Université de Poitiers
Faculté de Médecine et de Pharmacie

ANNÉE 2018 Thèse n°

THÈSE
POUR LE DIPLÔME D'ÉTAT
DE DOCTEUR EN PHARMACIE
(Arrêté du 17 juillet 1987)

et

MÉMOIRE
DU DIPLÔME D'ÉTUDES SPÉCIALISÉES
DE BIOLOGIE MÉDICALE
(Décret 88-996 du 19 octobre 1988)

présentée et soutenue publiquement
le 14 septembre 2018 à POITIERS
par Monsieur BRUNET Kévin

Activité de peptides antimicrobiens contre des levures et champignons filamenteux d'intérêt médical : la temporine [K3]SHa, un nouvel agent antifongique potentiel.

Composition du jury :

Président : - Madame le Professeur Rodier Marie-Hélène
Membres : - Monsieur le Professeur Dupuis Antoine
- Monsieur le Docteur Verdon Julien

Directeur de thèse : Madame le Docteur Perraud Estelle

UNIVERSITE DE POITIERS

Faculté de Médecine et de Pharmacie

Le Doyen,

Année universitaire 2017 - 2018

LISTE DES ENSEIGNANTS DE MEDECINE

Professeurs des Universités-Praticiens Hospitaliers

- AGIUS Gérard, bactériologie-virologie **(surnombre jusqu'en 08/2018)**
- ALLAL Joseph, thérapeutique
- BATAILLE Benoît, neurochirurgie
- BRIDOUX Frank, néphrologie
- BURUCOA Christophe, bactériologie – virologie
- CARRETIER Michel, chirurgie générale
- CHEZE-LE REST Catherine, biophysique et médecine nucléaire
- CHRISTIAENS Luc, cardiologie
- CORBI Pierre, chirurgie thoracique et cardio-vasculaire
- DAHYOT-FIZELIER Claire, anesthésiologie – réanimation
- DEBAENE Bertrand, anesthésiologie réanimation
- DEBIAIS Françoise, rhumatologie
- DROUOT Xavier, physiologie
- DUFOUR Xavier, Oto-Rhino-Laryngologie
- FAURE Jean-Pierre, anatomie
- FRASCA Denis, anesthésiologie-réanimation
- FRITEL Xavier, gynécologie-obstétrique
- GAYET Louis-Etienne, chirurgie orthopédique et traumatologique
- GICQUEL Ludovic, pédopsychiatrie
- GILBERT Brigitte, génétique
- GOMBERT Jean-Marc, immunologie
- GOUJON Jean-Michel, anatomie et cytologie pathologiques
- GUILLEVIN Rémy, radiologie et imagerie médicale
- HADJADJ Samy, endocrinologie, diabète et maladies métaboliques
- HAUET Thierry, biochimie et biologie moléculaire
- HOUETO Jean-Luc, neurologie
- INGRAND Pierre, biostatistiques, informatique médicale
- JAAFARI Nematollah, psychiatrie d'adultes
- JABER Mohamed, cytologie et histologie
- JAYLE Christophe, chirurgie thoracique t cardio-vasculaire
- KARAYAN-TAPON Lucie, cancérologie
- KEMOUN Gilles, médecine physique et de réadaptation **(en détachement)**
- KRAIMPS Jean-Louis, chirurgie générale
- LECRON Jean-Claude, biochimie et biologie moléculaire
- LELEU Xavier, hématologie
- LEVARD Guillaume, chirurgie infantile
- LEVEQUE Nicolas, bactériologie-virologie
- LEVEZIEL Nicolas, ophtalmologie
- LEVILLAIN Pierre, anatomie et cytologie pathologiques **(surnombre jusqu'en 12/2017)**
- MACCHI Laurent, hématologie
- MARECHAUD Richard, médecine interne **(émérite à/c du 25/11/2017)**
- MAUCO Gérard, biochimie et biologie moléculaire **(surnombre jusqu'en 08/2018)**
- MEURICE Jean-Claude, pneumologie
- MIGEOT Virginie, santé publique
- MILLOT Frédéric, pédiatrie, oncologie pédiatrique
- MIMOZ Olivier, anesthésiologie – réanimation
- NEAU Jean-Philippe, neurologie
- ORIOT Denis, pédiatrie
- PACCALIN Marc, gériatrie
- PERAULT Marie-Christine, pharmacologie clinique
- PERDRISOT Rémy, biophysique et médecine nucléaire
- PIERRE Fabrice, gynécologie et obstétrique
- PRIES Pierre, chirurgie orthopédique et traumatologique
- RICHER Jean-Pierre, anatomie
- RIGOARD Philippe, neurochirurgie
- ROBERT René, réanimation
- ROBLOT France, maladies infectieuses, maladies tropicales
- ROBLOT Pascal, médecine interne
- RODIER Marie-Hélène, parasitologie et mycologie
- SAULNIER Pierre-Jean, thérapeutique
- SILVAIN Christine, hépato-gastro- entérologie
- SOLAU-GERVAIS Elisabeth, rhumatologie
- TASU Jean-Pierre, radiologie et imagerie médicale
- THIERRY Antoine, néphrologie
- THILLE Arnaud, réanimation
- TOUGERON David, gastro-entérologie
- TOURANI Jean-Marc, cancérologie
- WAGER Michel, neurochirurgie

Bât. D1 - 6 rue de la Milétrie – TSA 51115 - 86073 POITIERS CEDEX 9 - France
☎05.49.45.43.43 - 🖷 05.49.45.43.05

Maîtres de Conférences des Universités-Praticiens Hospitaliers

- ALBOUY-LLATY Marion, santé publique
- BEBY-DEFAUX Agnès, bactériologie – virologie
- BEN-BRIK Eric, médecine du travail **(en détachement)**
- BILAN Frédéric, génétique
- BOURMEYSTER Nicolas, biologie cellulaire
- CASTEL Olivier, bactériologie - virologie – hygiène
- COUDROY Rémy, réanimation
- CREMNITER Julie, bactériologie – virologie
- DIAZ Véronique, physiologie
- FEIGERLOVA Eva, endocrinologie, diabète et maladies métaboliques
- FROUIN Eric, anatomie et cytologie pathologiques
- GARCIA Magali, bactériologie-virologie
- LAFAY Claire, pharmacologie clinique
- PERRAUD Estelle, parasitologie et mycologie **(mission 09/2017 à 03/2018)**
- RAMMAERT-PALTRIE Blandine, maladies infectieuses
- SAPANET Michel, médecine légale
- SCHNEIDER Fabrice, chirurgie vasculaire
- THUILLIER Raphaël, biochimie et biologie moléculaire

Professeur des universités de médecine générale

- BINDER Philippe
- GOMES DA CUNHA José

Maître de conférences des universités de médecine générale

- BOUSSAGEON Rémy **(disponibilité de 10/2017 à 01/2018)**

Professeurs associés de médecine générale

- BIRAULT François
- PARTHENAY Pascal
- VALETTE Thierry

Maîtres de Conférences associés de médecine générale

- AUDIER Pascal
- ARCHAMBAULT Pierrick
- BRABANT Yann
- FRECHE Bernard
- MIGNOT Stéphanie
- VICTOR-CHAPLET Valérie

Enseignants d'Anglais

- DEBAIL Didier, professeur certifié
- SIMMONDS Kevin, maître de langue étrangère

Professeurs émérites

- DORE Bertrand, urologie (08/2020)
- EUGENE Michel, physiologie (08/2019)
- GIL Roger, neurologie (08/2020)
- GUILHOT-GAUDEFFROY François, hématologie et transfusion (08/2020)
- HERPIN Daniel, cardiologie (08/2020)
- KITZIS Alain, biologie cellulaire (16/02/2019)
- MARECHAUD Richard, médecine interne **(émérite à/c du 25/11/2017 – jusque 11/2020)**
- POURRAT Olivier, médecine interne (08/2018)
- RICCO Jean-Baptiste, chirurgie vasculaire (08/2018)
- SENON Jean-Louis, psychiatrie d'adultes (08/2020)
- TOUCHARD Guy, néphrologie (08/2018)

Professeurs et Maîtres de Conférences honoraires

- ALCALAY Michel, rhumatologie
- ARIES Jacques, anesthésiologie-réanimation
- BABIN Michèle, anatomie et cytologie pathologiques
- BABIN Philippe, anatomie et cytologie pathologiques
- BARBIER Jacques, chirurgie générale (ex-émérite)
- BARRIERE Michel, biochimie et biologie moléculaire
- BECQ-GIRAUDON Bertrand, maladies infectieuses, maladies tropicales (ex-émérite)
- BEGON François, biophysique, médecine nucléaire
- BOINOTCatherine, hématologie – transfusion
- BONTOUX Daniel, rhumatologie (ex-émérite)
- BURIN Pierre, histologie
- CASTETS Monique, bactériologie -virologie – hygiène
- CAVELLIER Jean-François, biophysique et médecine nucléaire
- CHANSIGAUD Jean-Pierre, biologie du développement et de la reproduction
- CLARAC Jean-Pierre, chirurgie orthopédique
- DABAN Alain, cancérologie radiothérapie (ex-émérite)
- DAGREGORIO Guy, chirurgie plastique et reconstructrice
- DESMAREST Marie-Cécile, hématologie
- DEMANGE Jean, cardiologie et maladies vasculaires
- FAUCHERE Jean-Louis, bactériologie-virologie (ex-émérite)
- FONTANEL Jean-Pierre, Oto-Rhino Laryngologie (ex-émérite)
- GRIGNON Bernadette, bactériologie
- GUILLARD Olivier, biochimie et biologie moléculaire
- GUILLET Gérard, dermatologie
- JACQUEMIN Jean-Louis, parasitologie et mycologie médicale
- KAMINA Pierre, anatomie (ex-émérite)
- KLOSSEK Jean-Michel, Oto-Rhino-Laryngologie
- LAPIERRE Françoise, neurochirurgie (ex-émérite)
- LARSEN Christian-Jacques, biochimie et biologie moléculaire
- MAGNIN Guillaume, gynécologie-obstétrique (ex-émérite)
- MAIN de BOISSIERE Alain, pédiatrie
- MARCELLI Daniel, pédopsychiatrie (ex-émérite)
- MARILLAUD Albert, physiologie
- MENU Paul, chirurgie thoracique et cardio-vasculaire (ex-émérite)
- MORICHAU-BEAUCHANT Michel, hépato-gastro-entérologie
- MORIN Michel, radiologie, imagerie médicale
- PAQUEREAU Joël, physiologie
- POINTREAU Philippe, biochimie
- REISS Daniel, biochimie
- RIDEAU Yves, anatomie
- SULTAN Yvette, hématologie et transfusion
- TALLINEAU Claude, biochimie et biologie moléculaire
- TANZER Joseph, hématologie et transfusion (ex-émérite)
- VANDERMARCQ Guy, radiologie et imagerie médicale

Bât. D1 - 6 rue de la Milétrie – TSA 51115 - 86073 POITIERS CEDEX 9 - France
☎05.49.45.43.43 - 🖷 05.49.45.43.05

UNIVERSITE DE POITIERS

Faculté de Médecine et de Pharmacie

Année universitaire 2017-2018

PHARMACIE

Professeurs

- CARATO Pascal, Chimie Thérapeutique
- COUET William, Pharmacie Clinique
- FAUCONNEAU Bernard, Toxicologie
- GUILLARD Jérôme, Pharmaco chimie
- IMBERT Christine, Parasitologie
- MARCHAND Sandrine, Pharmacocinétique
- OLIVIER Jean Christophe, Galénique
- PAGE Guylène, Biologie Cellulaire
- RABOUAN Sylvie, Chimie Physique, Chimie Analytique
- SARROUILHE Denis, Physiologie
- SEGUIN François, Biophysique, Biomathématiques

Maîtres de Conférences

- BARRA Anne, Immunologie-Hématologie
- BARRIER Laurence, Biochimie
- BODET Charles, Bactériologie
- BON Delphine, Biophysique
- BRILLAULT Julien, Pharmacologie
- BUYCK Julien, Microbiologie
- CHARVET Caroline, Physiologie
- DEBORDE Marie, Sciences Physico-Chimiques
- DEJEAN Catherine, Pharmacologie
- DELAGE Jacques, Biomathématiques, Biophysique
- DUPUIS Antoine, Pharmacie Clinique
- FAVOT Laure, Biologie Cellulaire et Moléculaire
- GIRARDOT Marion, pharmacognosie, botanique, biodiversité végétale
- GREGOIRE Nicolas, Pharmacologie
- HUSSAIN Didja, Pharmacie Galénique
- INGRAND Sabrina, Toxicologie
- MARIVINGT-MOUNIR Cécile Pharmaco chimie
- PAIN Stéphanie, Toxicologie
- RAGOT Stéphanie, Santé Publique
- RIOUX BILAN Agnès, Biochimie
- TEWES Frédéric, Chimie et Pharmaco chimie
- THEVENOT Sarah, Hygiène et Santé publique
- THOREAU Vincent, Biologie Cellulaire
- WAHL Anne, Pharmaco chimie, Produits naturels

AHU

- BINSON Guillaume

PAST - Maître de Conférences Associé

- DELOFFRE Clément, Pharmacien
- HOUNKANLIN Lydwin, Pharmacien

Professeur 2nd degré

- DEBAIL Didier

Maître de Langue - Anglais

- SIMMONDS Kévin

Poste d'ATER

- JUIN Camille

Poste de Doctorant

- BERNARD Clément
- DOUMAS Manon

Remerciements

À Madame le Professeur Marie-Hélène Rodier,

Merci de me faire l'honneur de présider ce jury. Merci de m'avoir permis de réaliser ma thèse dans votre laboratoire, de votre soutien et de m'accueillir pour la suite. Merci pour le temps que vous êtes toujours prête à m'accorder, pour votre avis toujours pertinent et votre bonne humeur dans le travail.

À Monsieur le Professeur Antoine Dupuis,

Je vous remercie de me faire l'honneur de juger ce travail et d'y apporter votre vision.

À Monsieur le Docteur Julien Verdon,

Je vous remercie de me faire l'honneur de juger ce travail, merci de votre disponibilité et de m'avoir encadré pour certaines expériences.

À Madame le Docteur Estelle Perraud,

Merci d'avoir accepté de diriger cette thèse. Je te remercie de m'avoir apporté ton aide tout au long de ce travail et d'avoir été disponible dès que j'en avais besoin. Merci également de me former quotidiennement à la parasitologie et à la mycologie et de me permettre de travailler et d'apprendre avec toi. Merci pour ton soutien, ton temps et ta bonne humeur.

Sommaire

Abstract

Activity of antimicrobial peptides against medically relevant yeasts and moulds: temporin [K3]SHa a potential antifungal compound.

Introduction

Fungi are an emerging cause of infections that can represent a significant health problem in immunocompromised patients. Unfortunately, few antifungal agents are available to fight these diseases. Antimicrobial peptides which are natural molecules and belong to immune system of many species represent a promising way of research. However, previous studies have mainly focused on their antibacterial activity. The aim of this study was to test the activity of warnericin RK, armadillidin H, magainin II and temporin [K3]SHa against some species of yeasts and moulds, and to explore temporin [K3]SHa activity against *Candida albicans*.

Materials and methods

In order to assess the antifungal activity of these peptides, MIC determinations were performed according to the EUCAST guidelines. Following that, activity of temporin [K3]SHa against *C. albicans* was explored by time kill curve experiment, membrane permeabilization assay and electron microscopy. Finally, activity of temporin [K3]SHa against *C. albicans* biofilm was investigated using XTT assay.

Results

No antifungal activity against tested fungi was observed for warnericin RK, armadillidin H and magainin II. Temporin [K3]SHa was found to be active against yeasts (most of the tested *Candida* species and *Cryptococcus neoformans*). However, *C. glabrata* seemed poorly sensitive to this antimicrobial peptide and its efficacy against moulds was very moderate. Temporin [K3]SHa was shown to have a rapid fungicidal activity against *C. albicans*. Mechanism could be due to membrane permeabilization and fungal structure alterations. However, only slight anti-biofilm activity of this molecule was found.

Conclusion

Temporin [K3]SHa could represent a potential new antifungal compound against non-*glabrata Candida* species and *C. neoformans*. However, further studies are needed to specify its mode of action, and to explore a potential synergy with conventional antifungal agents.

Introduction

Invasive fungal infections have increased this last decade mainly due to the use of immunosuppressive drugs and critical care therapies even though, few systemic antifungal drugs are available. Responsible for these invasive fungal infections, yeasts and filamentous fungi represent a major public health problem (1, 2). *Candida* species are involved in many invasive infections in the hospital, especially in critical care units. Amongst them, *Candida albicans* is a leading cause of nosocomial infections (2). *Cryptococcus neoformans*, another yeast, also represents a major source of systemic infections worldwide, causing 200 000 infections per year (3). While this yeast essentially affects patients with HIV, organ transplantation, chemotherapy and immunosuppressive regimens are other risk factors for this disease (4). As for fungal infections due to filamentous fungi, they cause major problems of morbidity and mortality in hematology departments (5). Concerning therapy, there exist only four families of usable systemic antifungal agents: polyenes, echinocandins, triazoles and pyrimidine analogs. In addition to the low number of available systemic antifungals, many cases of natural or acquired resistance to antifungals have been described (6). In this context, antimicrobial peptides (AMPs) could be an interesting alternative to conventional antifungal treatments (7, 8). For cryptococcosis, amphotericin B (AmB), fluconazole and 5-fluorocytosine are the only active drugs. That is why the discovery of new active molecules, such as AMPs, would be a major therapeutic advance (9).

Fungal agents can also cause local infections such as vulvovaginitis, onychomycosis, skin infections, ocular infections and oral or periodontal infections (10-15). Moreover, some fungi could cause chronic infections such as chronic aspergillosis, especially in cystic fibrosis patients (16, 17). AMPs could be used in these indications and some are presently being studied in clinical trials against local infections (12).

AMPs are natural molecules involved in the innate immune response of many living organisms. They are found in bacteria, archaea, protists, fungi, plants and animals. These peptides have antimicrobial properties on bacteria, viruses and fungi (7, 18). Moreover, their physicochemical mode of action is less susceptible to resistance, making them very interesting antimicrobial agents (19). Some also present immunomodulatory or antitumor properties (20-22). They are composed of a small number of amino acids, most of which are cationic and amphipathic, and they are grouped according to their secondary structures in α-helices, β-sheet or both (7, 18). While their mechanism of action mainly involves membrane permeabilization, some of them exert other modes of action: interactions with intracellular targets, cell wall binding, mediated internalization by receptor or induction of a signaling cascade (7). Their antibacterial effect has been well-studied but little is known about their antifungal activity.

The aim of our study was to test four AMPs: warnericin RK, armadillidin H, magainin II and temporin [K3]SHa for their potential antifungal activity. Warnericin RK is a cationic AMP extracted from *Staphylococcus warneri* RK with activity against some gram-positive and gram-negative bacteria, especially *Legionella sp* (23). Armadillidin is a glycine-rich cationic AMP isolated from *Armadillidium vulgare*, a crustacean isopod with activity against some gram-positive and gram-negative bacteria and some filamentous fungi (24, 25). Magainin II is also a cationic AMP extracted from frog skin (26). Temporins are an AMPs family extracted from amphibian skin which have a narrow spectrum activity predominantly against gram-positive bacteria (27). A member of this family, temporin-SHa was extracted from the skin of the Sahara frog *Pelophylax (Rana) saharica* originating in Tunisia, and it presents a broader spectrum of activity toward gram-positive and gram-negative bacteria, yeasts and *Leishmania* parasites (28). Temporin [K3]SHa was designed and synthesized from

the temporin-SHa. Some authors have demonstrated an improved activity of this modified peptide against yeasts compared to the temporin SHa (27).

In this study, the activity of warnericin RK, armadillidin H, magainin II and temporin [K3]SHa against some species of yeasts and moulds was first evaluated, following which temporin [K3]SHa activity against *C. albicans* was explored.

Materials and methods

Strains and culture conditions

The strains of yeasts used in this study were *Candida albicans* (ATCC 14053 and ATCC 90028), *Candida glabrata* (ATCC MYA 2950 and a clinical strain), *Candida parapsilosis* (ATCC 22019), *Candida krusei* (ATCC 6258 and a clinical strain), *Cryptococcus neoformans* var. *grubii* (clinical strain), *Candida lusitaniae* (ATCC 34449), *Candida utilis* (ATCC 9950), *Candida kefyr* (clinical strain), *Candida tropicalis* (clinical strain), *Candida dublinensis* (clinical strain). Studied strains of moulds were *Aspergillus fumigatus* (ATCC 16424), *Lichtheimia corymbifera* (CNRMA 2011.1047, Paris, France), *Fusarium oxysporum* (clinical strain) and *Scedosporium apiospermum* (clinical strain).

Identification of clinical strains was performed by mass spectrometry for yeasts and by ITS1-ITS4 sequencing for moulds.

Yeasts and moulds were stored at -20°C and cultured at 37°C on Sabouraud agar (BioMérieux) before use.

Antimicrobial peptides and antifungal agents

The antimicrobial peptides studied were warnericin RK, armadillidin H, magainin II and temporin [K3]SHa. Armadillidin H was purchased from ProteoGenix Corporation (Schitilgheim, France). Magainin II and temporin [K3]SHa were kindly provided by Pr. Ali Ladram (Sorbonne Universités, UPMC Univ Paris 06, CNRS, Institut de Biologie Paris-Seine, Biogenèse des Signaux Peptidiques, Paris, France). Warnericin RK was obtained from GenScript (Piscataway, USA).

Antifungal agents used as controls were fluconazole (Sigma-Aldrich, Saint-Louis, USA) and amphotericin B (Sigma-Aldrich). Stock solutions were performed in dimethylsulfoxide (final concentration <1%).

MIC determination

Minimum inhibitory concentrations (MICs) for yeasts were determined according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines (E.DEF 7.3.1) (29). Briefly, yeasts were grown for 24 h on Sabouraud agar. An inoculum of five representative colonies was performed in 3 mL of sterile water (Fresenius, Sèvres, France). Suspension was homogenized for 15 seconds using a vortex and density was adjusted to 0.5 Mc Farland. The suspension was then diluted in sterile water to 1:10 to obtain a concentration of 1-5 x 10^5 colony-forming units (CFU)/mL. AMPs were diluted in RPMI [2% dextrose; buffered with MOPS at PH7], to obtain concentrations ranging from 128 to 0.25 µg/mL. MICs were determined by spectrophotometry (500 nm) after 24 h of incubation at 37°C for *Candida* species or 48 h for *C. neoformans* by concentration inhibiting > 90% of growth.

MICs for moulds were determined according to the EUCAST guidelines (E.DEF 9.3) (29). Briefly, moulds were grown for 96 h on Sabouraud agar. Conidia were recovered with sterile water supplemented with 0.1% Tween 20 (Sigma-Aldrich). Suspension was filtered (70µm) (Falcon, Corning, USA) to remove hyphae and clumps. Suspension was then counted with a haemocytometer chamber (Kova, Hycor, Garden Grove, USA) and adjusted to 1-2.5 x 10^5 CFU/mL. AMPs were diluted in RPMI [2% dextrose; buffered with MOPS at PH7], to obtain concentrations ranging from 128 to 0.25 µg/mL. MICs were determined by the last

well, with no visible growth after 24 h of incubation at 37°C for *Lichtheimia corymbifera* and 48 h for other moulds.

Concentrations are expressed as the means of three independent experiments performed in triplicate.

After MIC determination, each fungal suspension present in the well corresponding to the MIC was collected. These suspensions were washed twice with distilled sterile water and 10 µL were plated on Sabouraud agar. Fungal colonies were counted after 24 h, 48 h and 72 h of incubation at 37°C.

Time killing assay

Time killing assay was assessed on *C. albicans.* A yeast suspension at 10^5 CFU/mL was performed in distilled sterile water and was incubated with 64, 32 or 16 µg/mL of temporin [K3]SHa diluted in RPMI [2% dextrose; buffered with MOPS at PH7]. After 30 min, 1, 2, 3, 4, 5, 6 and 7 h, 10µL of pure and diluted treated suspension (1:10 and 1:100) were plated on Sabouraud. After 24 h at 37°C, CFU were counted. Three independent experiments were performed in triplicate.

Membrane permeabilization assay

C. albicans was grown for 24 h on Sabouraud agar and diluted in distilled water to obtain a concentration of 1-5 x 10^5 CFU/mL, which was incubated at 37°C with temporin [K3]SHa at 128 µg/mL, 64 µg/mL, 32 µg/mL and 16 µg/mL. After 30 min, 1 h and 3 h of incubation, 100 µL of fungal suspension was collected and 0.5 µL of propidium iodide (IP) (1mg/mL) was added. PI is able to enter into permeabilized cells and to bind to DNA leading

to fluorescence, which was assessed by flow cytometry (Cytoflex, Beckman Coulter, Brea, USA) (excitation wavelength 448 nm, emission wavelength 610/20 nm).

Scanning (SEM) and transmission (TEM) electron microscopy

C. albicans was grown for 24 h on Sabouraud agar and diluted in sterile distilled water to obtain a concentration of 1-5 x 10^5 CFU/mL. Then suspension was treated for 3 h with temporin [K3]SHa diluted in RPMI [2% dextrose; buffered with MOPS at PH7] at 32 µg/mL. Suspension was centrifuged, supernatant was removed and yeasts were fixed for 1 h with 2.5% glutaraldehyde in 1 M phosphate buffer, pH 7.1. After phosphate buffered saline (PBS) washes, yeasts were post-fixed for 45 min in 1% osmium tetraoxyde in phosphate buffer. Dehydration was carried out using successive incubations of increasing ethanol concentrations (from 70 to 100%). Yeasts were then suspended in 100% ethanol and separated for SEM or TEM. Each part was centrifuged at 10,000 rpm for 10 min. For SEM, yeasts were deposited on a 12 mm cover glass and dried by hexamethyldisilazane treatment. The surface of the cover glass was sputter-coated in a vacuum with an electrically conductive 25 nm thick layer of gold–palladium alloy coating system (BALTEC SCD 005). SEM images were recorded with a scanning electron microscope (JEOL 840) at 15 kV. For TEM, pellet was included in epon epoxy resin and after 24 h of polymerization, 70 nm sections were performed using an ultramicrotome UC6 (Leica). Uranyl acetate (2% in 70% ethanol) and lead citrate were used as contrasting agents for electron microscopy (JEOL 1010 at 80 KV). TEM was recorded using Quemesa camera with iTem software (Olympus).

Activity against *C. albicans* biofilm

C. albicans suspension (10^4 CFU/mL) was incubated during 1 h in Yeast Nitrogen Base - glucose medium (YNB – glucose) in 96 well plates. Following that, the wells were washed to remove fungal planktonic forms. *C. albicans* biofilm was grown 12 h at 37°C in YNB – glucose, then treated during 24 h at 37°C with temporin [K3]SHa, fluconazole or AmB or not treated (negative control). Another *C. albicans* biofilm (10^6 CFU/mL) was grown during 24 h at 37°C in the same medium, and also treated in the same conditions with temporin [K3]SHa, fluconazole or AmB or not treated (negative control). Concentrations of temporin [K3]SHa and antifungal agents ranged from 0.25 to 128 µg/mL. After incubation, metabolic activities of the treated biofilms were measured using 2,3-bis(2-Methoxy-4-Nitro-5-Sulfo-phenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) reduction assay. Briefly, wells were washed twice with PBS. 50 µL of XTT-menadione were added in each well and incubated 3 h at 37°C. Following that, optic density (OD) was evaluated at 450 nm. Inhibition percentage of metabolic activity was calculated by: (OD negative control – OD temporin [K3]SHa)/(OD negative control). Two independent experiments were performed in triplicate. Results were expressed as the means ± SD of metabolic activity compared to negative control.

Results

Temporin [K3]SHa exerts antifungal activity against some yeasts and filamentous fungi.

Antifungal activity of the four AMPs against yeasts and moulds was first evaluated by MICs determination. Armadillidin H, warnericin RK and magainin II presented no antifungal activity with MICs greater than 128 µg/mL for all studied strains of yeasts and moulds (Table 1).

Temporin [K3]SHa presented antifungal activity against some yeast species with the following MICs (Table 1): *C. albicans* (32 µg/mL; 23µM), *C. parapsilosis* (16 µg/mL; 11µM), *C. krusei* (32 µg/mL; 23µM), *C. lusitaniae* (16 µg/mL; 11µM), *C. utilis* (8 µg/mL; 6µM), *C. kefyr* (16 µg/mL; 11µM), *C. tropicalis* (16 µg/mL; 11µM), *C. dublinensis* (32 µg/mL; 23µM). MIC obtained on *C. neoformans* was 32 µg/mL (23µM). However, no or weak activity was found against *C. glabrata* (≥ 128 µg/mL; ≥ 90µM) (Table 2)

For moulds, temporin [K3]SHa MICs were higher than for yeasts: 128 µg/mL (90µM) for *A. fumigatus*, 64 µg/mL (45µM) for *L. corymbifera*, and > 128 µg/mL (> 90µM) for *F. oxysporum* and *S. apiospermum* (Table 2).

Temporin [K3]SHa presents fungicidal activity.

The fungicidal or fungistatic nature of temporin [K3]SHa activity was then determined. After MICs determination, well suspensions corresponding to MIC were plated. No growth was displayed for any fungal strain after 24 h, 48 h and 72 h of incubation at 37°C, which meant that temporin [K3]SHa presented fungicidal activity against moulds and yeasts at MIC.

Temporin [K3]SHa is fungicidal within 30 min against *C. albicans*.

During the next step, temporin [K3]SHa activity against *C. albicans* was explored. *C. albicans* suspension (10^5 CFU/mL) was incubated with temporin [K3]SHa at 0.5 x MIC (16µg/mL), MIC (32µg/mL) and twofold MIC (64µg/mL) to assess kill curve (Figure 1).

At MIC, a rapid decrease appeared within 30 min. After 5 hours, no more CFUs were detected in culture. This curve showed rapid fungicidal activity. Kill curves were then assessed for 0.5 x MIC and 2 x MIC. Action seemed dose-dependent, indeed, for twofold MIC, decrease was faster and no more CFUs were detected after 3 hours. For 0.5 x MIC, a CFU fungal decrease of $1Log_{10}$ was highlighted at 5 hours but fungal regrowth was recovered after this time (Figure 1).

Temporin [K3]SHa alters permeability of *C. albicans* membrane.

Further, we assessed a test of membrane permeabilization to understand the mechanism of action of temporin [K3]SHa (Figure 2). Rapid membrane permeabilization was displayed by IP intercalation. Permeabilization of 45% of *C. albicans* cells was highlighted within 30 min at 4 x MIC. After 1 h, the percentage of permeabilized cells reached 80% at 4 x MIC, 33% at 2 x MIC and 7% at MIC. After 3 hours, about 95 % of cells were permeabilized for 4 x MIC and 2 x MIC and 80% for MIC. Permeabilization did not reach 10% at 0.5 x MIC.

Temporin [K3]SHa alters integrity of *C. albicans* structure.

In order to specify the mechanism of action of temporin [K3]SHa, we performed scanning electron microscopy (SEM) and transmission electron microscopy (TEM). *C. albicans* (10^5 CFU/mL) was incubated 3 h at 37°C in RPMI medium without peptide or with temporin [K3]SHa at 32 µg/mL. SEM showed appreciable modifications of fungal structures when temporin [K3]SHa was added. No hyphae were detectable and fungal surfaces seemed altered. Fungal wall appeared perforated and destructed (Figure 3). TEM highlighted thinned and distorted wall and a modification of cytoplasmic density and content (Figure 4).

Temporin [K3]SHa presents weak activity on *C. albicans* biofilm.

To assess temporin [K3]SHa antibiofilm activity, two *C. albicans* biofilms were performed. For a *C. albicans* biofilm with low yeast density (10^4 CFU/mL) incubated 12 h at 37°C, metabolic activity significantly decreased after 24 h of contact up to 128 µg/mL of temporin [K3]SHa and up to 1µg/mL of amphotericin B but no reduction was shown for fluconazole. Reduction of *C. albicans* biofilm metabolic activity was 31.14 % for temporin [K3]SHa at 128 µg/mL compared to control ($p<0.01$). (Figure 5).

Concerning *C. albicans* biofilm with higher density (10^6 CFU/mL) incubated 24h at 37°C, no metabolic activity reduction appeared for temporin [K3]SHa after 24h .

Discussion

Invasive fungal infections are an important and increasing cause of morbidity and mortality (30). In addition, resistant species are emerging while few systemic antifungals are available to control these diseases. AMPs are interesting molecules that could enrich the therapeutic arsenal, moreover, due to their simple structure and their physical mode of action, they would be less subject to resistance emergence (19).

The first step of our study was to evaluate the antifungal activity of warnericin RK, armadillidin H, magainin II and temporin [K3]SHa. We evaluated MICs of these four AMPs against five yeast and four mould species.

Warnericin RK is an AMP extracted from *S. warneri* with activity almost limited to *Legionella* sp (23). This amphipatic alpha-helical structure AMP presents a detergent-like mode of action to which *Legionella* sp are very sensitive (31). To our knowledge, warnericin had never been tested against yeasts or moulds. Our study showed no antifungal activity, which is consistent with the limited spectrum of this peptide described, and is perhaps explained by differences in membrane composition between *Legionella sp* and other bacteria or fungi. Indeed, some authors have shown that fatty acid composition of membranes modulates sensitivity to warnericin RK (32).

Armadillidin H is a linear glycine-rich cationic peptide extracted from *Armadillidium vulgare*, a crustacean isopod (25). In a previous study, this peptide presented activity against gram-positive and gram-negative bacteria by membrane damage, but no activity against *C. albicans, C. glabrata and C. parapsilosis* was detected (24). Our results confirmed these findings and showed that *C. krusei* was not sensitive. Lack of activity against *Candida* species was surprising, indeed most glycine-rich peptides are active against yeasts (24). For moulds, some authors have shown that armadillidin H possessed no activity against *Aspergillus*

fumigatus, Aspergillus terreus, Botrytis cinerea and Scedosporium apiospermum (24). Our study confirms a lack of activity of this AMP against *A. fumigatus* and *S. apiospermum*. We have added that armadillidin H has no activity against *Lichtheimia corymbifera* and *Fusarium oxysporum*.

Magainin II, isolated in 1973 from frog skin, is a linear amphipathic cationic alpha-helical AMP which presents activity against gram-positive and gram-negative bacteria and moderate activity against *C. albicans* (MIC = 80 µg/mL) (33). Some authors have shown modest antifungal activity against *Penicillium digitatum* (MIC = 60 µg/mL), *Alternaria solani* (MIC > 100 µg/mL) and *Phytophthora infestans* (MIC > 100 µg/mL). These results can be explained by the difference between fungal and bacterial membrane composition, with fungi lacking acidic phospholipids and possessing sterols which reduce their sensitivity to lytic peptides (34, 35). Our study showed no activity of this AMP against the yeasts and moulds tested.

Temporin SHa was isolated from the *Pelophylax saharicus* frog and found to be a potentially strong antimicrobial peptide. Temporin SHa is active against gram-positive and gram-negative bacteria, yeasts and *Leishmania* (36). Some authors have modified the positive charge of this molecule by replacing a serine with a lysine in order to increase its antimicrobial activity. This modified peptide was named temporin [K3]SHa (27). A previous study demonstrated that temporin [K3]SHa was active against yeasts with MICs of 6 µM for *C. albicans*, 25 µM for *S. cerevisiae* and 25 µM for *C. parapsilosis* (27). In our study, activity against *C. albicans* and *C. parapsilosis* was confirmed. *C. lusitaniae, C. utilis, C. kefyr, C. tropicalis, C. dublinensis* and *C. krusei* were also sensitive to this AMP. However, we showed lower activity of temporin [K3]SHa on moulds than on yeasts.

Interestingly, temporin [K3]SHa presented activity against every *Candida* species except *C. glabrata* for which MICs were higher. Other authors have reported similar results with cationic AMPs such as histatin 5 or β-defensin 2 and 3 (37, 38). This phenomenon is poorly explained but could be due to the ability of *C. glabrata* to increase its efflux pumps encoded by CR1 and PDH1 and different expressions of cell transporters (37, 39).

The temporin family is known to be active essentially against gram-positive bacteria (40-42). However, some AMPs of this family have shown antifungal activity. Amino-acid composition and especially proline may explain antimicrobial different activities among AMPs of the temporin family (42). For example, temporin TL has shown antifungal activity against yeasts with reported MICs from 2.7 to 6 µM against *C. albicans* (40, 41). Another study explored the activity of 6 temporins (temporin CPa, temporin CPb, temporin-1Ga, temporin-1Ola, temporin-1Spa, temporin-1Oc) against yeasts. Temporin-1Ga and temporin-1Ola presented activity against *C. albicans* with a MIC of 12.5µM (42). According to our results, temporin [K3]SHa seems to be another active AMP against some yeast species.

Interestingly, temporin [K3]SHa presented antifungal activity against some species of yeasts with antifungal resistant phenotype. For example, we found *C. krusei and C. lusitaniae*, which present resistance to fluconazole and amphotericin B respectively (43, 44), sensitive to this temporin [K3]SHa. This molecule could be used in association with antifungal drugs to restore sensitivity or to avoid resistance emergence. In vulvovaginal candidiasis due to *C. krusei*, fluconazole use is not possible and development of new drugs like AMPs could be very useful (45).

Although several AMPs have shown interesting activity against *C. neoformans* (46), this is the first time to our knowledge that a peptide of the temporin family has been tested against this yeast. We showed anti-cryptococcal activity of temporin [K3]SHa with MIC of 32

µg/mL (23µM). Other peptides of temporin family could be tested and could represent an additional class of molecules helping to fight against this fungal infection.

We also explored activity of temporin [K3]SHa against *C. albicans* which is the main *Candida* species involved in human infections. This peptide induced rapid permeabilization of yeast, and electron microscopy showed alterations and perforations of fungal structures. This mechanism is known with cationic AMPs that are able to interact with membranes and to disturb cytoplasmic membrane permeability leading to cell death (27). Moreover, it has been shown that molecules of the temporin family present a detergent-like mode of action on bacteria with a rapid action (27, 47-49). However, other supplementary mechanisms of action cannot be excluded, such as intracellular targeting, DNA or mitochondrial interactions. Temporin [K3]SHa was fungicidal against *C. albicans* and this activity seemed to be dose-dependent. Its time of action was longer than those described for bacteria (5 min for *S. aureus* or 15 min for *E. coli*) (27); difference in membrane composition and presence of fungal wall could explain this delay compared to bacteria.

Finally, we showed only modest activity of temporin [K3]SHa against *C. albicans* biofilm. Thirty percent of metabolic inhibition was shown for 128µg/mL (90µM) against young biofilms with the lowest inoculum. The same experiment with a higher inoculum and twofold old biofilms highlighted no activity. Even if little is known about anti-*Candida* biofilm activity of AMPs, some of them have been found to be active against this fungal biofilm such as cathelicidins and their derivatives (50, 51).

In conclusion, temporin [K3]SHa showed interesting fungicidal activity against some yeasts and presented a rapid action against *C. albicans* by membrane permeabilization and structure alterations. Moreover, given the fact than temporin [K3]SHa presented low toxicity against human cells (27), it could be a promising drug against *Candida* non-*glabrata* and

Cryptococcus. Further investigations are needed to explore structure-activity relationships in view of improving its activity. Moreover, synergistic tests with conventional antifungal agents still need to be performed. Indeed, AMPs could represent good candidates for association because membrane permeabilization may facilitate drug penetration inside cells, including azoles that interact with intracellular targets (52). This association could be useful to decrease antifungal drug doses and drug resistance emergence (53).

References

1. Moura S, Cerqueira L, Almeida A. Invasive pulmonary aspergillosis: current diagnostic methodologies and a new molecular approach. Eur J Clin Microbiol Infect Dis. 2018.

2. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. Invasive candidiasis. Nat Rev Dis Primers. 2018;4:18026.

3. Taylor-Smith LM. *Cryptococcus*-Epithelial Interactions. J Fungi (Basel). 2017;3(4).

4. Guess TE, Rosen JA, McClelland EE. An Overview of Sex Bias in. J Fungi (Basel). 2018;4(2).

5. Bongomin F, Gago S, Oladele RO, Denning DW. Global and Multi-National Prevalence of Fungal Diseases-Estimate Precision. J Fungi (Basel). 2017;3(4).

6. Arendrup MC. Update on antifungal resistance in *Aspergillus* and *Candida*. Clin Microbiol Infect. 2014;20 Suppl 6:42-8.

7. van der Weerden NL, Bleackley MR, Anderson MA. Properties and mechanisms of action of naturally occurring antifungal peptides. Cell Mol Life Sci. 2013;70(19):3545-70.

8. de Oliveira Santos GC, Vasconcelos CC, Lopes AJO, de Sousa Cartágenes MDS, Filho AKDB, do Nascimento FRF, et al. Infections and Therapeutic Strategies: Mechanisms of Action for Traditional and Alternative Agents. Front Microbiol. 2018;9:1351.

9. Li Y, Yue Q, Jayanetti DR, Swenson DC, Bartholomeusz GA, An Z, et al. Anti-Cryptococcus Phenalenones and Cyclic Tetrapeptides from *Auxarthron pseudauxarthron*. J Nat Prod. 2017;80(7):2101-9.

10. Kühbacher A, Burger-Kentischer A, Rupp S. Interaction of Candida Species with the Skin. Microorganisms. 2017;5(2).

11. Piraccini BM, Alessandrini A. Onychomycosis: A Review. J Fungi (Basel). 2015;1(1):30-43.

12. Mirski T, Niemcewicz M, Bartoszcze M, Gryko R, Michalski A. Utilisation of peptides against microbial infections - a review. Ann Agric Environ Med. 2017;25(2):205-10.

13. Wu J, Zhang WS, Zhao J, Zhou HY. Review of clinical and basic approaches of fungal keratitis. Int J Ophthalmol. 2016;9(11):1676-83.

14. Lewis MAO, Williams DW. Diagnosis and management of oral candidosis. Br Dent J. 2017;223(9):675-81.

15. Hacioglu M, Guzel CB, Savage PB, Tan ASB. Antifungal susceptibilities, in vitro production of virulence factors and activities of ceragenins against *Candida spp*. isolated from vulvovaginal candidiasis. Med Mycol. 2018.

16. Maghrabi F, Denning DW. The Management of Chronic Pulmonary Aspergillosis: The UK National Aspergillosis Centre Approach. Curr Fungal Infect Rep. 2017;11(4):242-51.

17. Rivosecchi RM, Samanta P, Demehin M, Nguyen MH. Pharmacokinetics of Azole Antifungals in Cystic Fibrosis. Mycopathologia. 2018;183(1):139-50.

18. Biswaro LS, da Costa Sousa MG, Rezende TMB, Dias SC, Franco OL. Antimicrobial Peptides and Nanotechnology, Recent Advances and Challenges. Front Microbiol. 2018;9:855.

19. Patocka J, Nepovimova E, Klimova B, Wu Q, Kuca K. Antimicrobial Peptides: Amphibian Host Defense Peptides. Curr Med Chem. 2018.

20. Roudi R, Syn NL, Roudbary M. Antimicrobial Peptides As Biologic and Immunotherapeutic Agents against Cancer: A Comprehensive Overview. Front Immunol. 2017;8:1320.

21. Conlon JM, Mechkarska M, Radosavljevic G, Attoub S, King JD, Lukic ML, et al. A family of antimicrobial and immunomodulatory peptides related to the frenatins from skin secretions of the Orinoco lime frog *Sphaenorhynchus lacteus* (Hylidae). Peptides. 2014;56:132-40.

22. Jin G, Weinberg A. Human antimicrobial peptides and cancer. Semin Cell Dev Biol. 2018.

23. Verdon J, Berjeaud JM, Lacombe C, Héchard Y. Characterization of anti-*Legionella* activity of warnericin RK and delta-lysin I from *Staphylococcus warneri*. Peptides. 2008;29(6):978-84.

24. Verdon J, Coutos-Thevenot P, Rodier MH, Landon C, Depayras S, Noel C, et al. Armadillidin H, a Glycine-Rich Peptide from the Terrestrial Crustacean. Front Microbiol. 2016;7:1484.

25. Herbinière J, Braquart-Varnier C, Grève P, Strub JM, Frère J, Van Dorsselaer A, et al. Armadillidin: a novel glycine-rich antibacterial peptide directed against gram-positive bacteria in the woodlouse *Armadillidium vulgare* (Terrestrial Isopod, Crustacean). Dev Comp Immunol. 2005;29(6):489-99.

26. Bulet P, Stöcklin R, Menin L. Anti-microbial peptides: from invertebrates to vertebrates. Immunol Rev. 2004;198:169-84.

27. Raja Z, André S, Abbassi F, Humblot V, Lequin O, Bouceba T, et al. Insight into the mechanism of action of temporin-SHa, a new broad-spectrum antiparasitic and antibacterial agent. PLoS One. 2017;12(3):e0174024.

28. Abbassi F, Oury B, Blasco T, Sereno D, Bolbach G, Nicolas P, et al. Isolation, characterization and molecular cloning of new temporins from the skin of the North African ranid *Pelophylax saharica*. Peptides. 2008;29(9):1526-33.

29. Arendrup MC, Meletiadis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, et al. EUCAST technical note on isavuconazole breakpoints for *Aspergillus*, itraconazole breakpoints for *Candida* and updates for the antifungal susceptibility testing method documents. Clin Microbiol Infect. 2016;22(6):571.e1-4.

30. Ruhnke M, Behre G, Buchheidt D, Christopeit M, Hamprecht A, Heinz W, et al. Diagnosis of Invasive Fungal Diseases in Haematology and Oncology. 2018 Update of the Recommendations of the Infectious Diseases Working Party of the German Society for Hematology and Medical Oncology (AGIHO). Mycoses. 2018.

31. Verdon J, Falge M, Maier E, Bruhn H, Steinert M, Faber C, et al. Detergent-like activity and alpha-helical structure of warnericin RK, an anti-*Legionella* peptide. Biophys J. 2009;97(7):1933-40.

32. Verdon J, Labanowski J, Sahr T, Ferreira T, Lacombe C, Buchrieser C, et al. Fatty acid composition modulates sensitivity of *Legionella pneumophila* to warnericin RK, an antimicrobial peptide. Biochim Biophys Acta. 2011;1808(4):1146-53.

33. Zasloff M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci U S A. 1987;84(15):5449-53.

34. Alan AR, Earle ED. Sensitivity of bacterial and fungal plant pathogens to the lytic peptides, MSI-99, magainin II, and cecropin B. Mol Plant Microbe Interact. 2002;15(7):701-8.

35. Matsuzaki K, Sugishita K, Fujii N, Miyajima K. Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. Biochemistry. 1995;34(10):3423-9.

36. Abbassi F, Galanth C, Amiche M, Saito K, Piesse C, Zargarian L, et al. Solution structure and model membrane interactions of temporins-SH, antimicrobial peptides from amphibian skin. A NMR spectroscopy and differential scanning calorimetry study. Biochemistry. 2008;47(40):10513-25.

37. Helmerhorst EJ, Venuleo C, Beri A, Oppenheim FG. *Candida glabrata* is unusual with respect to its resistance to cationic antifungal proteins. Yeast. 2005;22(9):705-14.

38. Joly S, Maze C, McCray PB, Guthmiller JM. Human beta-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms. J Clin Microbiol. 2004;42(3):1024-9.

39. Tati S, Jang WS, Li R, Kumar R, Puri S, Edgerton M. Histatin 5 resistance of *Candida glabrata* can be reversed by insertion of *Candida albicans* polyamine transporter-encoding genes DUR3 and DUR31. PLoS One. 2013;8(4):e61480.

40. Grieco P, Carotenuto A, Auriemma L, Saviello MR, Campiglia P, Gomez-Monterrey IM, et al. The effect of d-amino acid substitution on the selectivity of temporin L towards target cells: identification of a potent anti-*Candida* peptide. Biochim Biophys Acta. 2013;1828(2):652-60.

41. Rinaldi AC, Mangoni ML, Rufo A, Luzi C, Barra D, Zhao H, et al. Temporin L: antimicrobial, haemolytic and cytotoxic activities, and effects on membrane permeabilization in lipid vesicles. Biochem J. 2002;368(Pt 1):91-100.

42. Mishra B, Wang X, Lushnikova T, Zhang Y, Golla RM, Narayana JL, et al. Antibacterial, antifungal, anticancer activities and structural bioinformatics analysis of six naturally occurring temporins. Peptides. 2018;106:9-20.

43. Atkinson BJ, Lewis RE, Kontoyiannis DP. *Candida lusitaniae* fungemia in cancer patients: risk factors for amphotericin B failure and outcome. Med Mycol. 2008;46(6):541-6.

44. Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V, et al. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* Species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. J Clin Microbiol. 2010;48(4):1366-77.

45. Bitew A, Abebaw Y. Vulvovaginal candidiasis: species distribution of *Candida* and their antifungal susceptibility pattern. BMC Womens Health. 2018;18(1):94.

46. Shenmar K, Sharma KK, Wangoo N, Maurya IK, Kumar V, Khan SI, et al. Synthesis, stability and mechanistic studies of potent anticryptococcal hexapeptides. Eur J Med Chem. 2017;132:192-203.

47. Abbassi F, Lequin O, Piesse C, Goasdoué N, Foulon T, Nicolas P, et al. Temporin-SHf, a new type of phe-rich and hydrophobic ultrashort antimicrobial peptide. J Biol Chem. 2010;285(22):16880-92.

48. Abbassi F, Raja Z, Oury B, Gazanion E, Piesse C, Sereno D, et al. Antibacterial and leishmanicidal activities of temporin-SHd, a 17-residue long membrane-damaging peptide. Biochimie. 2013;95(2):388-99.

49. Mangoni ML, Rinaldi AC, Di Giulio A, Mignogna G, Bozzi A, Barra D, et al. Structure-function relationships of temporins, small antimicrobial peptides from amphibian skin. Eur J Biochem. 2000;267(5):1447-54.

50. de Alteriis E, Maselli V, Falanga A, Galdiero S, Di Lella FM, Gesuele R, et al. Efficiency of gold nanoparticles coated with the antimicrobial peptide indolicidin against biofilm formation and development of. Infect Drug Resist. 2018;11:915-25.

51. De Brucker K, Delattin N, Robijns S, Steenackers H, Verstraeten N, Landuyt B, et al. Derivatives of the mouse cathelicidin-related antimicrobial peptide (CRAMP) inhibit fungal and bacterial biofilm formation. Antimicrob Agents Chemother. 2014;58(9):5395-404.

52. Hollmann A, Martinez M, Maturana P, Semorile LC, Maffia PC. Antimicrobial Peptides: Interaction With Model and Biological Membranes and Synergism With Chemical Antibiotics. Front Chem. 2018;6:204.

53. Bondaryk M, Staniszewska M, Zielińska P, Urbańczyk-Lipkowska Z. Natural Antimicrobial Peptides as Inspiration for Design of a New Generation Antifungal Compounds. J Fungi (Basel). 2017;3(3).

Acknowledgements

We thank E. Bere (ImageUP, University of Poitiers) and M.L. Mollichella (Laboratoire MDE, University of Poitiers) for expert technical advice with electron microscopy and flow cytometry assays, respectively.

We thank J. Ashram for English revision.

Figures and tables

Table 1: MICs of armadillidin H, warnericin RK and magainin II against yeasts and filamentous fungi according to EUCAST guidelines.

Results are expressed in µg/mL (µM).

	Armadillidin H	Warnericin RK	Magainin II
Yeasts			
Candida albicans (ATCC 14053)	>128 (>90)	>128 (>90)	>128 (>90)
Candida glabrata (ATCC MYA 2950)	>128 (>90)	>128 (>90)	>128 (>90)
Candida parapsilosis (ATCC 22019)	>128 (>90)	>128 (>90)	>128 (>90)
Candida krusei (ATCC 6258)	>128 (>90)	>128 (>90)	>128 (>90)
Cryptococcus neoformans (CS)	ND	>128 (>90)	>128 (>90)
Moulds			
Aspergillus fumigatus (ATCC 16424)	>128 (>90)	>128 (>90)	>128 (>90)
Lichtheimia corymbifera (CNRMA 2011.1047)	>128 (>90)	>128 (>90)	>128 (>90)
Fusarium oxysporum (CS)	>128 (>90)	>128 (>90)	>128 (>90)
Scedosporium apiospermum (CS)	>128 (>90)	>128 (>90)	>128 (>90)

ND: not determined
CS: clinical strain

Table 2: MICs of temporin [K3]SHa against yeasts and filamentous fungi according to EUCAST guidelines.

Results are expressed in µg/mL (µM).

	Temporin [K3]SHa
Yeasts	
Candida albicans (ATCC 14053)	32 (23)
Candida albicans (ATCC 90028)	32 (23)
Candida glabrata (ATCC MYA 2950)	>128 (>90)
Candida glabrata (CS)	128 (90)
Candida krusei (ATCC 6258)	32 (23)
Candida krusei (CS)	64 (45)
Candida parapsilosis (ATCC 22019)	16 (11)
Candida kefyr (CS)	16 (11)
Candida lusitaniae (ATCC 34449)	16 (11)
Candida dublinensis (CS)	32 (23)
Candida tropicalis (CS)	16 (11)
Candida utilis (ATCC 9950)	8 (6)
Cryptococcus neoformans (CS)	32 (23)
Moulds	
Aspergillus fumigatus (ATCC 16424)	128 (90)
Lichtheimia corymbifera (CNRMA 2011.1047, Paris, France)	64 (45)
Fusarium oxysporum (CS)	>128 (>90)
Scedosporium apiospermum (CS)	>128 (>90)

CS: clinical strain

Figure 1: Time-killing curve of temporin [K3]SHa against *C. albicans*. *C. albicans* (10^5 CFU/mL) was incubated with temporin [K3]SHa at 2 x MIC (64 µg/mL), MIC (32 µg/mL) and 0.5 x MIC (16 µg/mL). Data are shown as the means ± SD of three independent experiments performed in triplicate.

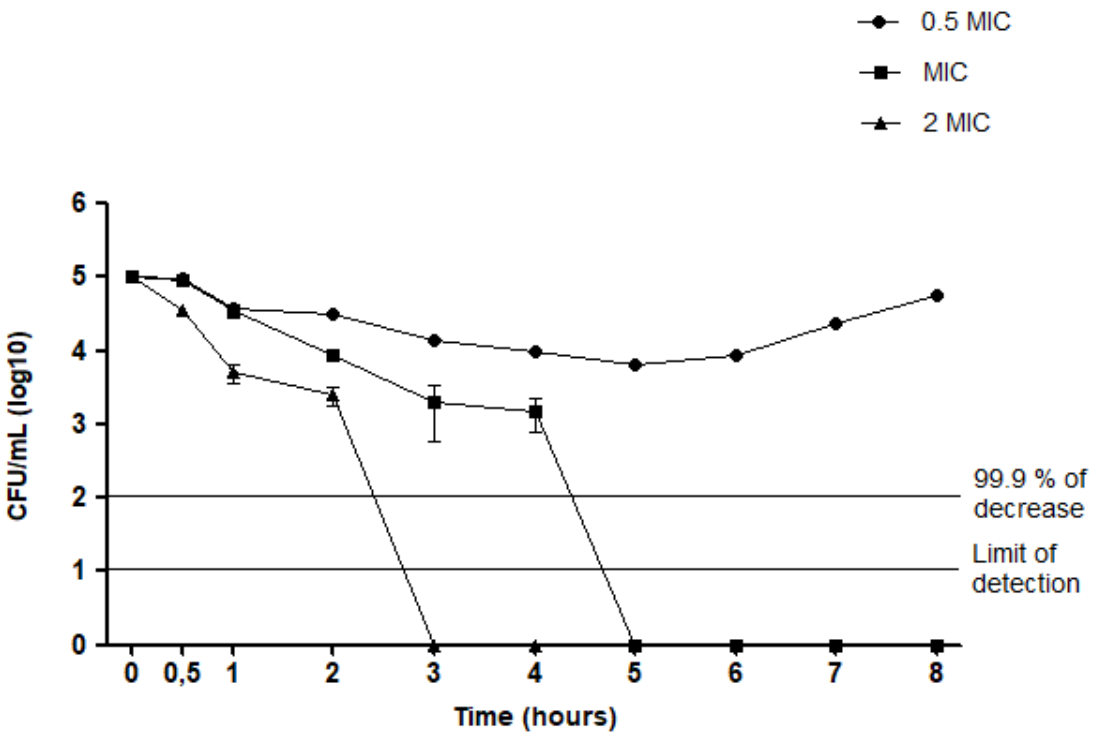


Figure 2: Fungal membrane permeabilization induced by temporin [K3]SHa against *C. albicans*. *C. albicans* (105 CFU/mL) was incubated with temporin [K3]SHa at 4 x MIC (128 µg/mL), 2 x MIC (64 µg/mL), MIC (32 µg/ml), 0.5 x MIC (16 µg/mL). Propidium iodide was added at 30 min, 1h and 3h and fluorescence was assessed by flow cytometry. Light grey curve represents non-permeabilized cells and dark grey curve (IP+) permeabilized cells. Percentage of permeabilized cells was expressed above dark grey curve.

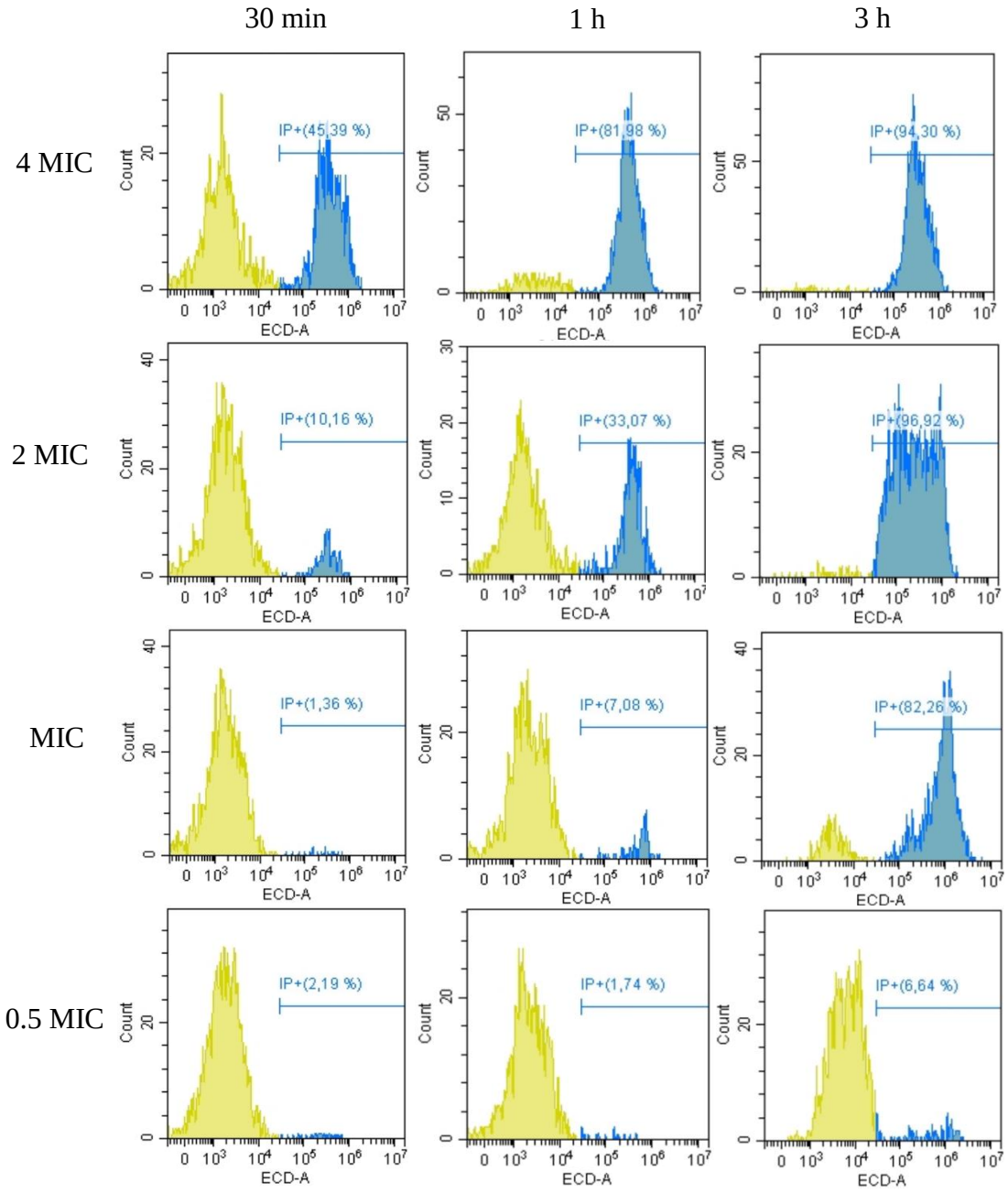


Figure 3: Scanning electron microscopy of *C. albicans* treated by temporin [K3]SHa. *C. albicans* (10^5 CFU/mL) was incubated 3h at 37°C in RPMI medium without peptide (A) or with temporin [K3]SHa at 32 µg/mL (B, C and D). No more hyphae were visible and fungal structures were altered and perforated when *C. albicans* was treated with temporin [K3]SHa.

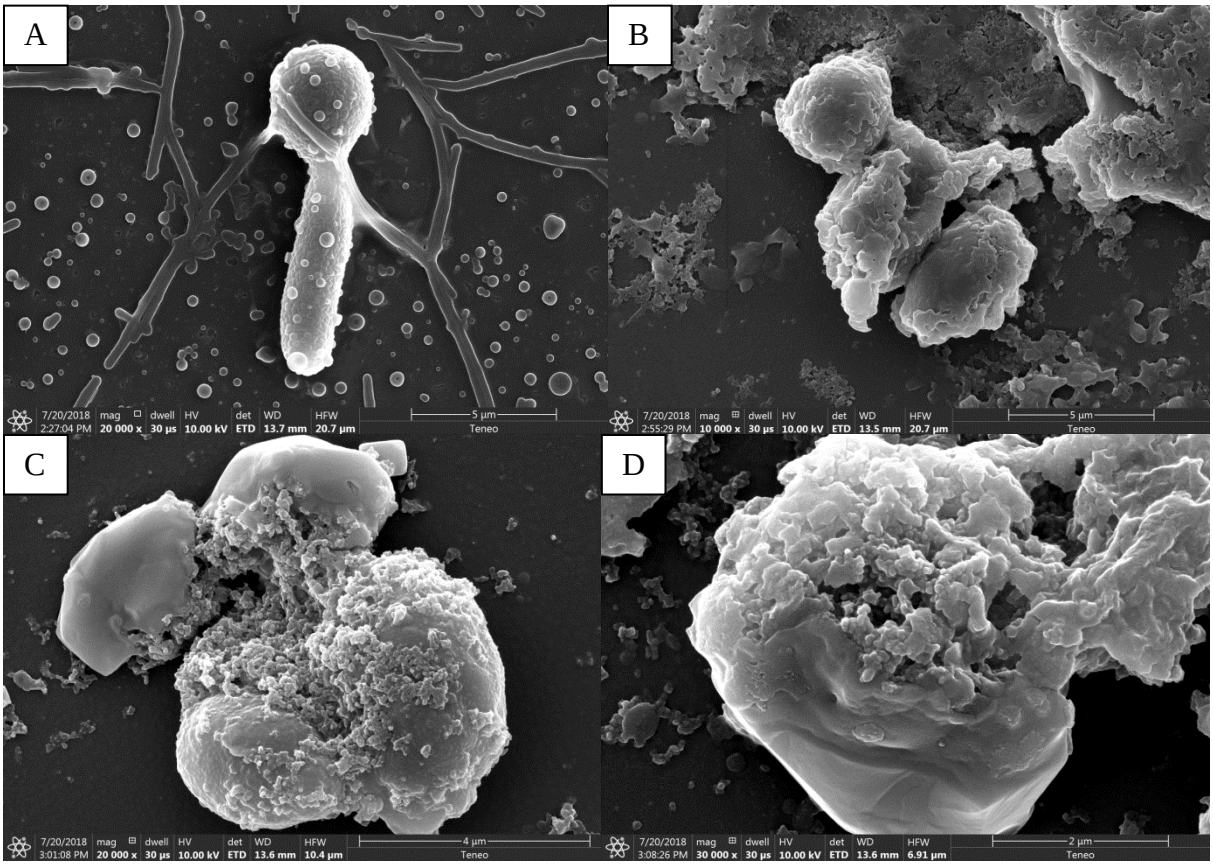


Figure 4: Transmission electron microscopy of *C. albicans* treated by temporin [K3]SHa. *C. albicans* (10^5 CFU/mL) was incubated 3h at 37°C in RPMI medium without peptide (A) or with temporin [K3]SHa at 32 µg/mL (B, C and D). Fungal wall was thinned and distorted (arrows) when *C. albicans* was treated with temporin [K3]SHa. Moreover, a modification of cytoplasmic density and content was shown.

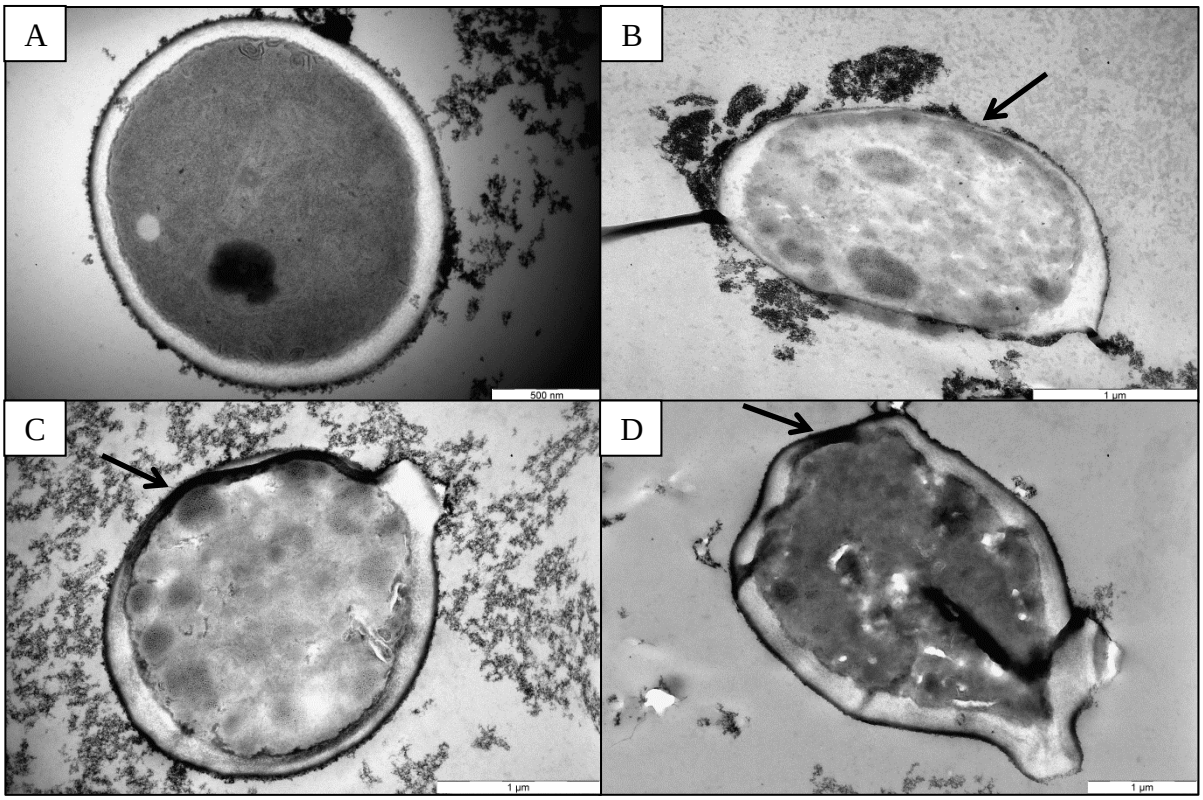
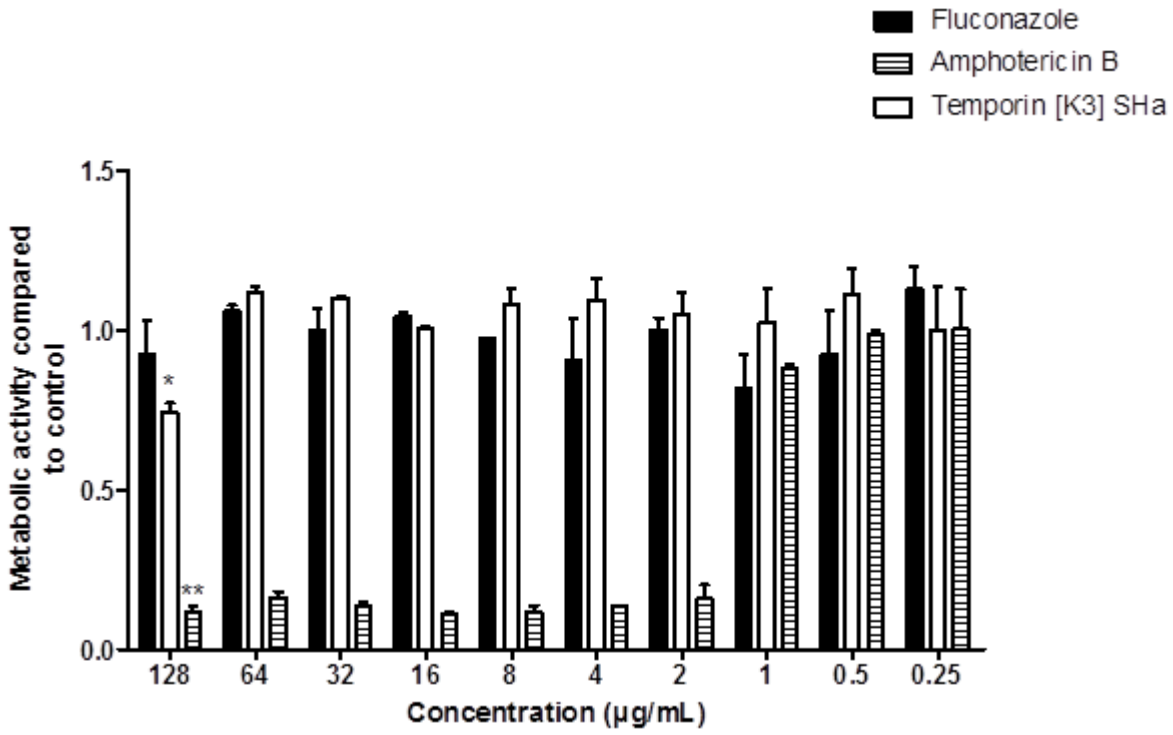


Figure 5: Metabolic activity of *C. albicans* biofilm incubated with temporin [K3]SHa. *C. albicans* 12h biofilm (10^4 CFU/mL) was incubated 24h with temporin [K3]SHa, amphotericin B, fluconazole or without drug (negative control). Metabolic activity was evaluated by XTT-assay. Data are shown as the means ± SD of metabolic activity compared to negative control. Three independent experiments were performed in triplicate. (*p < 0.01; **p < 0.001).



Résumé

Les levures et champignons filamenteux sont une cause émergente d'infections et représentent un problème important de santé publique chez les patients immunodéprimés. Cependant, le nombre d'antifongiques disponibles pour lutter contre ces maladies graves reste très faible. Les peptides antimicrobiens, qui sont des molécules faisant partie du système immunitaire de nombreuses espèces, représentent une voie de recherche prometteuse mais c'est surtout leur effet antibactérien qui a été jusque-là étudié. Le but de notre étude a été de tester l'activité de la warnericine RK, de l'armadillidine H, de la magainine II et de la temporine [K3]SHa contre certaines levures et moisissures puis d'explorer l'activité de la temporine [K3]SHa sur *C. albicans*.

Afin d'évaluer l'activité antifongique de ces peptides, leurs CMI ont été déterminées selon les recommandations de l'EUCAST. Puis, l'activité de la temporine [K3]SHa contre *C. albicans* a été évaluée par une courbe de mortalité, un test de perméabilisation membranaire et une observation en microscopie électronique. Enfin, l'action de la temporine [K3]SHa contre les biofilms de *C. albicans* a été étudiée.

La warnericine RK, l'armadillidine H et la magainine II n'ont présenté aucune efficacité antifongique contre les champignons testés. En revanche, la temporine [K3]SHa a exercé une activité contre *Cryptococcus neoformans* et la plupart des souches de *Candida* testées, excepté *C. glabrata*. L'efficacité contre les moisissures s'est révélée quant à elle très modérée. La temporine [K3]SHa a été rapidement fongicide contre *C. albicans* avec un mécanisme d'action qui semble dû à une perméabilisation membranaire et à des altérations des structures fongiques. En revanche, seule une légère activité anti-biofilm a pu être observée.

La temporine [K3] SHa pourrait représenter un nouveau composé antifongique potentiel contre les levures du genre *Candida* non *glabrata* et *C. neoformans*. D'autres études sont cependant nécessaires pour préciser son mode d'action et pour explorer une synergie potentielle avec les agents antifongiques conventionnels.

Mots clés : peptides antimicrobiens, temporine [K3]SHa, levures, champignons filamenteux, *Candida albicans*

SERMENT DE GALIEN

~~~~

Je jure, en présence des maîtres de la faculté et de mes condisciples :

D'honorer ceux qui m'ont instruit dans les préceptes de mon art et de leur témoigner ma reconnaissance en restant fidèle à leur enseignement.

D'exercer, dans l'intérêt de la santé publique, ma profession avec conscience et de respecter non seulement la législation en vigueur, mais aussi les règles de l'honneur, de la probité et du désintéressement.

De ne jamais oublier ma responsabilité, mes devoirs envers le malade et sa dignité humaine, de respecter le secret professionnel.

En aucun cas, je ne consentirai à utiliser mes connaissances et mon état pour corrompre les mœurs et favoriser des actes criminels.

Que les hommes m'accordent leur estime si je suis fidèle à mes promesses.

Que je sois couvert d'opprobre et méprisé de mes confrères si j'y manque.
~~~~