic interactions. As a consequence, transient permeability of membranes and leakage of cellular constituents may occur, leading to cell lysis.¹ Preferential binding of AMPs to pathogens over mammalian cells may be explained by the segregation of lipids with negatively charged headgroups into the inner leaflet of the membranes in mammalian cells.²⁷ Additionally, some studies have demon-

strated that the presence of cholesterol in eukaryotic membranes reduces AMP binding and suppresses the disruption of lipid bilayer structure by AMPs.^{28,29}

AMPs with antifungal activities can bind to fungal-specific negatively charged constituents of the cell wall, such as mannoproteins or interact with specific receptors on the plasma membrane.³⁰ In the case of *C. gattii* and *C. neoformans*, which are known to produce a polysaccharide capsule,^{31,32} we hypothesized that a specific interaction of the AMP with this negatively charged component and/or cellular membrane may take place. Although membrane permeabilization by AMPs is an essential step in the killing of fungi, certain antifungal peptides exert their antimicrobial action through formation of reactive oxygen species.^{33–37} Oxidative burst also plays a crucial role in the antifungal activity of some drugs, such as itraconazole and amphotericin B against *C. gattii*.³⁸ On the basis of these reports, the production of endogenous ROS in the fungus *C. gattii* induced by the peptide–coumarin conjugate **4** and by the coumarin–triazole derivative **6** was investigated using fluorometric assays.³⁸ The measured fluorescence intensity was directly proportional to the accumulation of ROS.

ROS levels were measured in *C. gattii* cells from the strain ATCC 32608 (control group) and also in the peptide–conjugate **4** and the coumarin–triazole derivative **6**, in order to obtain baseline levels. Subsequently, the intracellular ROS production after treatment of *C. gattii* with the MIC values of the compounds **4** and **6** was also measured. The results are shown in Figure 4 and expressed in arbitrary units (AU) of fluorescence.

The treatment of *C. gattii* with the compounds **4** and **6** increased the ROS levels compared with the control group or individual compounds. The highest ROS level was reached for the peptide–coumarin conjugate **4**, which exhibited the strongest antifungal activity among the synthesized compounds. Although the compound **4** has been shown to be able to produce ROS alone, maybe as a result of an autocatalytic process, the amount of ROS measured in this case was significantly lower than that observed in compound **4**-treated cells. These results suggest that, for the compounds **4** and **6**, a

Figure 4. Amounts of ROS detected in *C. gattii* cells (ATCC 32608), compound 4, *C. gattii* cells treated with 4 (4-treat), compound 6, and *C. gattii* cells treated with 6 (6-treat). “a” indicates a statistically significant difference between the group and *C. gattii*. “b” indicates a statistically significant difference between compound 4-treated cells and compound 4. “c” indicates a statistically significant difference between compound 6-treated cells and compound 6. “d” indicates a statistically significant difference between the two treatments (compound 4- and 6-treatment). Statistical analyses were conducted using GraphPad Prism version 6. The statistical comparisons were performed using the one-way analysis of variance (ANOVA) test at a global significance of 95%. The Bonferroni significance correction was adopted for pairwise comparisons.

correlation between the induction of ROS production and antifungal activity is present.

A valuable synthetic strategy for the *N*-terminal modification of the peptide UBI 31–38 through CuAAC is presented in this work. The peptide–coumarin conjugate 4 was successfully prepared by coupling the fully protected alkyne-decorated peptidyl resin 1 to the 3-azido-7-diethylaminocoumarin 3. The novel conjugate 4 exhibited antimicrobial activity against *C. gattii* and *C. neoformans*, including a fluconazole-resistant strain of *C. gattii*, at a concentration range of 0.04 to 0.18 $\mu\text{mol}\cdot\text{mL}^{-1}$. In addition, results of a cytotoxicity assay demonstrated that the conjugate 4 was nontoxic up to a concentration of 0.21 $\mu\text{g}\cdot\text{mL}^{-1}$, indicating a moderate to high selectivity index for anticryptococcal activity over cytotoxicity. UBI 31–38, 3-azido-7-diethylaminocoumarin 3, and the coumarin–triazole derivative 6 administered individually exhibited lower activities against the assayed strains, suggesting that the antifungal activity of 4 may be due to different properties of this compound, which act synergistically. Electrostatic and hydrophobic interactions of the peptide moiety with the polysaccharide capsule and cellular membrane of the fungi, as well as the induction of endogenous ROS production, may have important roles in the antifungal activity of 4. Further studies to elucidate the detailed mechanism of action of the conjugate 4 against *C. gattii* and *C. neoformans* are underway. These will hopefully contribute to the design and development of new antifungal agents.

■ ASSOCIATED CONTENT

Supporting Information

Chemicals, general experimental methods, procedures for synthesis, inoculum preparation, determination of MIC, cytotoxicity assay, measurement of ROS, and spectral data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ANOVA, analysis of variance; AMP, antimicrobial peptide; AU, arbitrary units; CuAAC, copper(I)-catalyzed azide–alkyne cycloaddition; DCM, dichloromethane; EDT, 1,2-ethanedithiol; EDTA, ethylene-diaminetetraacetic acid; equiv, equivalent; FCZ, fluconazole; Fmoc, *N*-9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; MIC, minimum inhibitory concentration; MS, mass spectrum; MTT, methylthiazoltetrazolium; ROS, reactive oxygen species; rpm, rotations per minute; rt, room temperature; TFA, trifluoroacetic acid; TIS, triisopropylsilane; UBI, ubiquitin

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