UNIVERSITÉ DE LA MÉDITERRANÉE AIX-MARSEILLE II
FACULTE DE MÉDECINE ET DE PHARMACIE DE MARSEILLE
ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

THÈSE DE DOCTORAT

Présentée par

ALHANOUT Kamel

En vue de l'obtention du grade de **DOCTEUR** de **L'UNIVERSITÉ** de la MÉDITERRANÉE
Spécialité : Maladies Transmissibles et Pathologies Tropicales

Evaluation de l'activité antimicrobienne de nouveaux composés aminostéroïdiens dans le contexte de la mucoviscidose

Soutenue le :

22/06/2010

Composition du Jury :

Professeur Bernard La Scola	Président de jury
Professeur Alain Philippon	Examinateur
Docteur Michel Arthur	Rapporteur
Professeur Hélène Marchandin	Rapporteur
Professeur Jean-Marc Rolain	Directeur de thèse
Docteur Jean-Michel Brunel	Co-Directeur de thèse

Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes
URMITE CNRS-IRD UMR 6236

Sommaire

Avant propos……1

Résumé…….3

Summary……..5

Introduction……..7

Chapitre 1: Revue……....13

Article 1: Aminosterols as a new class of antimicrobial agents: a critical review……...17

Chapitre 2: Partie I

Evaluation de l'activité antimicrobienne des dérivés aminostéroïdiens contre des souches microbiennes cliniques……49

Article 2: In vitro antibacterial activity of aminosterols against multidrug-resistant bacteria from patients with cystic fibrosis.....51

Article 3: In vitro antifungal activity of aminosterols against moulds isolated from cystic fibrosis patients.....……..61

Article 4: In vitro activity of aminosterol compounds against Yeasts Isolated from Blood Stream infections……..67

Chapitre 2 : Partie II
Analyse du mécanisme d'action antibactérienne de la squalamine……..99

Article 5: New Insights on the Antibacterial Mechanism of Action of Squalamine……..…101

Conclusions générales et perspectives……...……..113

Annexes……...121

AVANT PROPOS :

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

Résumé

Les dérivés aminostéroïdiens (DASs) comme la squalamine et ses analogues ont démontré une activité antimicrobienne évaluée *in vitro* contre des bactéries et des champignons de référence et jamais contre des souches cliniques et/ou multi résistantes. Le mécanisme d'action de la squalamine vis-à-vis des bactéries à Gram-négatif a été partiellement démontré suggérant que cette molécule agit en perturbant l'intégrité de la membrane externe de ces bactéries. A l'inverse, son mécanisme d'action contre les bactéries à Gram-positif n'a pas été étudié. Ainsi, l'objectif de ce travail a été d'évaluer d'une part l'activité antimicrobienne *in vitro* de la squalamine et de dérivés aminostéroïdiens contre un panel de souches cliniques de bactéries et de champignons filamenteux isolés de crachats de patients atteints de mucoviscidose et de levures impliquées dans des fongémies, et d'autre part de mieux comprendre leur mécanisme d'action vis-à-vis des bactéries à Gram-positif et à Gram-négatif. Nous avons trouvé que les DASs possèdent une activité antibactérienne intéressante qui était d'autant plus importante chez les bactéries non mucoïde et/ou sensible à la colistine laissant supposer qu'une corrélation existait entre l'activité des DASs et celle de la colistine vis-à-vis des bactéries à Gram-négatif. Malgré cette corrélation, les DASs ont été plus actifs contre les bactéries à Gram-positif suggérant la présence de deux mécanismes d'action différents à l'égard des deux groupes de bactéries. Alors que les agents antifongiques classiques ont montré des activités hétérogènes vis-à-vis des champignons filamenteux et des levures

testés d’une manière dépendante de l’espèce, les DASs ont présenté des activités homogènes indiquant que ces molécules possèdent un mécanisme d’action différent de ceux rapportés avec les antifongiques classiques. Ainsi, nos données préliminaires ont indiqué que la squalamine induit une rupture de la membrane de levures par, probablement, un effet "mécanique". L’analyse du mécanisme d’action antibactérien de la squalamine a montré que cette molécule agit en perturbant l’intégrité de bactéries à Gram-négatif par un mécanisme comparable à celui d’un détergeant et en dépolarisant la membrane des bactéries à Gram-positif conduisant à la rupture totale de cette membrane et au drainage de la matière cytoplasmique.

Mots clés : Squalamine, mucoviscidose, aminostérol, multi résistance, infections pulmonaires.

Summary

Aminosterol derivatives (ASDs) such as squalamine and its analogs have demonstrated an interesting *in vitro* antimicrobial activity evaluated against bacterial and fungal reference strains and never against clinical and / or multi-resistant pathogens. It was shown that squalamine acts against Gram-negative bacteria by disrupting the integrity of their outer membrane. Instead, its mechanism of action against Gram-positive bacteria has never been studied. Thus, the aim of this study was firstly to evaluate *in vitro* antimicrobial activity of squalamine and two synthesized ASDs against a panel of clinical strains of bacteria and filamentous fungi isolated from sputum of cystic fibrosis patients and yeasts involved in fungemia, and secondly to better understand their mechanism of action against Gram-positive and Gram-negative bacteria. We found that ASDs possess an interesting *in vitro* antibacterial activity which was more important against non-mucoid and/or colistin sensitive strains suggesting that a correlation exists between the activity of ASDs and that of colistin against Gram-negative bacteria. Despite this correlation, ASDs were more active against Gram-positive bacteria indicating the presence of two different mechanisms of action against both groups of bacteria. While classical antifungal agents showed heterogeneous activities against tested filamentous fungi and yeasts specie-dependent manner, ASDs displayed homogeneous activity indicating that these molecules may possess a mechanism of action different from those reported with conventional antifungals. Thus, in our work

we have demonstrated that squalamine induced the disruption of yeast memb rane, probably via a "mechanical" effect. Analysis of the antibacterial mechanism of action of squalamine has shown that this molecule acts by disrupting the integrity of bacterial membrane of Gram-negative bacteria via a detergent-like mode of action and by depolarizing the membrane of Gram-positive bacteria leading to a total disruption of this membrane.

Mots clés : Squalamine, cystic fibrosis, aminosterol, multi resistance, lung infections.

Introduction :

L'introduction des antibiotiques en médecine humaine a considérablement amélioré le traitement des maladies infectieuses et ce au niveau mondial. [1-4] Toutefois, avec cet usage de plus en plus important, le problème de la résistance aux antibiotiques ne cesse d'augmenter et ceci est particulièrement inquiétant dans le contexte de la mucoviscidose où les patients sont, et à cause d'un environnement pulmonaire fragile, exposés à de nombreux pathogènes pouvant engendrer des infections pulmonaires récurrentes. Les pathogènes les plus fréquemment rencontrés dans ce contexte sont des bactéries comme *Staphylococcus aureus, Pseudomonas aeruginosa* et *Burkholderia cepacia* et/ou des champignons comme *Candida albicans*, *Aspergillus fumigatus, Aspergillus terreus* et *Scedosporium apiospermum*. [5-10] L'usage d'agents antimicrobiens par voie générale pour le traitement des infections pulmonaires dans le contexte de la mucoviscidose a diminué la fréquence de ces infections et les taux d'hospitalisation tout en améliorant la qualité et l'espérance de vie de ces patients. [11-15] Cependant, les données cliniques actuelles montrent que l'éradication totale des pathogènes du milieu pulmonaire est irréalisable du fait de l'impossibilité à assurer une biodisponibilité pulmonaire suffisamment élevée du médicament sans engendrer de graves effets secondaires. [14, 16, 17] En revanche, l'administration locale de tels médicaments sous forme d'aérosols a permis d'obtenir des concentrations pulmonaires élevées du médicament sans passage systémique et donc sans effets secondaires. [11, 13, 14] C'est pour cette raison que

des antibiotiques de la famille des aminosides comme la tobramycine ont été et sont encore utilisés sous forme d'aérosols, tout comme la colistine qui avait été abandonnée pour cause de toxicité en usage systémique, retrouvent un regain d'intérêt dans le contexte de la mucoviscidose. [13-15] Toutefois, l'émergence de bactéries et/ou de champignons multi résistants limite le choix thérapeutique avec les antimicrobiens disponibles. [16-20] Ainsi, la recherche de nouvelles molécules antimicrobiennes constitue une approche essentielle pour lutter contre le phénomène de résistance. La squalamine, un dérivé aminostéroïdien naturel, a été découverte en 1993 dans le foie du requin du genre *Squalus acanthias.* [21] L'activité antimicrobienne évaluée *in vitro* contre des bactéries et des champignons a très vite démontrée de fortes potentialités pour ce dérivé aminostéroïdien. [22, 23] Néanmoins, c'est essentiellement son activité antiangiogénique qui a été le plus étudiée dans des études cliniques. [24-26] Un inconvénient à l'utilisation de cette molécule réside dans le fait que sa synthèse est assez complexe et onéreuse avec des rendements de synthèse médiocres. [24-28] L'alternative la plus intéressante a consisté à synthétiser des molécules analogues à la squalamine à partir de produits beaucoup moins coûteux en utilisant des protocoles de synthèse plus simples et plus économiques que ceux mis en œuvre dans la synthèse de la squalamine. [22, 29] L'activité antimicrobienne de ces molécules a été exclusivement déterminée vis-à-vis de souches bactériennes et fongiques de référence et n'a jamais été évaluée contre des souches cliniques multi résistantes. [21, 22] Il a été montré que la squalamine agit

contre les bactéries à Gram négatif en perturbant l'intégrité de leur membrane externe après interaction avec les groupements de phosphates qui sont chargés négativement. [30] Cependant, ceci ne peut pas expliquer son action contre les bactéries à Gram positif étant donné que ce groupe de bactéries ne possède pas de groupes phosphates avec lesquels la squalamine pourrait interagir. Malgré leur large spectre d'activité antimicrobienne, ces molécules ont montré des concentrations minimales inhibitrices (CMIs) relativement élevées indiquant que leur utilisation par voie générale serait probablement toxique. Ainsi, le développement de ces molécules ne peut être envisagé que pour une administration par voie locale telle que la voie pulmonaire sous forme d'aérosol qui pourrait représenter une stratégie intéressante dans le traitement des infections pulmonaires des patients atteints de mucoviscidose. Notre travail a ainsi porté dans une première partie sur l'évaluation de l'activité antimicrobienne de la squalamine et de deux dérivés aminostéroïdiens (DAS 1-2, Figure 1) contre un panel de souches cliniques de bactéries contenant des souches mucoïdes et des souches non mucoïdes (selon le caractère phénotypique de croissance sur les milieux de culture) ainsi que des souches multi et/ou pan-résistantes aux différents antibiotiques. De plus, l'activité antifongique *in vitro* de ces molécules a été testée contre une collection de souches cliniques de champignons filamenteux isolés de patients atteints de mucoviscidose et de levures impliquées dans des fongémies. L'ensemble des souches fongiques a été identifié par des techniques phénotypiques et moléculaires. Dans une seconde

partie, de nouveaux éléments concernant le mécanisme d'action antibactérienne et antifongique de la squalamine ont été mis en évidence particulièrement vis-à-vis des bactéries à Gram positif et des levures. Finalement, la faisabilité d'une formulation d'aérosol à partir d'une solution aqueuse contenant un des composés testés (DAS1) a été évaluée.

Concernant la première partie de notre travail, nous avons montré que les aminosterols testés possèdent des valeurs de CMIs variant de 2 à 128 mg/L pour les bactéries à Gram négatif et de 0,5 à 8 mg/L pour les isolats à Gram- positif, excepté pour les isolats de *S. pneumoniae* (souches capsulées) qui avaient des valeurs de CMI de 32 mg/L (Article **2**). Nous avons montré dans cette partie que l'évaluation de l'activité antibactérienne des DASs contre des souches de référence ne permet pas d'estimer correctement leurs effets antimicrobiens. En effet, les DASs se sont révélés plus actifs contre les souches non mucoïdes et/ou les souches sensibles à la colistine en comparaison avec les souches mucoïdes et/ou celles qui sont résistantes à cet antibiotique. Malgré la corrélation entre l'activité des DASs et celle de la colistine contre les bactéries à Gram-négatif, les DASs ont montré une activité nettement supérieure contre les bactéries à Gram-positif suggérant que ces molécules aient deux mécanismes d'action différents vis-à-vis de ces deux groupes de bactéries. Ces résultats nous ont mené à dédier la seconde partie de notre travail à l'analyse du mécanisme d'action de la squalamine qui nous sembler original. Par ailleurs, des CMIs variant de 8 à 16 mg/L et de 2 à 4 mg/L contre tous les isolats des champignons

filamenteux testés, ont été trouvées pour respectivement, la squalamine et le DAS1 (Article **3**). De la même manière, des CMIs homogènes variant de 8 à 16 mg / L et de 1 à 2 mg / L contre tous les isolats des levures testés, ont été trouvées pour respectivement, la squalamine et le DAS1 (Article **4**). A l'inverse, les agents antifongiques classiques ont présenté des activités *in vitro* hétérogènes (CMIs variant de <0.5 à > 32 mg/L) dans les deux études (les résultas de l'identification de souches des champignons filamenteux par les techniques phénotypiques et moléculaires ont été discutés dans l'Article **6**, Annexe). Ceci suggère que les DASs probablement possèdent un mécanisme d'action antifongique différent de ceux rapportés avec les agents antifongiques classiques. L'analyse du mécanisme d'action antibactérien de la squalamine a démontré la présence de deux modes d'action différents de cette molécule contre les bactéries à Gram-négatif et à Gram-positif (Article **4**). Dans le cas des bactéries à Gram-négatif, la squalamine partage avec la colistine le besoin d'interagir avec les groupes phosphates chargés négativement pour produire une série d'effets qui seraient spécifiques à chaque composé. En effet, la colistine est connue pour agir en créant des pores dans les membranes des bactéries à Gram-négatif (Article **5**). D'après les résultats obtenus dans ce travail, la squalamine agit en perturbant l'intégrité des membranes des bactéries à Gram-négatifs par un mécanisme d'action comparable à celui d'un détergent et en créant des lésions beaucoup plus importantes que celles produites par la colistine. Ceci a été révélé en analysant les effets de chaque composé sur la morphologie de la membrane

bactérienne et en mesurant leurs effets sur le flux d'ATP intracellulaire des bactéries. D'une manière intéressante, il a été trouvé que la squalamine produit une rupture complète de la membrane bactérienne des bactéries à Gram-positif. Cet effet s'est révélé comme étant le résultat d'une action dépolarisante de la squalamine spécifiquement sur la membrane des bactéries à Gram-positif et pas sur les bactéries à Gram-négatif. Par ailleurs, nous avons pu obtenir des éléments préliminaires concernant le mécanisme d'action antifongique de la squalamine contre les levures suggérant que la squalamine agit en perturbant l'intégrité de la membrane fongique comme démontrée par la vitesse de l'effet fongicide de ce composé et son effet sur l'efflux d'ATP intracellulaire (Article **4**). Finalement, dans un travail préliminaire nous avons démontré, par l'analyse des caractères aérodynamiques de formulations contenant le composé DAS1 dans une solution aqueuse, la possibilité d'intégrer ces molécules dans des formulations à visée pulmonaire locale (Article **7**, Annexe).

Chapitre 1

Article 1: Revue

Aminosterols as a new class of antimicrobial agents: a critical review.

Kamel Alhanout, Jean Marc Rolain and Jean Michel Brunel*

* Corresponding author, e-mail: bruneljm@yahoo.fr.

Submitted to **Current Medicinal Chemistry**

Résumé

La recherche de nouveaux composés ayant une activité antimicrobienne représente une stratégie importante pour contourner le problème de la résistance aux antimicrobiens. Dans ce contexte, les dérivés aminostéroïdiens comme la squalamine, une molécule naturelle, et les dérivés aminostéroïdiens synthétiques comme ceux ayant une structure semblable à celle de la squalamine et les ceragenins (composé désignés pour mimer l'activité des antibiotiques poly peptidiques comme la colistine), ont suscité un intérêt en raison de leur large spectre d'action antibactérien et antifongique. Nous avons analysé dans cette revue les activités antimicrobiennes de ces composés et les éléments disponibles concernant leur mécanisme d'action antibactérienne. Ainsi, en dépit de leur bonne activité contre des souches bactériennes de référence, ces composés se sont révélés actifs contre des bactéries multi résistantes. En outre, leur mécanisme d'action reste tout à fait singulier à l'égard de tous les antibiotiques connus puisqu'il est maintenant démontré que ces molécules agissent en perturbant l'intégrité de la membrane bactérienne suggérant aussi une potentialité pour leur développement en tant que décontaminants ou désinfectants.

Aminosterols as a new class of antimicrobial agents: a critical review

Kamel Alhanout, Jean Marc Rolain and Jean Michel Brunel*

Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes (URMITE) UMR 6236 CNRS, Faculté de Pharmacie et de Médecine, Université de la Méditerranée, 27 boulevard Jean Moulin, 13385 Marseille 05, France.

* Corresponding author, e-mail: bruneljm@yahoo.fr.

Abstract:

An important strategy to circumvent the problem of antimicrobial resistance is to search for new compounds with antimicrobial activity. In this context, aminosterols, which include squalamine-like compounds and ceragenins, have gained interest due to their wide spectrum of antibacterial and antifungal properties. In light of recently reported data, we decided to analyze the mechanism of action of these compounds as well as their antimicrobial properties. Aminosterols are active against both bacterial reference strains and multidrug-resistant antibiotics as they disrupt the integrity of the bacterial membrane. Thus, these compounds could be useful in the development of new topical decontaminants or disinfecting agents.

Introduction

Antimicrobial agents have always played a crucial role in the management of microbial infections and the improvement of human health. On the other hand, the emergence of antimicrobial resistance constitutes a serious, life-threatening problem that limits treatment options and stimulates the discovery of new antimicrobial compounds.[1,2] In this context, aminosterol derivatives (ASDs) have recently gained interest due to their effective antimicrobial activities.[3,4] Generally, ASDs designate molecules that are structurally composed of a sterol core substituted with one or more polyamine side chains (Figure **1**). ASDs are usually divided into squalamine parent derivatives and cholic-acid-derived ceragenins (Figure 1), which are studied separately.[5]

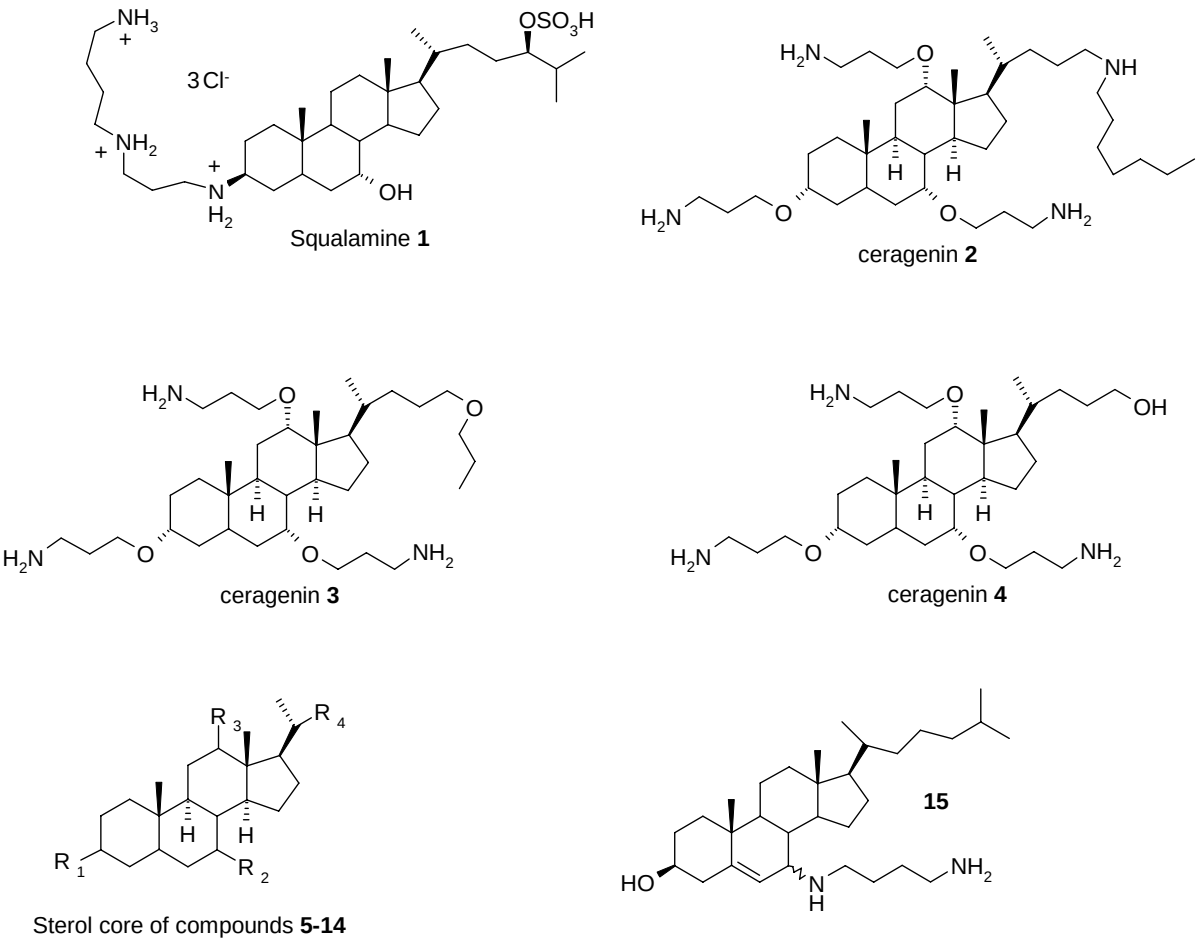


Figure **1**: Structures of squalamine **1**, ceragenins **2-4**, the sterol core of compounds **5-14**, and ASD **15.**

ASDs were previously studied for their antiangiogenic and antiobesity properties[6-15], They were also shown to exhibit interesting *in vitro* antibacterial and antifungal activities, thereby suggesting that they may be potent candidates for antimicrobial drug development.[3,4] While previous reviews [3-5,16] essentially focused on the *in vitro* activity of ASDs and their large antimicrobial spectrum, the goal of our study was to evaluate the utility of these compounds from a clinical and microbiological standpoint. Thus, we determined the *in vitro*

activities of various ASDs against relevant multidrug-resistant (MDR) pathogens and analyzed their mechanism of action.

Squalamine and squalamine-like aminosterols (SLAs)

While looking for new antimicrobial agents in nature, Michael Zasloff discovered squalamine **1** in 1993 in the tissues of the Dogfish shark (*Squalus acanthias)*.[17,18] Squalamine was first isolated from the shark liver and gallbladder, which correspond to the sites of bile synthesis.[19] The structure of squalamine consists of a sterol core substituted with a sulfate-containing moiety, a hydroxyl group, and a spermidine moiety attached (Figure **1** and Tables **1** and **2**).[20]

Table **1**: Antimicrobial activities of various aminosterols selected from the literature against reference bacterial and fungal strains.

Side chains	Compound (Ref)	MICs mg/L									
		Gram-positive bacteria				Gram-negative bacteria				Fungi	
		S. aureus CIP2	*S. aureus* ATCC 25923	*S. pneumoniae* ATCC 6305	*S pyogenes* ATCC 19615	*E. coli* CIP54127	*E. coli* ATCC25922	*P. aeruginosa* ATCC 27853	*K. pneumoniae* ATCC 27853	*C albicans* ATCC90028	*A. fumigatus* H1120
Squalamine **1**	[3,42]	2	2	-	-	2	1-2	4-8	-	4-8	-
R_1=R_2 =H, R_3=OH R_4:CO-spermine	**5**[28]	-	1.56	3.13	0.78	-	3.13	3.13	3.13	3.13	12.5
R_1=R_2=H, R_3=OH R_4=CO-putrescine	**6**[28]	-	25	25	6.25	-	50	100	50	25	50
R_1= R_2=R_3=H R_4=CO-spermine	**7**[28]	-	3.13	0.78	3.13	-	3.13	12.5	6.25	3.13	3.13
R_1=OH, R_2=R_3=H R_4=CO-spermine	**8**[28]	-	0.78	3.13	0.78	-	1.56	0.78	> 100	3.13	12.5
R_1= R_2=OH,	**9**[28]	-	1.56	3.13	0.78	-	3.13	3.13	3.13	3.13	12.5

R_3=H R_4=CO-spermine R_1=R_2 =R_3=OH R_4=CO-spermine	**10**[28]	-	6.25	25	3.13	-	25	25	>100	12.5	25
R_1=OH, R_2=NH_2 R_3 =R_4=H	**11**[26]	>100	-	-	-	>100	-	-	-	-	-
R_1=OH, R_3=R_4=H R_2= putrescine	**12**[26]	2.5	-	-	-	2.5	-	-	-	-	-
R_1=OH, R_3=R_4=H R_2= cadaverine,	**13**[26]	2.5	-	-	-	2.5	-	-	-	-	-
R_1=OH, R_3=R_4=H R_2= 1,12 diamine	**14**[26]	10	-	-	-	50	-	-	-	-	-
Ceragenin **2**[15,30]		-	2	-	2.3	-	3	3.2	5.8	45	-
Ceragenin **3**[15,30]		-	4.2	-	3	-	7.4	6.4	27	47	-
Ceragenin **4**[15,30]		-	9.2	-	5.8	-	40	36	50	29	-

Gram-negative bacteria	Compounds				Gram-positive bacteria	Compounds			
	1	**15**	**2**[#]	**3**[#]		**1**	**15**	**2**[#]	**3**[#]
Pseudomonas aeruginosa	2-8	2-8	2-32	30	*Staphylococcus aureus*	2-8	0.5-4	4	9-15
*Pseudomonas aeruginosa**	4	4	-	-	*Streptococcus pneumoniae*	32	32	4	9-15
Haemophilus influenzae	4-8	4-8	-	-	*Enterococcus faecalis*	-	-	3	25
Escherichia coli	8	16	2-37	53.5	*Aspergillus fumigatus*	8-16	4	-	-
Acinetobacter baumannii	8	16	-	-	Filamentous fungi	Compounds			
Achromobacter xylosoxidans	64	64	-	-		**1**	**15**	**2**[#]	**3**[#]
Klebsiella pneumoniae	8	8-16	1	41.3	*Aspergillus niger*	16	4	-	-
Burkholderia cepacia	16-64	16-64	-	-	*Fusarium* spp.	16	2-4	-	-
Inquilinus limosus	16-64	16-64	-	-	*Scedosporium prolificans*	8-16	2-4	-	-
Enterobacter aerogenes	32	32	-	-	*Rhizopus* spp.	16	2	-	-

Salmonella typhimurium	-	-	0.8	15-20	*Rhizomucor* spp.	16	2	-	-

Table **2**: Antimicrobial activities of selected aminosterols against clinical bacterial and fungal isolates.

***** mucoid isolates; Data designated with an # were obtained from Savage *et al.*[31] The rest is from Alhanout *et al.* [37]

Squalamine was first isolated and its original structure was determined. Its antimicrobial activity against a broad spectrum of bacteria and fungi was subsequently evaluated (Figure **1**).[19] The low abundance of squalamine in animal tissues rendered this natural resource insufficient for developing clinical and pharmaceutical trials. Moreover, the synthesis of squalamine was considered expensive as it involved sophisticated chemical strategies with low overall yields.[16,21] Hence, many researchers decided to synthesize squalamine-like aminosterols (SALs) in an attempt to develop simpler synthesis procedures from cheaper starting materials.[3] SALs were generally synthesized from a sterol precursor by attaching a polyamine side chain. By changing the nature of the sterol core, and the position of the polyamine side chain, numerous compounds with variable antimicrobial activities were prepared.[22-28] A list of all synthesized SALs would be exhaustive and boring. Thus, we have only selected representative compounds that possess interesting antimicrobial activities (Figure **1**) to analyze structure-activity relationships. It clearly appears that the antimicrobial properties of SALs mainly result from the synergistic combination of an anionic bile salt and a polyamine side chain as they lead to a considerably lower antibacterial effect individually.[24] In addition, it was demonstrated that the sulfate group of squalamine had no influence on the antimicrobial activity because the absence of this group led to comparable or better results.[3] Kikuchi *et al*. prepared an extensive series of sterol-polyamine

conjugates that were derived from combinations of bile acid bases (one out of seven) and polyamine side chains (one out of three) at position C24 of the sterol core (Figure **1,** Table **1,** compounds **5-12**).[29] This work suggested that for compounds with the same sterol backbone, those bearing a spermine group as a side chain were more active than those bearing a putrescine group (Figure **1,** compounds **5-6**). Moreover, spermine conjugates having either a deoxycholic acid, ursocholanic acid, lithocholic acid, or chenodeoxycholic acid backbone had a better activity than those with a cholic acid or hyodeoxycholic acid backbone (Figure **1,** compounds **7-10**).[29] It was concluded that the nature of the polyamine side chain, along with the number and the position of the hydroxyl groups on the sterol core, clearly influenced the antimicrobial activities of SALs.[29] Moreover, while squalamine contained a spermine attached to position C3,[20] spermine and spermidine chains were attached to positions C24 and C7 for derivatives **5-14** (Figure **1**). This suggests that changing the position of the polyamine side chain on the sterol core does neither affect the antimicrobial activity nor the mechanism of action of these molecules. Another feature of the structure-activity relationships of SALs was provided by the work of Loncle *et al.* [3,30] in which diamino or polyamino groups were attached to position C7 of the sterol core (Figure **1**, compounds **11-14**). Increasing the number of carbons between the two amino groups increased the length of the functionalized chain and enhanced the antimicrobial activity. Moreover, the presence of only two amino groups seemed to be sufficient to restore the antimicrobial activity as the

compounds substituted with putrescine and cadaverine each exhibited an activity comparable to those of the spermine conjugate and squalamine (Figure **1**).

Ceragenins

Ceragenins belong to a group of synthetic aminosterols that were designed to mimic the activity of cationic peptide antibiotics such as polymyxin B.[5,31] A prior study demonstrated that facially amphiphilic molecules, such as cationic peptide antibiotics (CPAs), can disrupt bacterial membranes.[32] Hence, Savage *et al.* synthesized compounds using cholic acid as a precursor for the sterol core and modified the length of the side chains attached to the hydroxyl group to evaluate structure-activity relationships.[4,5] The so-called cationic steroid antibiotics, later named ceragenins, exhibited antibacterial and antifungal activities.[5,33] Furthermore, it was clearly demonstrated that compounds possessing a long hydrophobic chain attached to position C27, such as ceragenin **2**, had greater antibacterial activities (Figure **1** and T**able 1**).[4,5,31,33] The authors attributed this finding to the fact that a long, hydrophobic side chain enhanced the insertion of the compound into the bacterial membranes and improved its antibacterial effect.[31]

Antimicrobial activities of ASDs

It is noteworthy that almost all ASDs were evaluated against reference bacterial and fungal strains while relatively few were tested against multidrug-resistant clinical isolates (Figure **1** and Tables **1-2**).[3-5,34-37] In fact, only squalamine and two synthetic ASDs have been investigated against reference strains and 135

multidrug-resistant clinical bacterial isolates recovered from cystic fibrosis (CF) patients.[37] According to published data, squalamine and other aminosterols exhibited good activity against reference strains of *P. aeruginosa* and *S. pneumoniae* (Tables **1**-**2**).[29] Indeed, while MICs for reference strains of *P. aeruginosa* ranged from 2 to 4 mg/L for ASDs,[29,37] MICs of 32-64 mg/L were found in clinical mucoid isolates of these bacteria (Table **2**)[37,35]. Furthermore, capsulated gram-positive bacteria, such as *Streptococcus pneumoniae,* exhibited reduced susceptibility to ASDs (MICs of 32 mg/L)[37] compared to data obtained against reference strains of such bacteria (MICs ranging from 0.5 to 4 mg/L) (Tables **1** and **2**).[29] Moreover, it has been observed that colistin-resistant bacteria were much less susceptible to ASDs than colistin-sensitive ones.[37] Indeed, mucoid growth patterns are the result of bacteria adapting to the pulmonary microenvironment of CF patients to protect themselves from host immunity and antibiotic therapy.[38] In the mucoid phenotype, the outer membrane structure is modified by overproduction of alginates, which are anionic exopolysaccharides (EPSs) consisting of mannuronic acid and guluronic acid monomers.[38] EPSs are also present in capsules of some bacteria, such as certain variants of *Streptococcus pneumoniae* and *Klebsiella* spp.[39] These EPSs act as anionic shields that could bind the polycationic aminosterols and reduce their activity.[39] Furthermore, resistance to colistin is reported to be mediated through modification of the lipid composition of LPS in the outer membrane of gram-negative bacteria. This modification reduces the negative charge of LPS

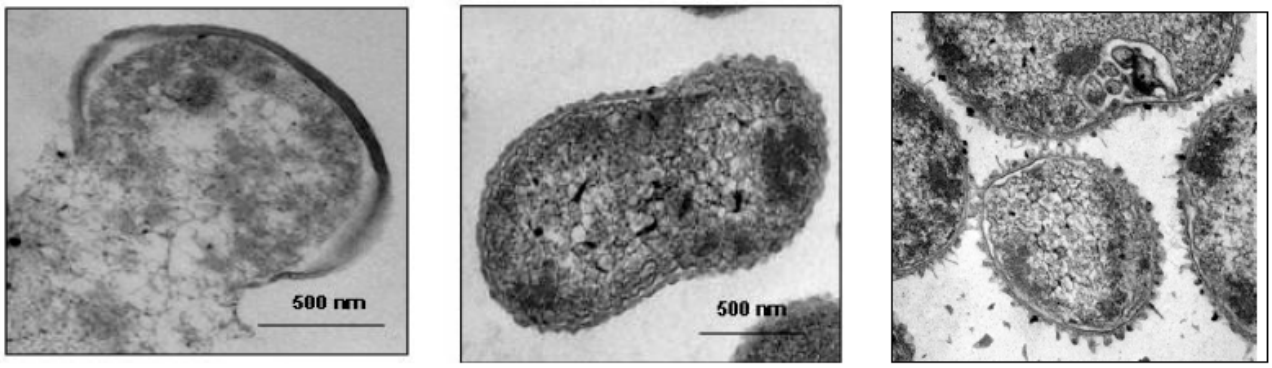
and disables the colistin-LPS interaction.[40] Since both ASDs and colistin interact with LPS, colistin resistance might intrinsically reduce the activity of ASDs.[41] It is also noteworthy that ASDs were reported to possess a remarkable activity against vancomycin-resistant and methicillin-resistant *S. aureus* (Tables **1** and **2**) [34,37]

Although MICs, varying from 2 to 50 μg/mL were obtained against reference bacterial strains, no information is available on the antibacterial activity of ceragenin compounds against MDR strains. As the concept behind the design and development of ceragenins is different from that of SALs, researchers generally focus on both groups of aminosterols separately. However, currently available arguments concerning the activity and mechanisms of action of all aminosterol derivatives tend to indicate that they might be regarded as a single family. The following section deals with the postulated antimicrobial mechanism of action of both groups.

Antibacterial mechanism of action of ASDs

In the last five years, numerous studies have rationalized the possible antibacterial mechanism of action of squalamine and its parent aminosterol derivatives. Initial observations on *in vitro* reconstituted bacterial membrane-mimicking liposomes or whole bacteria found that aminosterols were membrane-active molecules that disrupted the integrity of bacterial membranes.[30,42] This was clearly demonstrated for squalamine by measuring the release of intracellular ATP after exposing *E. coli* bacteria to this

compound.[30] This disruption was also demonstrated by a dose-dependent increase in the permeability of *E. coli* membranes to cell-impermeable dyes.[30] Furthermore, squalamine was shown to have a higher affinity for negatively charged bacterial lipopolysaccharides (LPSs) than for eukaryotic lipids.[30] These data suggest that the positively charged amino groups of squalamine interact with the negatively charged phosphate groups of LPS resulting in disruption of the outer membrane and subsequent cell death. While the mechanism of action of squalamine against gram-negative bacteria could be explained by the fact that the outer membranes of the bacteria are composed of negatively charged LPS, the mechanism of action of the same compound against gram-positive bacteria could not be explained by this fact as the membranes of gram-postive bacteria are essentially constituted of peptidoglycans that are less negatively charged. Alhanout *et al.* used transmission electron microscopy (TEM) to study *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923. They found that squalamine induces markedly different morphological changes in gram-negative and gram-positive bacteria.[41] The treatment of *P. aeruginosa* with squalamine resulted in bleb-like projections that originated from the outer membrane and indicated disturbed membrane integrity. On the other hand, the treatment of *S. aureus* with squalamine led to a dramatic disruption of the bacterial membrane causing drainage of the cytoplasmic material (Figure **2**). [41]



(**A**) (**B**) (**C**)

Figure **2:** TEM images demonstrating the morphological effects of squalamine on bacterial membranes of *Staphylococcus aureus* ATCC 25923 (**A**) and *Pseudomonas aeruginosa* ATCC 27853 (**B**), as well as the effect of colistin on *Pseudomonas aeruginosa* ATCC 27853 (**C**).

Moreover, by investigating the kinetics of intracellular ATP release from squalamine-treated *P. aeruginosa* and *S. aureus*, we found a strong ATP efflux from *S. aureus*, while a gradually increasing ATP efflux was noted in *P. aeruginosa* (Figure **3**). [41]

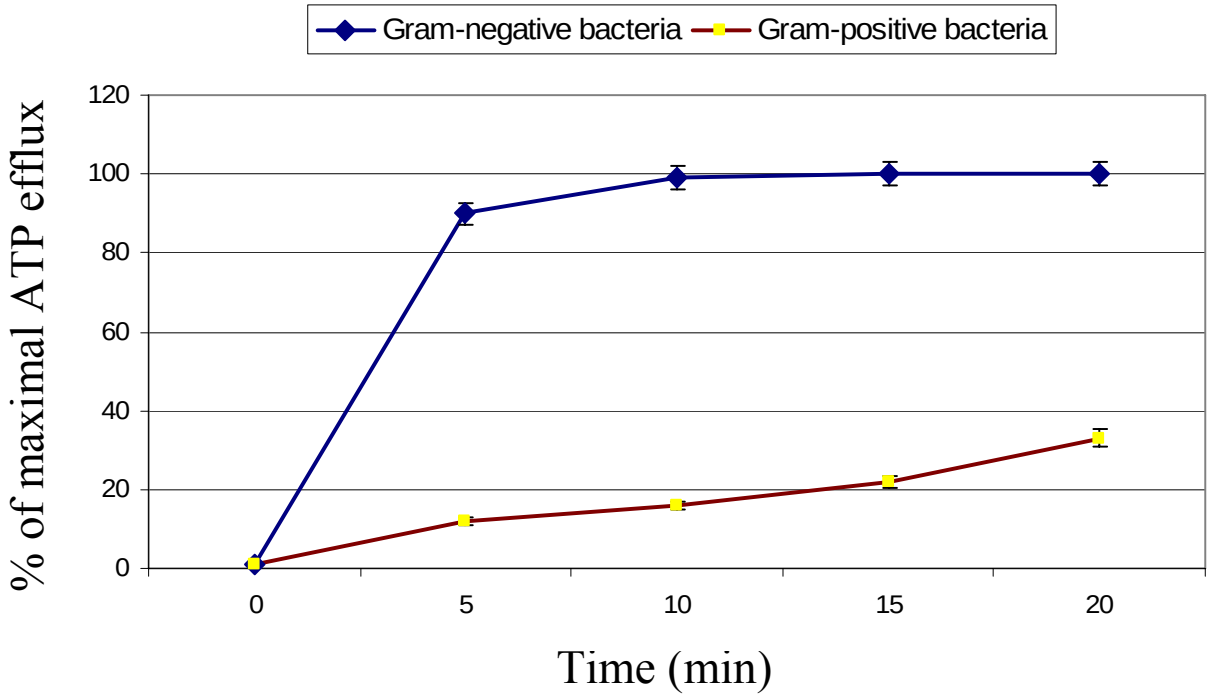
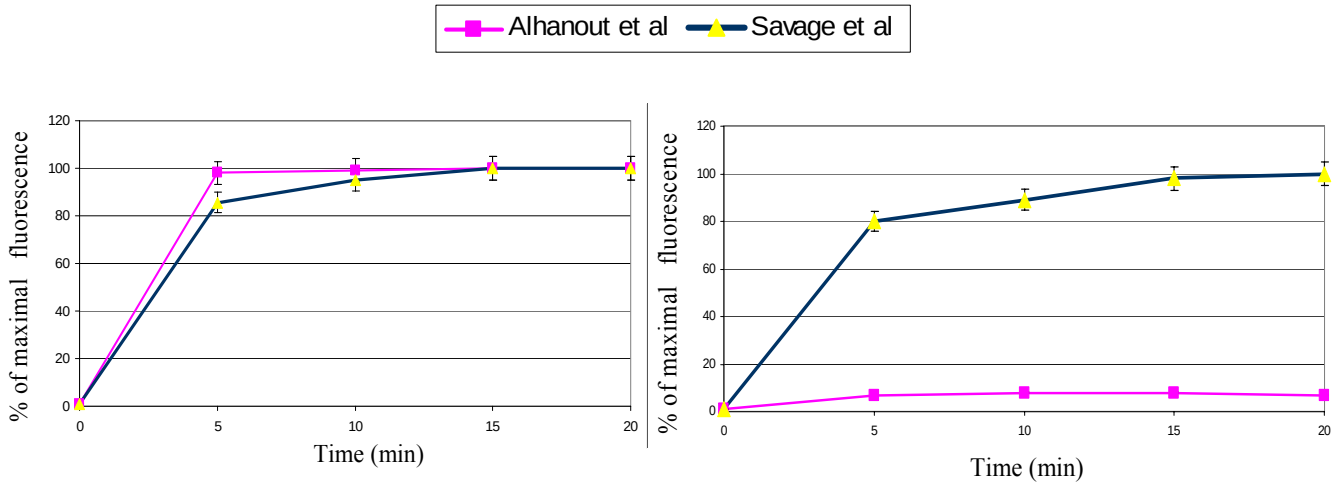


Figure **3:** This graph illustrates the effect of squalamine on ATP efflux in gram-positive and gram-negative bacteria, as reported by Alhanout *et al*. [41]

Thus, it was speculated that a specific phenomenon may be involved in the mode of action of squalamine against gram-positive bacteria. Indeed, squalamine led to a strong depolarization of the bacterial membrane of *S. aureus* while no depolarization was observed for gram-negative bacteria (Figure **4**).[41]



(**A**) (**B**)

Figure **4**: Comparison of data on the squalamine depolarizing effect on gram-positive bacteria (**A**) and gram-negative bacteria (**B**), as reported by Alhanout *et al*. [41] and Savage *et al.* [31-33].

Ceragenins were also reported to act by destabilizing the integrity of bacterial membranes, increasing the permeability of the outer membranes of gram-negative bacteria, and producing bleb-like projections similar to those observed for squalamine.[5,31,43] Historically, ceragenins were designed to mimic the amphiphilic morphology of cationic peptides and were reported to possess a depolarizing effect on bacterial membranes that was similar to that of CPAs. [32,44] Although polymyxin B targets lipid A, a component of LPS in the outer membrane of gram-negative bacteria, Savage *et al.* demonstrated that ceragenins possess a higher affinity to lipid A than polymyxin B.[44] The authors suggested that due to their affinity to lipid A, ceragenins would be highly selective for gram-negative bacterial membranes over gram-positive bacteria or eukaryotic

cell membranes.[32,44] From a mechanistic point of view, this may be confusing as ceragenins generally possess activities against gram-positive bacteria that are similar to or higher than those against gram-negative bacteria.[5,35,36,44] Moreover, correlating the activities of ceragenins with those of CPAs such as polymyxins are questionable because, contrary to ceragenins, polymyxins have no effect on gram-positive bacteria.[32,32,43,45] However, in the presence of squalamine, bacterial membrane depolarization is restricted to gram-positive bacteria (Figure **4**).[41] Moreover, it has been shown that ASDs may share certain mechanistic aspects with polymyxins.[41] Polymyxins were reported to act by disrupting the outer membranes of gram-negative bacteria after interacting with the negatively charged LPS and displacing divalent cations, such as Ca^{2+} and Mg^{2+}, that stabilize the outer membrane structure.[40,46] We found that divalent cations inhibited the negative effects on membrane integrity and considerably increased the MICs of squalamine and colistin against these bacteria. These results suggest that the interactions with LPSs represent a common prerequisite for both compounds. However, a comparison of the intracellular ATP efflux kinetics of tested gram-negative bacteria indicated that squalamine induced a faster and larger ATP efflux than colistin. In this context, Di Pasquale *et al.* recently studied the interaction of squalamine, colistin, and the detergent hexadecyltrimethylammonium bromide (CTAB) with eukaryotic and prokaryotic lipid bilayers, as well as the consequences of these interactions on the electrical properties of the membranes. [47] The results clearly established

that these three compounds act differently on lipid bilayers., Squalamine and colistin acted similarly on bacterial membranes as they led to electrically active lesions. However, these lesions differed in their diameter sizes (33.3±5 versus 9.1±1 nm for squalamine and colistine, respectively). Conversely, the prototypal detergent CTAB did not cause electrical fluctuations and was only able to disrupt prokaryotic membranes, which is consistent with the effects of a detergent. By comparison, these data show that squalamine and colistine do not possess detergent-like activity on bacterial membranes. Colistine was the only compound that was able to lead to electrically active lesions (diameter of 2.8±0.5 nm) on eukaryotic bilayers; however, they were 3.2-fold smaller than the lesions caused on bacterial membranes. Squalamine and CTAB both caused membrane ruptures on eukaryotic bilayers. Taken together, these results demonstrate that squalamine, but not colistine, possesses detergent-like activity on eukaryotic membranes. This was also evidenced by TEM imaging of bacteria, which showed an important, substantial difference between colistin and squalamine regarding their interactions with the membranes (Figure **2**).[41] Therefore, ASDs seem to possess a mechanism of action against gram-negative bacteria that is different from that against gram-positive bacteria. Indeed, in the former case, ASDs seem to act by disintegrating the outer membrane in a detergent-like manner, while in the latter case they seem to act as depolarizing agents that disrupt the bacterial membrane (Figure **5**).

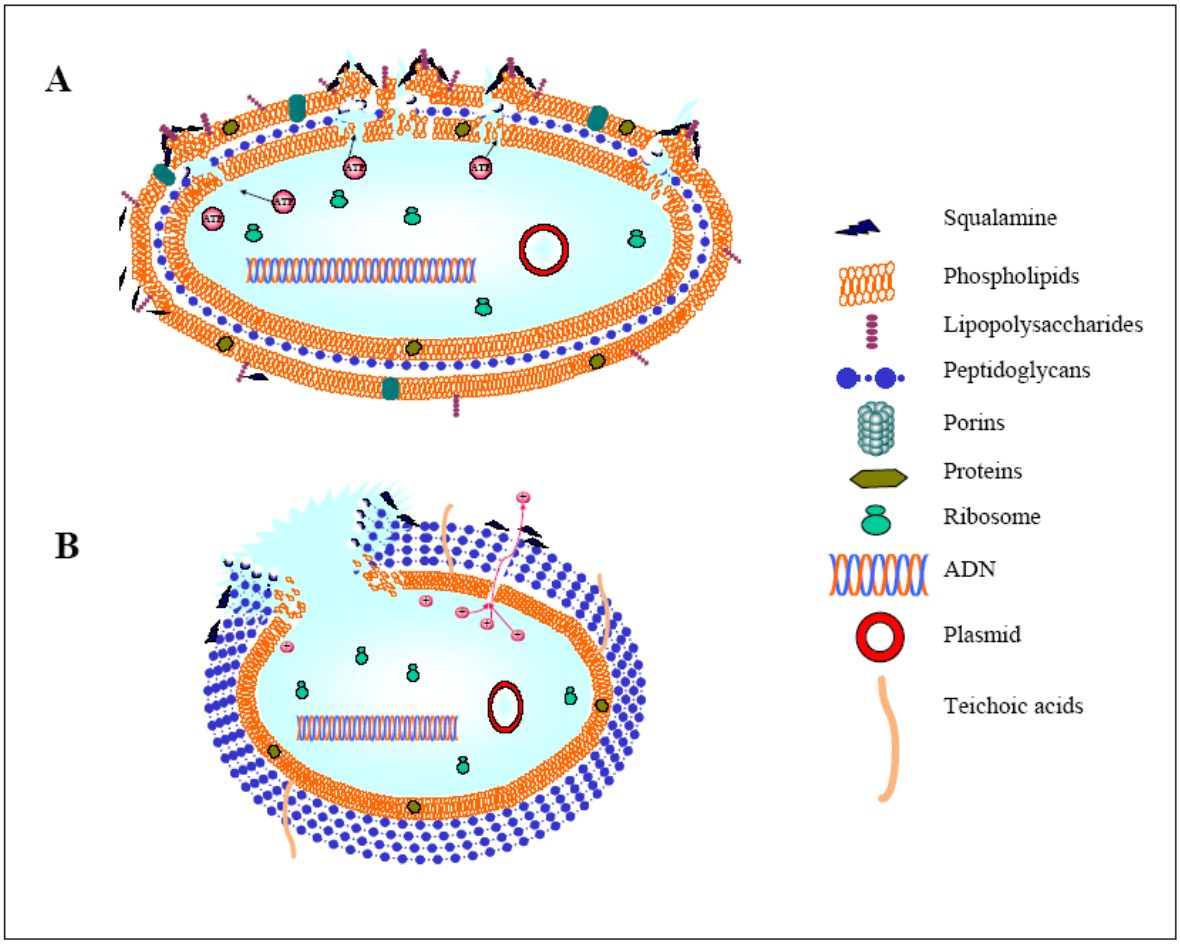


Figure 5: Representation of the speculated mechanism of action of squalamine against (**A**) gram-negative bacteria, demonstrating membrane disintegration, and (**B**) gram-positive bacteria, showing the depolarizing effect of squalamine that leads to membrane disruption.[41]

Miscellaneous activities

In addition to their antibacterial activities, numerous data in the literature indicate that ASDs possess interesting antifungal activities against reference strains of yeasts and filamentous fungi.[3,5,29] Furthermore, Alhanout *et al.* recently evaluated the efficiency of ASDs against MDR clinical fungal isolates,

including yeasts isolated from hemocultures of immunocompromised patients[48] and filamentous fungi[49] recovered form sputa of CF patients. Squalamine and ASD **15** were similarly active against all isolates (Table **2**) as compared to standard tested antifungal agents that displayed disparate activities (MICs ranging from 0.5 to 32 mg/L).[49] Nevertheless, no data are currently available regarding the potential antifungal mechanism of action of ASDs because these compounds were active against fungal species that were resistant to various antifungal drugs.

On the other hand, Salmi *et al*. reported that 3-aminosterol compounds exhibited antimalarial properties against chloroquine-susceptible and chloroquine-resistant *Plasmodium falciparum*. These derivatives possess a good therapeutic index compared to chloroquine (the IC50 varied from 2 to 22 μM) indicating their potential for the discovery and development of new antimalarial drugs.[50]

Recent data have shown that ceragenin compound **2** was active against *Helicobacter pylori* as well as cariogenic and periodontopathic bacteria, as indicated by MICs ranging from 0.275 to 8.9 and 1 to 16 μg/mL, respectively.[51] Additionally, the combination of ceragenins with other antibiotics like erythromycin and rifampin increased their activity.[29,43] A similar effect was also recently reported regarding the activity of squalamine on susceptible bacterial strains (*E. coli*, *E. aerogenes* and *P. aeruginosa*), suggesting that this compound may chemosensitize the membranes of nonresistant bacteria and decrease the amount of antibiotic needed.[52] Consequently, the use of

squalamine against polymyxin-resistant isolates selected during colistin treatment, a therapy that is now proposed for the MDR phenotype, may be an attractive hypothesis for the development of future drug combinations. Squalamine may be a fruitful candidate for the development of combinations, such as β-lactams/β-lactamase inhibitors, to combat MDR pathogens because (i) it could minimize bacterial resistance, (ii) it has potent antimicrobial actions, and (iii) it is relatively insensitive to efflux resistance mechanisms.

Aminosterols toxicity

In the early stages of development, molecules designed for biological use must display an acceptable *in vitro* toxicity/activity ratio to evolve from chemicals to drugs that are further tested *in vivo* (go/nogo strategy).[53] Therefore, selecting the proper therapeutic target and examining toxicity by *in vitro* screening are pivotal for an accurate and reliable estimation of the activity of tested compounds. In the case of ASDs, toxicity might be a serious problem since aminosterols act in a non-specific detergent-like manner in eukaryotic cell membranes .[19,32,41]

The hemolytic effect of aminosterols was used as an indicator of toxicity for these compounds.[4,29] To date, few synthesized ASDs[29,36] have shown an encouraging *in vitro* safety index, which is defined as the ratio of Minimal Hemolytic Concentration (MHC) versus the MIC value. Squalamine has reached phase III trials for the treatment of age-related macular degeneration (AMD), also known as “wet” AMD, and prostate cancer disease without any major side

effects as it appears to be well tolerated, even at doses around 250 mg/day in adults.[6] Two phase II dose escalation studies have been completed in subjects with advanced solid tumors refractory to other therapy or for which other therapy was not available.[7] The results suggest that squalamine lactate was well tolerated as a monotherapy in this subject population.

Conclusions

In conclusion, ASD compounds were found to possess interesting permeabilization properties and antimicrobial activities that could lead to the development of an efficient new class of antimicrobial agents against MDR bacteria. In the coming years, numerous researchers in this field will work towards the development of these molecules. Thus, we envision an exploration of the relationship between the structure and biologic function of a series of cationic steroids obtained by simple and inexpensive methods. Development in this field could lead to the preparation of various derivatives of squalamine with a mechanism of action that is different from that of polymyxin analogs but, presumably, easily accessible in regards to the structure of this natural product. Because of their potencies, broad spectra of antimicrobial activity and potential for systemic toxicity, squalamine derivatives seem to be good candidates for the development of topical antimicrobial agents.

References

[1] Kaier, K.; Wilson, C.; Chalkley, M.; Davey, P. G.; Suetens, C.; Grundmann, H.; de, K. M.; Schumacher, M.; Wolkewitz, M.;

Frank, U. Health and economic impacts of antibiotic resistance in European hospitals--outlook on the BURDEN project. *Infection* **2008,** *36* (5), 492-494.

[2] Tacconelli, E. Antimicrobial use: risk driver of multidrug resistant microorganisms in healthcare settings. *Curr. Opin. Infect. Dis.* **2009,** *22* (4), 352-358.

[3] Salmi, C.; Brunel, J. M. Therapeutic potential of cationic steroid antibacterials. *Expert. Opin. Investig. Drugs* **2007,** *16* (8), 1143-1157.

[4] Savage, P. B.; Li, C.; Taotafa, U.; Ding, B.; Guan, Q. Antibacterial properties of cationic steroid antibiotics. *FEMS Microbiol. Lett.* **2002,** *217* (1), 1-7.

[5] Savage, P. B.; Li, C. Cholic acid derivatives: novel antimicrobials. *Expert. Opin. Investig. Drugs* **2000,** *9* (2), 263-272.

[6] Hao, D.; Hammond, L. A.; Eckhardt, S. G.; Patnaik, A.; Takimoto, C. H.; Schwartz, G. H.; Goetz, A. D.; Tolcher, A. W.; McCreery, H. A.; Mamun, K.; Williams, J. I.; Holroyd, K. J.; Rowinsky, E. K. A Phase I and pharmacokinetic study of squalamine, an aminosterol angiogenesis inhibitor. *Clin. Cancer Res.* **2003,** *9* (7), 2465-2471.

[7] Herbst, R. S.; Hammond, L. A.; Carbone, D. P.; Tran, H. T.; Holroyd, K. J.; Desai, A.; Williams, J. I.; Bekele, B. N.; Hait, H.; Allgood, V.; Solomon, S.; Schiller, J. H. A phase I/IIA trial of continuous five-day infusion of squalamine lactate (MSI-1256F) plus carboplatin and paclitaxel in patients with advanced non-small cell lung cancer. *Clin. Cancer Res.* **2003,** *9* (11), 4108-4115.

[8] Li, D.; Williams, J. I.; Pietras, R. J. Squalamine and cisplatin block angiogenesis and growth of human ovarian cancer cells with or without HER-2 gene overexpression. *Oncogene* **2002,** *21* (18), 2805-2814.

[9] Sills, A. K., Jr.; Williams, J. I.; Tyler, B. M.; Epstein, D. S.; Sipos, E. P.; Davis, J. D.; McLane, M. P.; Pitchford, S.; Cheshire, K.; Gannon, F. H.; Kinney, W. A.; Chao, T. L.; Donowitz, M.; Laterra, J.; Zasloff, M.; Brem, H. Squalamine inhibits angiogenesis and solid tumor growth in vivo and perturbs embryonic vasculature. *Cancer Res.* **1998,** *58* (13), 2784-2792.

[10] Teicher, B. A.; Williams, J. I.; Takeuchi, H.; Ara, G.; Herbst, R. S.; Buxton, D. Potential of the aminosterol, squalamine in combination therapy in the rat 13,762 mammary carcinoma and the murine Lewis lung carcinoma. *Anticancer Res.* **1998,** *18* (4A), 2567-2573.

[11] Chernova, M. N.; Vandorpe, D. H.; Clark, J. S.; Williams, J. I.; Zasloff, M. A.; Jiang, L.; Alper, S. L. Apparent receptor-mediated activation of Ca2+-dependent conductive Cl- transport by shark-derived polyaminosterols. *Am. J. Physiol Regul. Integr. Comp Physiol* **2005,** *289* (6), R1644-R1658.

[12] Ahima, R. S.; Patel, H. R.; Takahashi, N.; Qi, Y.; Hileman, S. M.; Zasloff, M. A. Appetite suppression and weight reduction by a centrally active aminosterol. *Diabetes* **2002,** *51* (7), 2099-2104.

[13] Higgins, R. D.; Yan, Y.; Geng, Y.; Zasloff, M.; Williams, J. I. Regression of retinopathy by squalamine in a mouse model. *Pediatr. Res.* **2004,** *56* (1), 144-149.

[14] Higgins, R. D.; Sanders, R. J.; Yan, Y.; Zasloff, M.; Williams, J. I. Squalamine improves retinal neovascularization. *Invest Ophthalmol. Vis. Sci.* **2000,** *41* (6), 1507-1512.

[15] Zasloff, M.; Williams, J. I.; Chen, Q.; Anderson, M.; Maeder, T.; Holroyd, K.; Jones, S.; Kinney, W.; Cheshire, K.; McLane, M. A spermine-coupled cholesterol metabolite from the shark with potent appetite suppressant and antidiabetic properties. *Int. J. Obes. Relat Metab Disord.* **2001,** *25* (5), 689-697.

[16] Brunel, J. M.; Salmi, C.; Loncle, C.; Vidal, N.; Letourneux, Y. Squalamine: a polyvalent drug of the future? *Curr. Cancer Drug Targets.* **2005,** *5* (4), 267-272.

[17] Rao, M. N.; Shinnar, A. E.; Noecker, L. A.; Chao, T. L.; Feibush, B.; Snyder, B.; Sharkansky, I.; Sarkahian, A.; Zhang, X.; Jones, S. R.; Kinney, W. A.; Zasloff, M. Aminosterols from the dogfish shark *Squalus acanthias*. *J. Nat. Prod.* **2000,** *63* (5), 631-635.

[18] Senior, K. New aminosterols from the dogfish shark. *Drug Discov. Today* **2000,** *5* (7), 267-268.

[19] Moore, K. S.; Wehrli, S.; Roder, H.; Rogers, M.; Forrest, J. N., Jr.; McCrimmon, D.; Zasloff, M. Squalamine: an aminosterol antibiotic from the shark. *Proc. Natl. Acad. Sci. U. S. A* **1993,** *90* (4), 1354-1358.

[20] Wehrli, S. L.; Moore, K. S.; Roder, H.; Durell, S.; Zasloff, M. Structure of the novel steroidal antibiotic squalamine determined by two-dimensional NMR spectroscopy. *Steroids* **1993,** *58* (8), 370-378.

[21] Brunel, JM.; Letourneux , Y. Recent Advances in the Synthesis of Spermine and Spermidine Analogs of the Shark Aminosterol Squalamine. *Eur. J. Org. Chem.* **2003,** (20), 3897-3907.

[22] Chen, W. H.; Shao, X. B.; Moellering, R.; Wennersten, C.; Regen, S. L. A bioconjugate approach toward squalamine mimics: Insight into the mechanism of biological action. *Bioconjug. Chem.* **2006,** *17* (6), 1582-1591.

[23] Choucair, B.; Dherbomez, M.; Roussakis, C.; El-kihel, L. Synthesis of 7 alpha- and 7 beta-spermidinylcholesterol, squalamine analogues. *Bioorg. Med. Chem. Lett.* **2004,** *14* (16), 4213-4216.

[24] Jones, S. R.; Kinney, W. A.; Zhang, X.; Jones, L. M.; Selinsky, B. S. The synthesis and characterization of analogs of the antimicrobial compound squalamine: 6 beta-hydroxy-3-aminosterols synthesized from hyodeoxycholic acid. *Steroids* **1996,** *61* (10), 565-571.

[25] Kim, H. S.; Choi, B. S.; Kwon, K. C.; Lee, S. O.; Kwak, H. J.; Lee, C. H. Synthesis and antimicrobial activity of squalamine analogue. *Bioorg. Med. Chem.* **2000,** *8* (8), 2059-2065.

[26] Shu, Y.; Jones, S. R.; Kinney, W. A.; Selinsky, B. S. The synthesis of spermine analogs of the shark aminosterol squalamine. *Steroids* **2002,** *67* (3-4), 291-304.

[27] Salmi, C.; Loncle, C.; Vidal, N.; Laget, M.; Letourneux, Y.; Brunel, J. M. Antimicrobial activities of 3-amino- and polyaminosterol analogues of squalamine and trodusquemine. *J. Enzyme Inhib. Med. Chem.* **2008,** *23* (6), 860-865.

[28] Salmi, C.; Loncle, C.; Vidal, N.; Letourneux, Y.; Brunel, J. M. New stereoselective titanium reductive amination synthesis of 3-amino and polyaminosterol derivatives possessing antimicrobial activities. *Eur. J. Med. Chem.* **2008,** *43* (3), 540-547.

[29] Kikuchi, K.; Bernard, E. M.; Sadownik, A.; Regen, S. L.; Armstrong, D. Antimicrobial activities of squalamine mimics. *Antimicrob. Agents Chemother.* **1997,** *41* (7), 1433-1438.

[30] Salmi, C.; Loncle, C.; Vidal, N.; Letourneux, Y.; Fantini, J.; Maresca, M.; Taieb, N.; Pages, J. M.; Brunel, J. M. Squalamine: an appropriate

strategy against the emergence of multidrug resistant gram-negative bacteria? *PLoS. ONE.* **2008,** *3* (7), e2765.

[31] Lai, X. Z.; Feng, Y.; Pollard, J.; Chin, J. N.; Rybak, M. J.; Bucki, R.; Epand, R. F.; Epand, R. M.; Savage, P. B. Ceragenins: cholic acid-based mimics of antimicrobial peptides. *Acc. Chem. Res.* **2008,** *41* (10), 1233-1240.

[32] Ding, B.; Guan, Q.; Walsh, J. P.; Boswell, J. S.; Winter, T. W.; Winter, E. S.; Boyd, S. S.; Li, C.; Savage, P. B. Correlation of the antibacterial activities of cationic peptide antibiotics and cationic steroid antibiotics. *J. Med. Chem.* **2002,** *45* (3), 663-669.

[33] Epand, R. M.; Epand, R. F.; Savage, P. B. Ceragenins (cationic steroid compounds), a novel class of antimicrobial agents. *Drug News Perspect.* **2008,** *21* (6), 307-311.

[34] Chin, J. N.; Rybak, M. J.; Cheung, C. M.; Savage, P. B. Antimicrobial activities of ceragenins against clinical isolates of resistant Staphylococcus aureus. *Antimicrob. Agents Chemother.* **2007,** *51* (4), 1268-1273.

[35] Chin, J. N.; Jones, R. N.; Sader, H. S.; Savage, P. B.; Rybak, M. J. Potential synergy activity of the novel ceragenin, CSA-13, against clinical isolates of *Pseudomonas aeruginosa,* including multidrug-resistant P. aeruginosa. *J. Antimicrob. Chemother.* **2008,** *61* (2), 365-370.

[36] Schmidt, E. J.; Boswell, J. S.; Walsh, J. P.; Schellenberg, M. M.; Winter, T. W.; Li, C.; Allman, G. W.; Savage, P. B. Activities of cholic acid-derived antimicrobial agents against multidrug-resistant bacteria. *J. Antimicrob. Chemother.* **2001,** *47* (5), 671-674.

[37] Alhanout, K.; Brunel, J. M.; Raoult, D.; Rolain, J. M. *In vitro* antibacterial activity of aminosterols against multidrug-resistant bacteria from patients with cystic fibrosis. *J Antimicrob Chemother* **2009,** *64* (4), 810-814.

[38] Pritt, B.; O'Brien, L.; Winn, W. Mucoid *Pseudomonas* in cystic fibrosis. *Am. J. Clin. Pathol.* **2007,** *128* (1), 32-34.

[39] Llobet, E.; Tomas, J. M.; Bengoechea, J. A. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. *Microbiology* **2008,** *154* (Pt 12), 3877-3886.

[40] Zavascki, A. P.; Goldani, L. Z.; Li, J.; Nation, R. L. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J. Antimicrob. Chemother.* **2007,** *60* (6), 1206-1215.

[41] Alhanout, K.; Malesinki, S.; Vidal, N.; Peyrot, V.; Rolain, J. M.; Brunel, J. M. New insights on the antibacterial mechanism of action of squalamine. *Bioconjug. Chem.* **2010**. Submitted

[42] Selinsky, B. S.; Zhou, Z.; Fojtik, K. G.; Jones, S. R.; Dollahon, N. R.; Shinnar, A. E. The aminosterol antibiotic squalamine permeabilizes large unilamellar phospholipid vesicles. *Biochim. Biophys. Acta* **1998,** *1370* (2), 218-234.

[43] Saha, S.; Savage, P. B.; Bal, M. Enhancement of the efficacy of erythromycin in multiple antibiotic-resistant gram-negative bacterial pathogens. *J. Appl. Microbiol.* **2008,** *105* (3), 822-828.

[44] Ding, B.; Yin, N.; Liu, Y.; Cardenas-Garcia, J.; Evanson, R.; Orsak, T.; Fan, M.; Turin, G.; Savage, P. B. Origins of cell selectivity of cationic steroid antibiotics. *J. Am. Chem. Soc.* **2004,** *126* (42), 13642-13648.

[45] Li, J.; Nation, R. L.; Milne, R. W.; Turnidge, J. D.; Coulthard, K. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. *Int. J. Antimicrob. Agents* **2005,** *25* (1), 11-25.

[46] Jenssen, H.; Hamill, P.; Hancock, R. E. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* **2006,** *19* (3), 491-511.

[47] Di Pasquale E.; Salmi-Smail, C.; Brunel, J. M.; Sanchez, P.; Fantini, J.; Maresca, M. Biophysical studies of the interaction of squalamine and other cationic amphiphilic molecules with bacterial and eukaryotic membranes: importance of the distribution coefficient in membrane selectivity. *Chem. Phys. Lipids* **2010,** *163* (2), 131-140.

[48] Alhanout, K.; Djouhri, L.; Vidal, N.; Brunel, J. M.; Piarroux, R.; Ranque, S. *In Vitro* activity of aminosterols against yeasts isolated from blood stream infections. *Medical Mycology* **2010,** Submitted.

[49] Alhanout, K.; Brunel, J. M.; Ranque, S.; Rolain, J. M. *In vitro* antifungal activity of aminosterols against moulds isolated from cystic fibrosis patients. *J. Antimicrob. Chemother.* **2010,** Submitted.

[50] Salmi, C.; Loncle, C.; Vidal, N.; Rogier, C.; Letourneux, Y.; Pradines, B.; Brunel, J. M. 3-Aminosterol Compounds as a New Class of Anti-Malarial Agents against Chloroquine-Susceptible and -Resistant

Plasmodium falciparum. *Lett. Drug Design Discovery* **2008,** *6* (2), 163-166.

[51] Leszczynska, K.; Namiot, A.; Fein, D. E.; Wen, Q.; Namiot, Z.; Savage, P. B.; Diamond, S.; Janmey, P. A.; Bucki, R. Bactericidal activities of the cationic steroid CSA-13 and the cathelicidin peptide LL-37 against Helicobacter pylori in simulated gastric juice. *BMC. Microbiol.* **2009,** *9*, 187.

[52] Lavigne, J. P; Brunel, J. M; Chevalier, J; Pages J. M. Squalamine, an original chemosensitizer to combat antibiotic-resistant Gram-negative bacteria. *J. Antimicrob. Chemother.* **2010,** Under press.

[53] Singh, S. S. Preclinical pharmacokinetics: an approach towards safer and efficacious drugs. *Curr. Drug Metab* **2006,** *7* (2), 165-182.

Chapitre 2

- **Evaluation de l'activité antimicrobienne des dérivés aminostéroïdiens contre des souches microbiennes cliniques**

➢ Activité antibactérienne *in vitro* des dérivés aminostéroïdiens contre des bactéries multi résistantes isolées des patients atteints de mucoviscidose

K. Alhanout, Journal of Antimicrobial Chemotherapy (2009) 64, 810–814

➢ Activité antifongique *in vitro* des dérivés aminostéroïdiens contre des champignons filamenteux isolés des patients atteints de mucoviscidose.

K. Alhanout, Journal of Antimicrobial Chemotherapy
doi:10.1093/jac/dkq089

➢ Activité *in vitro* de dérivés aminostéroïdiens contres des levures impliquées dans des fongémies.

K. Alhanout, en révision dans Medical Mycology

- **Analyse du mécanisme d'action antibactérienne de la squalamine**

Article 2

In vitro antibacterial activity of aminosterols against multidrug-resistant bacteria from patients with cystic fibrosis

Kamel Alhanout, Jean-Michel Brunel, Didier Raoult and Jean-Marc Rolain*

Journal of Antimicrobial Chemotherapy (2009) 64, 810–814

Résumé de l'article

Cette étude a examiné pour la première fois l'activité de la squalamine et de dérivés aminostéroïdiens (DASs 1-2) contre des souches bactériennes cliniques multi résistantes isolées des crachats de patients atteints de mucoviscidose. L'activité antibactérienne de la squalamine et DAS 1 et 2 a été évaluée contre 135 bactéries à Gram négatif et à Gram positif contenant des isolats ayant un phénotype multi résistant aux antibiotiques en utilisant la méthode de la microdilution pour la détermination de la concentration minimale inhibitrice (CMI). La colistine a été testée également pour des fins de comparaison. En fonction du phénotype de croissance sur les milieux de culture, les bactéries ont été réparties en deux groupes, des souches mucoïdes et des souches non mucoïdes.

Pour les bactéries à Gram-négatif, les CMIs ont varié de 2 à 128 mg /L. la résistance à la colistine et le phénotype mucoïde étaient significativement associés à des CMIs plus élevées pour la squalamine et les DASs1- 2. Les DASs étaient actifs contre l'ensemble de bactéries à Gram positif en présentant des valeurs de CMI variant de 0,5 à 8 mg/L, à l'exception de deux isolats capsulés de *Streptococcus pneumoniae* présentant une CMI de 32 mg /L.

Dans cette étude, nous avons présenté des nouveaux éléments concernant le potentiel antibactérien des DASs contre des souches bactériennes cliniques contenant des pathogènes multi résistants. La résistance à la colistine, le phénotype mucoïde et la présence de capsule semble contrarier l'effet des DASs.

Ceci pourrait indiquer que ces composés peuvent partager certains aspects mécanistiques avec la colistine vis-à-vis des bactéries à Gram-négatif. Toutefois, les DASs ont été remarquablement actifs contre les bactéries à Gram positif suggérant la présence de deux mécanismes distincts d'action pour ces molécules à l'égard des bactéries à Gram-positif et à Gram-négatif.

Comme les DASs testés ont présenté des CMIs relativement élevées dans certains cas, nous proposons que ces composés puissent être développés pour être administrés localement sous forme d'aérosols plutôt que par voie d'administration systémique. L'évaluation de l'efficacité *in vivo* de ce groupe de molécules dans des formulations d'aérosols serait toute à fait envisageable en utilisant un modèle animal d'infection pulmonaire.

Journal of Antimicrobial Chemotherapy (2009) **64**, 810–814
doi:10.1093/jac/dkp281
Advance Access publication 7 August 2009

JAC

In vitro antibacterial activity of aminosterols against multidrug-resistant bacteria from patients with cystic fibrosis

Kamel Alhanout, Jean-Michel Brunel, Didier Raoult and Jean-Marc Rolain*

URMITE UMR 6236, CNRS-IRD, Faculté de Médecine et de Pharmacie, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France

Received 13 March 2009; returned 27 April 2009; revised 29 May 2009; accepted 12 July 2009

***Objectives*: Respiratory infections with multidrug-resistant (MDR) bacteria are life-threatening in patients with cystic fibrosis (CF). Squalamine and aminosterol derivatives (ASDs) have previously demonstrated interesting antibacterial activity against bacterial reference strains. This study investigated for the first time their activity against MDR clinical isolates recovered from the sputa of CF patients.**

***Methods*: Antibacterial activity of squalamine and two ASDs (1 and 2) was evaluated against 135 MDR Gram-negative and Gram-positive bacteria using the broth microdilution method for MIC determination.**

***Results*: For Gram-negative bacteria, MICs ranged from 2 to 128 mg/L. Resistance to colistin and mucoidity were significantly associated with higher MICs of squalamine and ASDs 1 and 2. Tested compounds were active against various Gram-positive bacteria with MIC values varying from 0.5 to 8 mg/L, with the exception of two capsulated isolates of *Streptococcus pneumoniae* demonstrating MICs of 32 mg/L.**

***Conclusions*: In this study, we present new findings concerning the antibacterial potential of ASDs against MDR bacteria. Colistin-resistant, mucoid and capsulated bacteria were found to exhibit decreased susceptibility to ASDs indicating that these compounds might share some mechanistic aspects with polymyxins towards Gram-negative bacteria. However, ASDs were remarkably active against Gram-positive species suggesting different mechanisms of action towards Gram-positive and Gram-negative bacteria. As tested ASDs exhibited elevated MICs in some cases, we believe that these compounds may be developed to be locally administrated as aerosols rather than via systemic administration routes. Further work is warranted to evaluate their *in vivo* efficacy in aerosol formulations using a lung-infected animal model.**

Keywords: squalamine, antibiotics, infections

Introduction

Bacterial pulmonary infections represent a major risk for patients with hereditary diseases such as cystic fibrosis (CF). Although the diversity of bacteria that can be recovered from sputa of patients with CF has recently expanded,[1] *Pseudomonas aeruginosa* and *Staphylococcus aureus* remain the most common pathogens in CF lung infections.[1] Antibiotic administration by inhaled aerosols may be beneficial in the management of lung infections in CF patients since antibiotics are delivered to the site of infection at high local concentrations; in addition, systemic exposure is minimized.[2] Nevertheless, multidrug resistance and biofilm formation continue to reduce the efficacy of antibiotic therapy and may render bacterial eradication unachievable.[1] Consequently, there is a great need to develop new classes of antibiotics and new therapeutic strategies that can improve therapeutic efficacy in CF patients. Squalamine, a natural aminosterol, and related synthesized aminosterol derivatives (ASDs) (Figure 1a), demonstrated interesting *in vitro* antibacterial activities against reference bacterial strains.[3] The evaluation of the anti-angiogenic effect of squalamine has been investigated in a Phase I/IIA trial, demonstrating its safety after continuous 5 day infusion of 300 mg/m^2/day.[4] However, the clinical usefulness of squalamine as an antibiotic remains uncertain. The evaluation of the antibacterial effect of these compounds against bacteria involved in clinical pathologies rather than reference strains would be more relevant. Thus, we provide herein the first study focusing on the antibacterial effect of squalamine and two ASDs synthesized in our laboratory towards a large panel of Gram-positive and Gram-negative planktonic

*Corresponding author: Tel: +33-4-91-32-43-75; Fax: +33-4-91-38-77-72; E-mail: jean-marc.rolain@univmed.fr

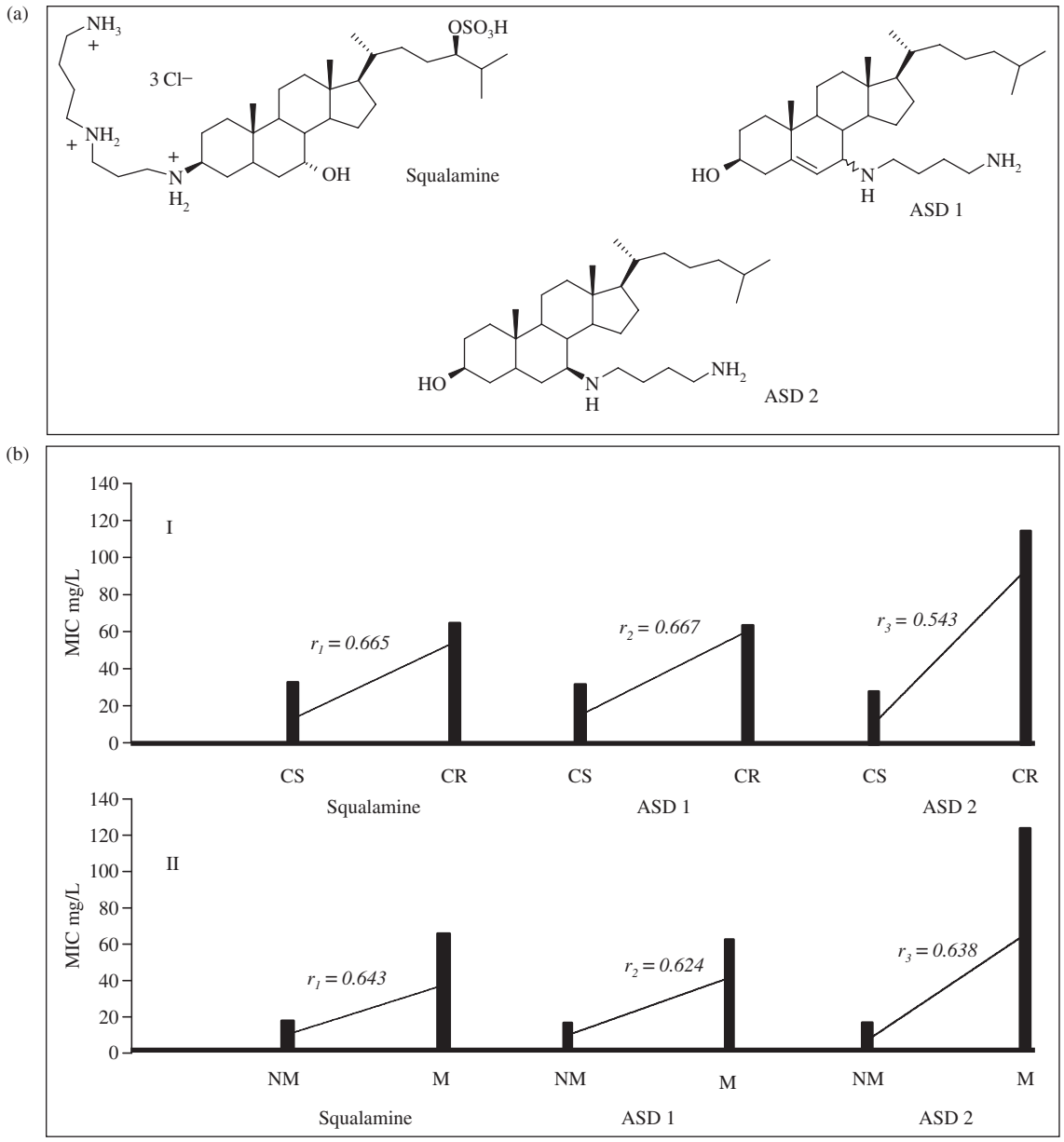


Figure 1. (a) Structure of squalamine and synthesized ASDs 1 and 2. (b) Susceptibility of Gram-negative isolates to squalamine and ASDs 1 and 2 was significantly associated with resistance to colistin (part I) and the presence of the mucoid phenotype (part II) in tested isolates. $P<0.0001$. CS, colistin-susceptible; CR, colistin-resistant; NM, non-mucoid; M, mucoid.

bacteria containing multidrug-resistant (MDR) and emergent bacteria recovered from sputa of CF patients.

Materials and methods

Compounds: squalamine, ASDs 1 and 2 and antibiotic controls

Squalamine was a gift from Professor M. Zasloff (Georgetown University, Washington, DC, USA). ASD 1 [7-(1,4-diaminobutane)-cholest-5-ene-3β-ol] and ASD 2 [7β-(1,4-diaminobutane)-cholestan-3β-ol] were synthesized as previously reported.[5] Stock solutions were prepared in water for squalamine and in methanol for ASDs 1 and 2. Antibiotic controls were: colistin (Sanofi Aventis, Paris, France); amoxicillin (Bouchara-Recordati Laboratory, Paris, France); and vancomycin (Merck génériques, Lyon, France).

Bacterial strains

Reference strains used were *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. Clinical strains were isolated from the sputa of CF patients; 137 clinical strains consisting of Gram-negative ($n=92$) and Gram-positive ($n=45$) bacteria were tested. Phenotype identification was performed using Vitek 2 Auto systems (bioMérieux, Marcy l'Étoile, France) and, when necessary, 16S rDNA or *rpoB* PCR[6] was employed to confirm identification. Antibiotic susceptibility testing was performed by using Vitek 2 Auto systems, Etest (AB Biodisk,

Stockholm, Sweden) or the disc diffusion method (Mast Diagnostics, Merseyside, UK) as part of routine laboratory procedures. The breakpoints used for assigning the clinical category of resistance to antibiotics were those recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (http://www.sfm.asso.fr/). Resistance breakpoints were as follows: MIC > 2 mg/L for colistin; MIC > 16 mg/L for ticarcillin; MIC > 8 mg/L for ceftazidime and imipenem; MIC > 2 mg/L for methicillin; MIC > 4 mg/L for tobramycin; MIC > 1 mg/L for ciprofloxacin; and MIC > 4/76 mg/L for trimethoprim/sulfamethoxazole. Gram-negative isolates were divided into mucoid and non-mucoid isolates depending on phenotype growth pattern on agar plates. Mucoid-to-non-mucoid phenotype conversion in two clinical mucoid *P. aeruginosa* strains was achieved after 10 passages on trypticase soy agar (bioMérieux).

Susceptibility testing

MICs of squalamine, ASDs 1 and 2 and antibiotic controls were determined in duplicate by the broth microdilution method.[7] MICs were defined as the lowest concentrations inhibiting bacterial growth as compared with a growth control. Controls of water or methanol were included in each plate and found to have no effect on bacterial growth. In order to examine the reproducibility of our results, evaluation of squalamine, ASDs 1 and 2 and antibiotic control activities against used reference strains was repeated 10 times.

Statistical methods

Statistical analyses were performed using the correlation or the two-sample *t*-test options in Prism 5 for Windows (GraphPad Software).

Results

Evaluation of squalamine, ASDs 1 and 2 (collectively called ASDs for simplicity) and antibiotic control activities against reference strains was repeated and reproducible, and accurate MICs were obtained. For *E. coli* ATCC 25922, MICs were 2, 4, 4 and 2 mg/L for squalamine, ASD 1, ASD 2 and amoxicillin, respectively. For *P. aeruginosa* ATCC 27853, MICs were 8, 4, 8 and 1 mg/L for squalamine, ASD 1, ASD 2 and colistin, respectively. For *S. aureus* ATCC 25923, MICs were 2, 2, 4 and 0.5 mg/L for

Table 1. Tested bacteria with corresponding resistance phenotype as determined by Vitek 2, Etest or disc diffusion method and MICs of squalamine, ASDs 1 and 2 and colistin determined by the broth microdilution method

		MICs (mg/L)				Number of resistant isolates[a]					
Strains	*n*	squalamine	ASD 1	ASD 2	COL	TIC	CAZ	IMP	TOB	CIP	SXT
Non-mucoid Gram-negative isolates											
P. aeruginosa	12	2–8	2–8	2–8	0.5–1	3	4	4	8	4	10
P. aeruginosa[b]	2	4	4	8	1	2	2	2	1	0	0
Haemophilus influenzae	6	4–8	4–8	4	2	1	1	0	0	0	0
E. coli	3	8	16	4	0.5–1	1	0	0	0	0	2
A. baumannii	2	8	16	4	4	2	2	1	2	1	0
O. anthropi	3	8	8–16	8	4	2	2	0	3	3	3
Klebsiella pneumoniae	1	16	16	16	2	1	0	0	0	0	0
Klebsiella oxytoca	3	16	16	16	2	3	0	0	0	0	0
S. maltophilia	5	16	16	8–16	4	4	5	2	4	3	2
Mucoid Gram-negative isolates											
P. aeruginosa	28	16–32	16–32	16–32	4	8	10	5	16	10	25
P. aeruginosa[b]	2	16	32	16	2	2	2	2	1	0	0
Burkholderia cepacia	2	16–64	16–64	128	64	2	1	2	2	2	2
Inquilinus limosus	5	16–64	16–64	128	32	5	5	2	5	5	5
E. aerogenes	2	32	32	64	64	2	2	1	2	2	2
Serratia marcescens	2	32	32	32	16	2	0	0	2	0	0
Brevundimonas diminuta	1	64	64	32	16	0	1	0	1	1	1
Achromobacter xylosoxidans	15	64	64	128	16	7	6	8	15	15	15
Gram-positive isolates											
S. aureus[c]	33	2–8	0.5–4	2–8							
Corynebacterium spp.	8	2–4	2–4	2–4							
Nocardia spp.	2	4	4	8							
S. pneumoniae	2	32	32	32							
Total	137										

COL, colistin; TIC, ticarcillin; CAZ, ceftazidime; IMP, imipenem; TOB, tobramycin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole.
[a]Breakpoints for resistance were in accordance with CA-SFM recommendations (http://www.sfm.asso.fr/).
[b]Two mucoid strains of *P. aeruginosa* tested before and after suppressing their mucoid phenotype by successive cultures on TSA medium.
[c]Twelve strains were resistant to methicillin.

squalamine, ASD 1, ASD 2 and vancomycin, respectively. For the clinical isolates, the ASDs demonstrated MIC values varying from 2 to 128 mg/L for Gram-negative species (Table 1). According to CA-SFM breakpoints, non-mucoid strains were found to be susceptible to colistin except for *Acinetobacter baumannii*, *Ochrobactrum anthropi* and *Stenotrophomonas maltophilia*, while mucoid Gram-negative isolates were resistant to this antibiotic (Table 1). As reflected by MIC values in Table 1, ASDs possess a significantly higher activity against colistin-susceptible isolates (5.63 ± 4.98 mg/L) as compared with colistin-resistant ones (44.68 ± 31.30 mg/L, $P < 0.0001$). Indeed, resistance to colistin significantly correlated with increased MIC values of tested ASDs ($r_1 = 0.665$, $r_2 = 0.667$, $r_3 = 0.534$ for squalamine, ASD 1 and ASD 2, respectively, $P < 0.0001$; Figure 1b, part I). Furthermore, as determined from MICs, the ASDs demonstrated significantly higher activity against non-mucoid isolates (7.72 ± 4.12 mg/L) as compared with mucoid ones (48.11 ± 35.45 mg/L, $P < 0.0001$). Indeed, the mucoid phenotype was significantly correlated with increased MIC values of squalamine, ASD 1 and ASD 2 ($r_1 = 0.643$, $r_2 = 0.624$, $r_3 = 0.638$), respectively, $P < 0.0001$; Figure 1b, part II). In order to estimate more precisely how the mucoid character of *P. aeruginosa* strains may affect their susceptibility to ASDs, the mucoid mode of growth of two clinical isolates was successfully suppressed. Losing the mucoid phenotype significantly increased susceptibility to the ASDs as reflected by decreased MIC values for non-mucoid strains compared with mucoid strains (Table 1). Interestingly, ASDs were active against Gram-positive isolates with MICs ranging from 0.5 to 8 mg/L, except for *Streptococcus pneumoniae* isolates with MICs of 32 mg/L.

Discussion

We report herein the first evaluation of the activity of ASDs against various bacterial clinical isolates including MDR and emergent bacteria recovered from CF patients. As depicted by MIC values (Table 1), tested ASDs showed moderate activity against Gram-negative bacteria. However, we found that their activity was associated with resistance to colistin in this group of bacteria indicating that these compounds might probably share some mechanistic aspects with polymyxins. In agreement with our work, it was previously reported that resistance to colistin in MDR *Enterobacter aerogenes* isolates could also decrease the susceptibility to squalamine.[8] Resistance to colistin was reported to be due to a reduced net negative charge of the outer membrane lipopolysaccharides.[9] Recent reports indicated that this negative charge was necessary for the activity of squalamine against Gram-negative bacteria explaining the correlation with colistin activity observed in this work.[8] Furthermore, we found that the mucoid phenotype is significantly associated with higher MICs of tested ASDs and colistin. This finding had not been previously reported since the activity of ASDs against mucoid Gram-negative bacteria was evaluated for the first time in this work.[3] The mucoid phenotype is the result of an overproduction of alginates consisting of anionic exopolysaccharides (EPSs).[10] It was reported that EPSs can act as anionic shields that bind polycationic peptides such as polymyxin B leading to increased MICs of this agent for Gram-negative bacteria.[10] As tested ASDs are also cationic, the presence of EPSs might explain the elevated MICs of these compounds observed for mucoid Gram-negative isolates. On the other hand, the ASDs exhibited remarkable activity against Gram-positive isolates including methicillin-resistant *S. aureus* bacteria. The only exception was two isolates of *S. pneumoniae*. Previous reports dealing with the antibacterial activity of ASDs indicated good activity of these compounds against various Gram-positive reference strains including *S. pneumoniae* ATCC 6305.[3] However, it is noteworthy that the two *S. pneumoniae* clinical isolates tested herein were capsulated. As discussed above, the presence of EPSs in the capsule of *S. pneumoniae* might explain the increased MICs noticed for these bacteria as compared with other Gram-positive isolates. Hence, as reported in this study, the evaluation of ASD activity against clinical isolates provided more relevant data about the antibacterial effect of this family of compounds than previously reported using reference bacterial strains. ASDs might have the potential to be further developed as antibiotics. However, it should be noted that the MICs obtained were considerably high in some cases. Administration of antibiotics such as tobramycin and colistin in aerosols for CF patients enables the use of elevated drug doses with minimized systemic effects.[2] Thus we believe that ASDs may be developed for local administration as aerosols for the treatment of lung infections in CF patients rather than systemic administration. In conclusion, our work provides new insights into the antibacterial activities of ASDs against various Gram-negative and Gram-positive clinical isolates. Further work is warranted in order to evaluate their activity in biofilm conditions and as aerosol formulations in a lung-infected animal model.

Acknowledgements

We thank Paul Newton, Wellcome Trust-Mahosot Hospital-Oxford Tropical Medicine Research Collaboration, Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao PDR, for correcting our English.

Funding

This study was supported by the Centre National de la Recherche Scientifique, France.

Transparency declarations

None to declare.

References

1. Davies JC, Bilton D. Bugs, biofilms, and resistance in cystic fibrosis. *Respir Care* 2009; **54**: 628–40.

2. Touw DJ, Brimicombe RW, Hodson ME *et al.* Inhalation of antibiotics in cystic fibrosis. *Eur Respir J* 1995; **8**: 1594–604.

3. Salmi C, Brunel JM. Therapeutic potential of cationic steroid antibacterials. *Expert Opin Investig Drugs* 2007; **16**: 1143–57.

4. Herbst RS, Hammond LA, Carbone DP *et al.* A phase I/IIA trial of continuous five-day infusion of squalamine lactate (MSI-1256F) plus carboplatin and paclitaxel in patients with advanced non-small cell lung cancer. *Clin Cancer Res* 2003; **9**: 4108–15.

5. Loncle C, Salmi C, Vidal N *et al*. Synthesis of new 7-aminosterol squalamine analogues with high antimicrobial activities through a stereoselective titanium reductive amination reaction. *Eur J Med Chem* 2007; **43**: 540–7.

6. Adekambi T, Drancourt M, Raoult D. The *rpoB* gene as a tool for clinical microbiologists. *Trends Microbiol* 2009; **17**: 37–45.

7. Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001; **48** Suppl 1: 5–16.

8. Salmi C, Loncle C, Vidal N *et al*. Squalamine: an appropriate strategy against the emergence of multidrug resistant Gram-negative bacteria? *PLoS ONE* 2008; **3**: e2765.

9. Li J, Nation RL, Milne RW *et al*. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. *Int J Antimicrob Agents* 2005; **25**: 11–25.

10. Llobet E, Tomas JM, Bengoechea JA. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. *Microbiology* 2008; **154**: 3877–86.

Article 3

In vitro antifungal activity of aminosterols against moulds isolated from cystic fibrosis patients

Kamel Alhanout, Jean Michel Brunel, Stéphane Ranque and Jean Marc Rolain

J Antimicrob Chemother doi:10.1093/jac/dkq089

Résumé de l'article

Les infections et/ou colonisations fongiques des voies respiratoires dues aux champignons filamenteux pathogènes sensibles ou multi résistants aux antifongiques représentent un réel danger pour les patients atteints de mucoviscidose soulignant l'importance de la recherche de nouvelles molécules antifongiques. Ce travail a visé, pour la première fois, à évaluer l'activité antifongique de la squalamine et d'un dérivé aminostéroïdien (DAS 1) contre des champignons filamenteux isolés de patients atteints de mucoviscidose. Ainsi, une collection de 47 champignons filamenteux a été étudiée. L'identification moléculaire de ces champignons a été réalisée par l'analyse des séquences du gène ITS (internal transcribed spacer). Les CMIs de la squalamine et du DAS1 ont été déterminées par la méthode de référence de microdilution. Les CMIs des antifongiques classiques (l'amphotéricine B, le voriconazole, l'itraconazole et la caspofungine) couramment utilisés en clinique ont également été déterminées. Les antifongiques testés ont montré des profils d'activités hétérogènes à l'égard de l'ensemble de souches testées avec des valeurs de CMIs variant de < 0.5 to 32 mg/L selon l'espèce. A l'inverse, les DASs ont présenté des profils d'activité homogènes vis-à-vis de tous les isolats avec des CMIs variant de 2 à 4 et de 8 à 16 mg /L (squalamine et DAS1, respectivement) quelle que soit la sensibilité des souches aux antifongiques classiques. En conclusion cette étude démontre que les dérivés aminostéroïdiens possèdent un effet antifongique intéressant avec des CMIs relativement élevées justifiant le

développement de ce groupe de molécules pour une utilisation par voie locale sous forme d'aérosol comme nous l'avons souligné dans notre travail précédent. L'activité antifongique des DAS est probablement à relier à un mécanisme d'action original et différent des autres antifongiques connus qui méritera d'être étudié d'avantage ultérieurement.

Par ailleurs, l'utilisation de l'identification moléculaire des souches testées par l'analyse des séquences du gène ITS a permis l'indentification de la totalité des souches (47 souches) y compris 23 souches (48.9%) qui n'avaient pu être identifiées au niveau de l'espèce à l'aide des techniques phénotypiques conventionnelles. Parmi ces souches, 16 isolats appartenant à 9 espèces différentes n'ont jamais été décrites dans le contexte de la mucoviscidose, et ont aussi pu être identifiées dans ce travail et ont fait l'objet d'une publication présentée en Annexe (Article **6**).

Journal of Antimicrobial Chemotherapy Advance Access published March 24, 2010

Journal of Antimicrobial Chemotherapy

J Antimicrob Chemother
doi:10.1093/jac/dkq089

In vitro antifungal activity of aminosterols against moulds isolated from cystic fibrosis patients

Kamel Alhanout, Jean Michel Brunel, Stéphane Ranque and Jean Marc Rolain*

URMITE UMR 6236, CNRS-IRD, Faculté de Médecine et de Pharmacie, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France

*Corresponding author. Tel: +33-4-91-32-43-75; Fax: +33-4-91-38-77-72; E-mail: jean-marc.rolain@univmed.fr

Keywords: filamentous fungi, antifungal agents, lung infections

Sir,

We have recently demonstrated that aminosterol derivatives (ASDs) possess an interesting *in vitro* antibacterial activity against multidrug-resistant bacteria recovered from cystic fibrosis (CF) patients.[1] However, mould infections are also a serious threat for CF patients.[2] Indeed, the fragile pulmonary environment of these patients facilitates the development of bacterial and fungal infections.[2] Repeated bacterial infections, intensive antibiotic therapy and lung transplantation are considered as risk factors for airway infection and/or colonization by moulds in CF patients.[2] Major problems are related to *Aspergillus* spp. and *Scedosporium* spp. that may cause chronic disorders such as asthma, bronchitis and aspergilloma.[2] Amphotericin B, caspofungin and azoles are currently used in the management of fungal infections. However, in addition to acquired resistance resulting from antifungal treatment pressure as noted for *Aspergillus* spp., intrinsic resistance to available antifungals reported in some fungi such as *Fusarium*, *Rhizopus*, *Rhizomucor* and *Scedosporium* spp. represents a major issue, highlighting the need to develop new antifungal compounds.[2,3] ASDs have been evaluated only against reference fungal strains.[4] We evaluated herein for the first time the *in vitro* antifungal activity of squalamine and one of our compounds (ASD 1; the chemical structure is available in Alhanout *et al.*[1]) possessing an *in vitro* antibacterial effect,[1] against a panel of clinical mould isolates recovered from sputa of CF patients. All tested isolates were identified at the species level using partial internal transcribed spacer PCR amplification, and the sequencing procedure and sequences have been deposited in GenBank under accession numbers GU594733–GU594779. The *in vitro* antifungal activities of currently available systemic antifungal drugs were evaluated for comparison purposes. MICs of all tested compounds were determined by the reference broth dilution method as previously reported,[5] and found to be reproducible and in agreement with previous reports for standard antifungal agents as noted using reference fungal strains including four yeasts and one mould (*Aspergillus brasiliensis* ATCC 16404) (Table 1).[5] Squalamine and ASD 1 displayed MICs for all tested isolates ranging from 8 to 16 mg/L and from 2 to 4 mg/L, respectively. Since no internationally agreed antifungal resistance breakpoints are currently available, we adopted breakpoints proposed in a recent work suggesting MICs >2 mg/L for azoles to designate resistance in *Aspergillus* spp.[6] For simplicity, we used this MIC to assign resistance to other antifungal drugs for all fungal isolates. In keeping with previous studies, *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus flavus* isolates were susceptible to all tested antifungals.[5] Conversely, *Aspergillus terreus* isolates were resistant to amphotericin B as previously reported and, surprisingly, to itraconazole for our CF clinical isolates, which is unusual.[5] As has been documented for other *Aspergillus* spp., the resistance of *A. terreus* to itraconazole probably may be acquired since this fungus chronically colonizes the airways of CF patients that are exposed to various antimicrobial treatments.[3] According to previous reports, *Aspergillus ustus* was resistant to itraconazole, voriconazole and caspofungin.[6] Resistance to caspofungin and voriconazole was noted for *Emericella quadrilineata*. Antifungal resistance in *Penicillium* and *Cladosporium* spp. was previously reported.[5] Accordingly, two out of four *Penicillium griseofulvum* isolates were resistant to voriconazole and caspofungin while the other two isolates were resistant to all tested available antifungals. Moreover, *Cladosporium* spp. were resistant to all antifungals except amphotericin B, while *Alternaria* spp. were susceptible to all tested antifungals. In accordance with previous findings,[5] resistance to all antifungals was found for all tested isolates of *Fusarium proliferatum*, *Scedosporium prolificans*, *Pseudallescheria boydii*, *Scedosporium apiospermum*, *Rhizopus oryzae* and *Rhizomucor tauricus*. Hence, airway colonization with these moulds is quite worrying, calling for an effective therapy to be available. Conversely, tested aminosterols and particularly ASD 1 demonstrated a homogeneous *in vitro* activity against all susceptible and resistant isolates. This finding suggests that novel mechanistic aspects, distinct from those of available drugs, underlie the antifungal activity of ASDs. While it is known that squalamine acts by disrupting bacterial membranes,[1] the antifungal mechanism of action of ASDs remains unknown. Squalamine and ASD 1 previously demonstrated comparable activities against various bacterial isolates,[1] whilst the present data interestingly show that ASD 1 was superior to squalamine, indicating a probable different effect due to a different chemical structure of the two tested compounds. While the safety of squalamine has been demonstrated in clinical studies,[4] no *in vitro* or *in vivo* toxicity studies have been reported for synthesized aminosterols. Nevertheless, since ASDs were reported to act as membrane disruptors, such a wide antimicrobial spectrum may encompass a potential toxicity that should be further investigated. Overall, we have demonstrated herein that ASDs possess an interesting *in vitro* antifungal activity,[1] advocating their development for local administration, for example as aerosols especially in the context of CF. Further studies are warranted in order to evaluate their *in vivo* antimicrobial activities in animal models.

Table 1. MICs of squalamine, ASD 1 and standard antifungal agents for reference fungal strains and clinical mould isolates

Strains	Number of tested isolates	MICs (mg/L)					
		Sq	ASD 1	AMB	ITC	VRC	CAS
Candida albicans ATCC 90028	1	16	2	<0.5	1	<0.5	1
Candida glabrata ATCC 90030	1	8	1	2	1	1	1
Candida krusei ATCC 6258	1	8	1	1	1	<0.5	1
Candida parapsilosis ATCC 22019	1	8	1	1	2	1	0.5
Aspergillus brasiliensis ATCC 16404	1	8	2	1	<0.5	1	1
Aspergillus fumigatus complex	10	8–16	4	1–2	2	1–2	<0.5–1
Aspergillus niger complex	2	16	4	1	1	2	<0.5
Aspergillus flavus complex	8	8–16	2–4	<0.5–1	1–2	<0.5–1	1
Aspergillus terreus complex	5	8	4	**8 to >32**	**16 to >32**	<0.5	<0.5
Aspergillus ustus complex	1	16	2	2	**>32**	**16**	**16**
Emericella quadrilineata	1	8	2	1	1	**16**	**>32**
Penicillium griseofulvum	2	8–16	2–4	2	2	**16**	**16**
Penicillium griseofulvum	2	8	2	**8**	**>32**	**16**	**16**
Cladosporium cucumerinum	1	16	4	2	**>32**	**8**	**16**
Cladosporium cladosporioides	2	16	4	2	**>32**	**8**	**16**
Alternaria triticina	1	8	4	<0.5	2	2	2
Alternaria alternata	2	8	4	<0.5	2	2	1
Alternaria alternata/tenuissima	1	16	2	1	2	2	1
Fusarium proliferatum	2	16	2–4	**>32**	**>32**	**16**	**16**
Scedosporium prolificans	1	16	2	**>32**	**>32**	**>32**	**16**
Pseudallescheria boydii	2	16	4	**8**	**16**	**16**	**8**
Scedosporium apiospermum	1	8	2	**>32**	**16**	**16**	**16**
Rhizopus oryzae	1	16	2	**8**	**16**	**8**	**16**
Rhizomucor tauricus	2	16	2	**16**	**8**	**16**	**16**

Sq, squalamine; AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; CAS, caspofungin.
MICs >2 mg/L are presented in bold, since they were considered to designate resistance.[6]

Funding

Supported by the Centre National de la Recherche Scientifique, France and the French Association Vaincre la Mucoviscidose, France.

Transparency declarations

None to declare.

References

1 Alhanout K, Brunel JM, Raoult D *et al*. *In vitro* antibacterial activity of aminosterols against multidrug-resistant bacteria from patients with cystic fibrosis. *J Antimicrob Chemother* 2009; **64**: 810–4.

2 Pihet M, Carrere J, Cimon B *et al*. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis—a review. *Med Mycol* 2009; **47**: 387–97.

3 Qiao J, Liu W, Li R. Antifungal resistance mechanisms of *Aspergillus*. *Nippon Ishinkin Gakkai Zasshi* 2008; **49**: 157–63.

4 Brunel JM, Salmi C, Loncle C *et al*. Squalamine: a polyvalent drug of the future? *Curr Cancer Drug Targets* 2005; **5**: 267–72.

5 Cuenca-Estrella M, Gomez-Lopez A, Mellado E *et al*. Head-to-head comparison of the activities of currently available antifungal agents against 3,378 Spanish clinical isolates of yeasts and filamentous fungi. *Antimicrob Agents Chemother* 2006; **50**: 917–21.

6 Verweij PE, Howard SJ, Melchers WJ *et al*. Azole-resistance in *Aspergillus*: proposed nomenclature and breakpoints. *Drug Resist Updat* 2009; **12**: 141–7.

Article 4

In vitro activity of aminosterols against yeasts involved in blood stream infections

Kamel Alhanout, Lamia Djouhri, Nicolas Vidal, Jean Michel Brunel, Renaud Piarroux and Stéphane Ranque *

En révision à **Medical Mycology**

***corresponding author**:

Dr Stéphane RANQUE

Laboratoire de Parasitologie-Mycologie

AP-HM Timone, F-13385 MARSEILLE CEDEX 5

Tel. +33 491 38 60 90

Fax. +33 491 38 49 58

Email: stephane.ranque@ap-hm.fr

Résumé de l'article

La squalamine et les dérivés aminostéroïdiens ont fait preuve d'activités antifongiques intéressantes vis-à-vis des isolats cliniques de bactéries et un nombre limité de souches de référence de levures. La sécurité d'emploi de la squalamine a été démontrée dans des études clinque de phase II indiquant une tolérance parfaite de cette molécule administrée à une dose de 300 mg/m^2/jour par perfusion lente durant 5 jours. Ainsi, notre objectif était d'évaluer l'activité antifongique de la squalamine et un dérivé aminostéroïdien DAS1 contre un panel d'isolats cliniques de levures. Les antifongiques systémiques couramment disponibles ont été également testés à des fins de comparaison. L'effet hémolytique *in vitro* des DASs a été mesuré afin d'évaluer leur toxicité. Ce test est préconisé dans la littérature pour l'estimation *in vitro* de la toxicité des aminostérols. Les CMIs de la squalamine, du DAS1 et des antifongiques classiques ont été déterminées, contre 21 isolats de levures impliquées dans des fongémies. Des CMIs homogènes variant de 8 à 16 mg/L et de 1 à 2 mg/L ont été notées pour respectivement, la squalamine et le DAS1. En revanche, des profils d'activités hétérogènes ont été notés pour les antifongiques systémiques avec des CMIs variant de < 0.5 à > 32 mg/L. La survenue d'hémolyse avec les DASs a été notée à des concentrations au-dessus de leur CMIs. Dans les études de survie, l'effet fongicide avec DAS1 s'est produit aussi rapidement qu'avec l'amphotéricine B (4h) alors que celui de la squalamine était moins rapide (8h). Les DASs ont engendré une rupture de la membrane des levures qui était

dépendante du temps, comme le démontre la hausse progressive de l'efflux d'ATP intracellulaire constaté après l'ajout de chaque composé à une concentration égale à la CMI. En conclusion, nos données préliminaires indiquent que les DASs possèdent une activité antifongique intéressante *in vitro* qui méritera d'être là encore évaluée *in vivo* en utilisant des modèles animaux.

In vitro activity of aminosterol compounds against Yeasts Isolated from Blood Stream infections

Kamel Alhanout[a], Jean Michel Brunel, Lamia Djouhri, Stéphane Ranque[a,b]*

a. URMITE UMR 6236, CNRS-IRD, Faculté de Médecine et de Pharmacie, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France.

b. Laboratoire de Parasitologie-Mycologie, AP-HM Timone, 264 Boulevard Saint Pierre, 13385 Marseille cedex 05, France.

***corresponding author**:

Dr Stéphane RANQUE
Laboratoire de Parasitologie-Mycologie
AP-HM Timone
F-13385 MARSEILLE CEDEX 5
Tel. +33 491 38 60 90
Fax. +33 491 38 49 58
Email: stephane.ranque@ap-hm.fr

Keywords: squalamine, antifungal, resistance, therapy.

Abstract: 250

Abstract

Squalamine and squalamine-related aminosterol derivatives (ASDs) have demonstrated interesting antibacterial and antifungal activities against clinical bacterial and mold isolates and references yeast strains. Squalamine was reported in a phase II study to be safely administrated to cancer patients as antiangiogenic agent. This work determined the *in vitro* antifungal activity of squalamine and a synthesized aminosterol ASD against clinical yeasts isolates from blood cultures. For comparison, available antifungals were evaluated. MICs were determined and a time-kill study was performed. Toxicity of tested aminosterols was evaluated by determining their haemolytic effects. The effect of squalamine and ASD on membrane integrity of yeast was investigated by measuring intracellular ATP efflux from strains treated with aminosterols. MICs ranging from 8 to 16 mg/L and from 1 to 2 mg/L were noted for squalamine and ASD, respectively, while antifungal agents demonstrated inconsistent activity according to the strains. The haemolytic effect of tested aminosterols was highly above their MIC values suggesting a good *in vitro* safety index. The killing rate of ASD (4h) was equal to that of amphotericin B and faster than that of squalamine (8h). Remarkably, tested aminosterols induced a time-dependant disruption of yeast membrane as evidenced by gradual increase of ATP efflux. In conclusion, aminosterols may be further investigated as antifungal agents due to their good activity and low toxicity evaluated *in vitro* with probably a novel mechanism of action. Further works are warranted to fully elucidate their

antifungal mechanism of action and evaluate them *in vivo* using animal models for fungal infections.

Introduction

Squalamine is a natural aminosterol first isolated from the tissues of the dogfish shark *Squalus acanthias* (fig. 1) possessing numerous therapeutic virtues such as antiangiogenic and antimicrobial properties (2). The synthesis of squalamine is known to be sophisticated and expensive while many squalamine-related compounds, namely aminosterol derivatives (ASDs), have been synthesized from easily available and cheaper starting materials and have also demonstrated interesting antimicrobial activities (20).We have recently reported that aminosterol compounds possess interesting antibacterial and antifungal effects as determined against various bacterial clinical isolates containing multidrug resistant (MDR) species [1]. However, invasive fungal infections due to yeast species also represent a major threat particularly for immunocompromised patients (5,9). Such infections are known to be associated with high morbidity and mortality especially when involved pathogens present a natural or acquired resistance to available antifungal agents (9,10,14). Interestingly, aminosterols compounds had been reported to possess interesting antifungal activity against reference yeast isolates. Moreover, a phase II study evaluating the antiangiogenic effect of squalamine demonstrated that this aminosterol compound was safely administrated to cancer patients in a continuous five-day infusion of 300 mg/m^2/day (6). Hence, we aimed in this work to investigate their potential for the first time against a panel of clinical yeast isolates recovered from blood cultures containing multidrug resistant species. We evaluated the he

in vitro activity of squalamine and a synthetic aminosterol compound (ASD, fig.1) along with available antifungal agents such as amphotericin B, fluconazole, itraconazole, voriconazole for comparison purposes. A time kill study was also performed using squalamine, ASD and amphotericin B. In order to estimate the *in vitro* toxicity of tested aminosterols, their haemolytic effect was determined since this test is constantly used as an indicator of aminosterols toxicity (3,4,8,12). Moreover, we had insight into their mechanism of action using time-kill study and the measure of intracellular ATP efflux form yeast strains induced by tested compounds.

MATERIALS AND METHODS

Squalamine, ASD and antifungal agents

Squalamine was a generous gift from Pr. M. Zasloff (Georgetown University, Washington). ASD was synthesized as previously reported (21). Stock solutions were prepared in water for squalamine and in methanol for ASD. Tested antifungal agents were amphotericin B (Bristol Myers Squibb, Rueil-Malmaison, France), fluconazole (Pfizer, Paris, France), itraconazole (Cipharmed, Paris, France), voriconazole (Pfizer, Paris, France) and caspofungin (Merck Sharp & Dohme-Chibret, Paris, France).

Fungal strains

Control strains used in this work were *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida krusei* (ATCC 6258) and *Candida*

parapsilosis (ATCC 22019). A collection of 21 bloodstream yeast isolates were tested including: *C. albicans* (n=2), *C. glabrata* (n=2), *C. guilliermondii* (n=1), *C. krusei* (n=3), *C. lusitaniae* (n=3), *C. parapsilosis* (n=5), *C. tropicalis* (n=3), and *Cryptococcus neoformans* (n=2). Isolates were cultured on Mycosis IC/F bottles (Becton Dickinson Diagnostics, France). Purity was checked on CHROM agar Candida chromogenic medium (Bio-Rad, France). Species identification was based on direct immunological tests (Bichrolatex Albicans and Krusei Color, Fumouze, France), microscopical morphology on PCB medium, carbohydrate assimilation profile using Auxacolor (Sanofi Diagnostic Pasteur, France) and, when necessary, ID 32C (Biomerieux, France). All identifications have been confirmed by sequence analysis of the internal transcribed spacers (ITS) of the rDNA gene using previously described procedure (19). The ITS sequences of different fungi were aligned, to each other as well as the sequences retrieved from NCBI databases, using multiple sequence alignment software (ClustalX). A phylogenic tree was constructed using the neighbor-joining clustering method implemented using MEGA software - version 4. The ITS sequence data of *Candida tropicalis AB43708, Candida guilliermondii FJ515181, Candida lusitaniae EF136370, Cryptococcus neoformans FJ011544* and *Ornithonyssus bacoti AM903318* were obtained from GenBank and added in the phylogenic tree.

Antifungal susceptibility tests

MICs of squalamine, ASD and classical antifungal agents were determined in duplicate by the broth microdilution method according to CLSI standard documents M27-A2 (15).

Red blood cell lyses

The haemolytic activity of squalamine, ASD, and for comparison purpose amphotericin B was evaluated and the half maximal haemolytic contraction (IC_{50}) was determined using previously reported protocol (12). Maximal haemolysis was considered to be produced by 50 µg/ml solution of Cetyl trimethylammonium bromide (CTAB). Human red blood cells from fresh blood (Hematocrit ~5%) were suspended in PBS and incubated for 1.5 h at 37°C after addition of tested compounds. After centrifugation at 2000 × g, relative haemoglobin concentration in supernatants was monitored by measuring the absorbance at 540 nm.

Time-kill assays

Time-kill assays for squalamine, ASD and amphotericin B were performed using previously reported protocol (7). Two reference strains were tested: *Candida albicans* (ATCC 90028) and *Candida glabrata* (ATCC 90030). A fungicidal effect was considered to designate a reduction of >99.9% of the number of CFU/ml compared to starting inoculum. Results are presented as means ± standard deviation from triplicate time-kill assays.

Measurement of ATP efflux

The kinetics of intracellular ATP efflux was determined using previously reported procedure (22). Briefly, a suspension of growing *Candida albicans* (ATCC 90028) to be studied in Müller Hinton broth was prepared and incubated at 32 ° C. *C. albican*s in suspension was added to solutions of 1x MIC of squalamine and 1x MIC ASD and for comparison, to solutions of 1 x amphotericin B and CTAD (5 µg/ml). The kinetic of ATP efflux was measured during 60 min at intervals of 10 min. results are presented as means ± standard deviation from triplicate time-kill assays.

Results

The MICs of squalamine, ASD and antifungal agents were determined using a reproducible microdilution method and the MICs of the antifungal agents tested against control strains were within the expected ranges (fig. **2**). MICs for squalamine and ASD against tested isolates ranged form 8 to 16 mg/L and 1 to 2 mg/L, respectively (Fig. **2**). On the other hand, standard antifungal drugs tested in this work showed inconsistent activities according to the species as reflected by MIC values (fig. **2**). Indeed, high MIC values suggestive of *in vitro* resistance could be noted for many isolates such as *Candida guilliermondii* and *Candida lusitaniae*, *C. krusei* and *C. neoformans* (fig. **2**). Squalamine and ASD showed IC_{50} of 90 and 40 µg/ml, respectively, while that of amphotericin B

taken as control was > 100 mg/L. Thus, mean ratios (IC_{50} /MIC) of 8 and 30 could be observed for squalamine and ASD, respectively. The killing rates of squalamine, ASD and amphotericin B against *C. albicans* and *C. glabrata* are presented in fig. **3**. Treatment of both strains of *C. albicans* and *C. glabrata* with squalamine and ASD at their MIC values resulted in a fungicidal effect after 8 hr and 4 hr, respectively**.** Amphotericin B fungicidal effect was noted after 4 hr (fig. **3 a** and **b**). The kinetics of the intracellular ATP efflux resulting from the treatment of *C. albicans* strain with squalamine, ASD, and for comparison purposes CTAB and amphotericin B was measured. For squalamine and ASD, the maximum of ATP efflux was reached within 40 min. CTAB gave a rapid ATP efflux reaching the maximum with 10 min while amphotericin B produced on ATP efflux.

Discussion

In agreement with previous reports (7,17) some isolates presented clearly high MIC values indicating *in vitro* resistance (fig. 2). Amphotericin B was considered for a long time as the drug of choice for the treatment of invasive fungal infections. However, emerging fungal pathogens such as *C. lusitaniae* and *C. guilliermondii* might be resistant to amphotericin B as found in this work (11,13). Azoles resistance is increasingly reported in *Candida* species which is in keeping with this study for *C. krusei*, *C. glabrata* and *C. neoformans* isolates (7,9,13). Moreover, the elevated MICs for caspofungin noted for *C. neoformans* isolates were expected since *C. neoformans* species are known to be naturally

resistant to this antifungal agent (18). However, caspofungin demonstrated low *in vitro* activity against *C. guilliermondii* as compared to other tested isolates which also agrees with previous report (16,18). On the other hand, MICs of squalamine and ASD indicate that these compounds hold a remarkable *in vitro* antifungal activity with ASD being clearly more active than squalamine. Unlike used antifungal drugs, squalamine and ASD exhibited interesting homogeneous MICs against all tested isolates. More interestingly, the IC_{50} of squalamine and ASD were quite higher than their MIC values. The higher mean ratio (IC_{50} /MIC) of ASD as compared to squalamine suggest the haemolytic and the antifungal activity might be correlated or produced by a similar mechanism. Tested aminosterols showed a fungicidal effect with ASD having a killing equal that was faster than that of squalamine and equal to that of amphotericin B. However, being active against yeast strains that are resistant to amphotericin B and other antifungal agents may indicate that the antifungal effect of ASDs encloses new mechanistic aspects different form those reported with known antifungal drugs. It has been demonstrated that squalamine acts by disrupting the outer membrane of Gram-negative bacteria as reflected by increased permeability to hydrophobic dyes and induced intracellular ATP efflux by squalamine (22). To investigate whether a similar effect may be observed for yeasts, the effect of squalamine and ASD on yeast membrane integrity were evaluated. For comparison purposes, we included amphotericin B as well as a detergent agent, CTAB. Interestingly, squalamine and ASD resulted in

graduated intracellular ATP efflux reaching the maximum with 40 min while the detergent agent was clearly more rapid with maximum effect obtained within 10 min. On the other hand and as expected amphotericin B had no effect on ATP efflux. This probably indicates the aminosterols act by disturbing yeast membrane in a detergent-like manner that may also be responsible for their haemolytic. In conclusion, our findings indicate that tested aminosterol compounds have a potential to be developed as antifungal agents since their in vitro toxicity/ activity was promising. Moreover, aminosterols probably present new mechanistic features making them a possible candidate for treating multidrug resistant yeast strains. However, further works are warranted to elucidate the mechanism of antifungal action of ASDs and evaluate their *in vivo* activity using animal models of fungal infection.

REFERENCES

1. **Alhanout, K., J. M. Brunel, D. Raoult, and J. M. Rolain**. 2009. *In vitro* antibacterial activity of aminosterols against multidrug-resistant bacteria from patients with cystic fibrosis. J.Antimicrob.Chemother. **64**:810-814.

2. **Brunel, J. M., C. Salmi, C. Loncle, N. Vidal, and Y. Letourneux**. 2005. Squalamine: a polyvalent drug of the future? Curr.Cancer Drug Targets. **5**:267-272.

3. **Chin, J. N., R. N. Jones, H. S. Sader, P. B. Savage, and M. J. Rybak**. 2008. Potential synergy activity of the novel ceragenin, CSA-13, against clinical isolates of Pseudomonas aeruginosa, including multidrug-resistant P. aeruginosa. J.Antimicrob.Chemother. **61**:365-370.

4. **Chin, J. N., M. J. Rybak, C. M. Cheung, and P. B. Savage**. 2007. Antimicrobial activities of ceragenins against clinical isolates of resistant Staphylococcus aureus. Antimicrob.Agents Chemother. **51**:1268-1273.

5. **Fisher, B. T. and T. E. Zaoutis**. 2008. Treatment of invasive candidiasis in immunocompromised pediatric patients. Paediatr.Drugs **10**:281-298.

6. **Herbst, R. S., L. A. Hammond, D. P. Carbone, H. T. Tran, K. J. Holroyd, A. Desai, J. I. Williams, B. N. Bekele, H. Hait, V. Allgood, S. Solomon, and J. H. Schiller**. 2003. A phase I/IIA trial of continuous

five-day infusion of squalamine lactate (MSI-1256F) plus carboplatin and paclitaxel in patients with advanced non-small cell lung cancer. Clin.Cancer Res. **9**:4108-4115.

7. **Izgu, F., D. Altinbay, and A. E. Tureli**. 2007. *In vitro* susceptibilities of Candida spp. to Panomycocin, a novel exo-beta-1,3-glucanase isolated from Pichia anomala NCYC 434. Microbiol.Immunol. **51**:797-803.

8. **Kikuchi, K., E. M. Bernard, A. Sadownik, S. L. Regen, and D. Armstrong**. 1997. Antimicrobial activities of squalamine mimics. Antimicrob.Agents Chemother. **41**:1433-1438.

9. **Klepser, M. E.** 2006. Candida resistance and its clinical relevance. Pharmacotherapy **26**:68S-75S.

10. **Krcmery, V. and A. J. Barnes**. 2002. Non-albicans Candida spp. causing fungaemia: pathogenicity and antifungal resistance. J.Hosp.Infect. **50**:243-260.

11. **Lass-Florl, C., A. Mayr, S. Perkhofer, G. Hinterberger, J. Hausdorfer, C. Speth, and M. Fille**. 2008. Activities of antifungal agents against yeasts and filamentous fungi: assessment according to the methodology of the European Committee on Antimicrobial Susceptibility Testing. Antimicrob.Agents Chemother. **52**:3637-3641.

12. **Leszczynska, K., A. Namiot, D. E. Fein, Q. Wen, Z. Namiot, P. B. Savage, S. Diamond, P. A. Janmey, and R. Bucki**. 2009. Bactericidal activities of the cationic steroid CSA-13 and the cathelicidin peptide LL-37 against Helicobacter pylori in simulated gastric juice. BMC.Microbiol. **9**:187.

13. **Masia, C. M. and R. F. Gutierrez**. 2002. Antifungal drug resistance to azoles and polyenes. Lancet Infect.Dis. **2**:550-563.

14. **Montravers, P. and K. Jabbour**. 2006. Clinical consequences of resistant Candida infections in intensive care. Int.J.Antimicrob.Agents **27**:1-6.

15. **NCCLS, W. P. U. 2.** 2002. National Committee for Clinical Laboratory Standards. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts: Approved Standard M27-A2.

16. **Pfaller, M. A., D. J. Diekema, M. Mendez, C. Kibbler, P. Erzsebet, S. C. Chang, D. L. Gibbs, and V. A. Newell**. 2006. Candida guilliermondii, an opportunistic fungal pathogen with decreased susceptibility to fluconazole: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program. J.Clin.Microbiol. **44**:3551-3556.

17. **Pfaller, M. A., S. A. Messer, L. Boyken, R. J. Hollis, C. Rice, S. Tendolkar, and D. J. Diekema**. 2004. In vitro activities of voriconazole,

posaconazole, and fluconazole against 4,169 clinical isolates of Candida spp. and *Cryptococcus neoformans* collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. Diagn.Microbiol.Infect.Dis. **48**:201-205.

18. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema**. 2004. Further standardization of broth microdilution methodology for in vitro susceptibility testing of caspofungin against Candida species by use of an international collection of more than 3,000 clinical isolates. J.Clin.Microbiol. **42**:3117-3119.

19. **Pryce, T. M., S. Palladino, I. D. Kay, and G. W. Coombs**. 2003. Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system. Medical Mycology **41**:369-381.

20. **Salmi, C. and J. M. Brunel**. 2007. Therapeutic potential of cationic steroid antibacterials. Expert.Opin.Investig.Drugs **16**:1143-1157.

21. **Salmi, C., C. Loncle, N. Vidal, M. Laget, Y. Letourneux, and J. M. Brunel**. 2008. Antimicrobial activities of 3-amino- and polyaminosterol analogues of squalamine and trodusquemine. J.Enzyme Inhib.Med.Chem. **23**:860-865.

22. **Salmi, C., C. Loncle, N. Vidal, Y. Letourneux, J. Fantini, M. Maresca, N. Taieb, J. M. Pages, and J. M. Brunel**. 2008. Squalamine: an appropriate strategy against the emergence of multidrug resistant gram-negative bacteria? PLoS.ONE. **3**:e2765.

FIGURE LEGENDS

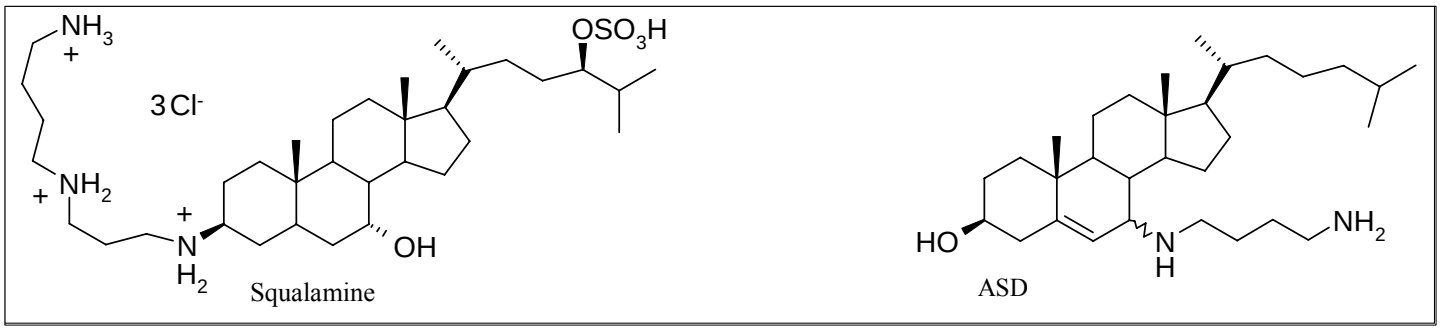
Figure 1: structures of squalamine and a synthesized aminosterol ASD.

Figure 2: Cladogram tree displaying the yeasts isolates tested in this work with the corresponding MICs and IC_{50} values of tested compounds.

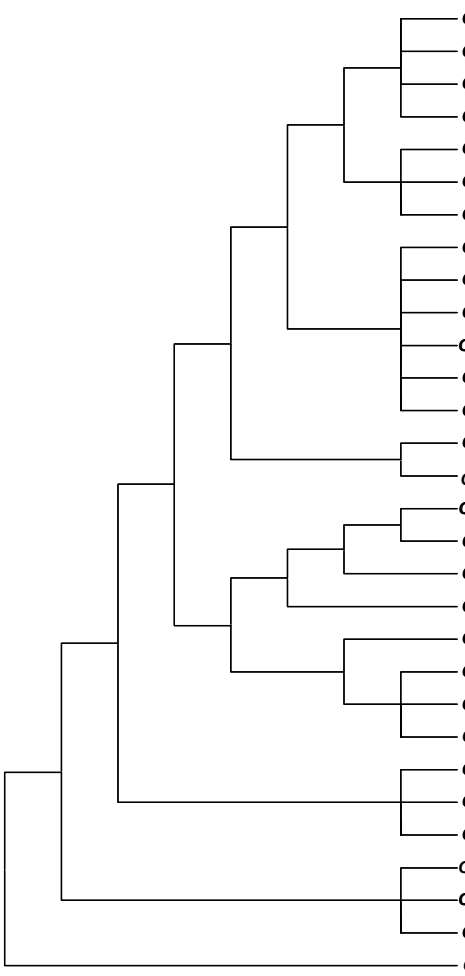
Sq: squalamine, ASD: aminosterol derivative, AMB: amphotericin B, FLC : fluconazole, ITC : itraconazole, CSN : caspofungin. *: designate the half maximal haemolytic concentration (IC_{50})

Figure 3: Time-Kill essays of squalamine, aminosterols derivative (ASD) and amphotericin B all tested at their MIC values on *Candida albicans* ATCC 90028 (**a**) and *C. glabrata* ATCC 90030 (**b**)

Figure 4: kinetics of intracellular ATP efflux from *Candida albicans* (ATCC 90028) treated with squalamine, aminosterol derivative (ASD), Cetyl trimethylammonium bromide (CTAB) and amphotericin B (AMB) all at their MIC values.



Fig; 1



Strains	MIC (mg/L)						
	Sq	**ASD**	**AMB**	**FLC**	**ITC**	**VRC**	**CSN**
Candida tropicalis	16	1	1	2	1	1	< 0.5
Candida tropicalis AB437087	---	---	---	---	---	---	---
Candida tropicalis	16	1	2	2	1	1	< 0.5
Candida tropicalis	16	1	2	2	1	1	< 0.5
Candida albicans	16	2	<0.5	1	1	< 0.5	< 0.5
Candida albicans	16	2	1	1	1	< 0.5	< 0.5
Candida albicans 90028	16	2	1	<0.5	1	< 0.5	< 0.5
Candida parapsilosis	16	1	2	<0.5	4	1	2
Candida parapsilosis	16	1	4	1	>32	1	2
Candida parapsilosis 22019	16	1	1	<0.5	1	1	<0.5
Candida parapsilosis	16	1	4	1	1	1	<0.5
Candida parapsilosis	16	1	2	<0.5	>32	1	<0.5
Candida parapsilosis	16	1	2	1	>32	1	<0.5
Candida guilliermondii	8	2	<0.5	2	2	<0.5	16
Candida guilliermondii FJ515181	---	---	---	---	---	---	---
Candida lusitaniae	8	1	1	1	<0.5	1	1
Candida lusitaniae EF136370	---	---	---	---	---	---	---
Candida lusitaniae	16	1	<0.5	1	<0.5	1	1
Candida lusitaniae	16	1	<0.5	1	<0.5	1	1
Candida krusei	8	2	4	>32	>32	1	<0.5
Candida krusei	8	2	4	>32	>32	1	<0.5
Candida krusei	8	2	4	>32	>32	1	<0.5
Candida krusei 6258	8	2	2	4	16	2	<0.5
Cryptococcus neoformans FJ011544	---	---	---	---	---	---	---
Cryptococcus neoformans	16	1	1	>32	16	2	>32
Cryptococcus neoformans	16	1	1	>32	16	2	>32
Candida glabrata 90028	8	2	2	1	2	<0.5	1
Candida glabrata	8	2	2	16	4	4	1
Candida glabrata	8	2	2	16	4	4	1
Ornithonyssus bacoti AM903318	---	---	---	---	---	---	---
	90*	40*	>100*	---	---	---	---

Fig 2

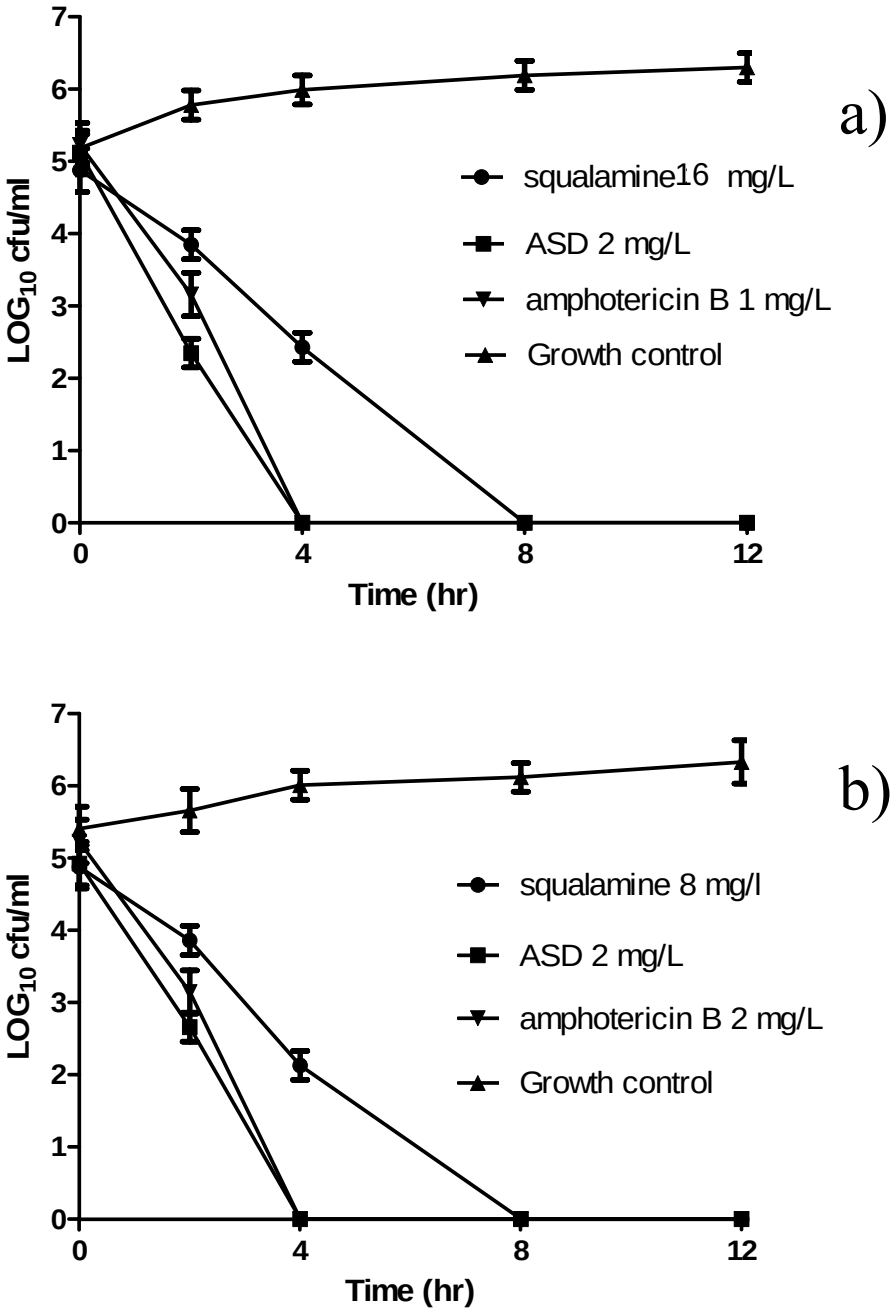

a)
LOG10 cfu/ml
Time (hr)
squalamine16 mg/L
ASD 2 mg/L
amphotericin B 1 mg/L
Growth control
b)
LOG10 cfu/ml
Time (hr)
squalamine 8 mg/l
ASD 2 mg/L
amphotericin B 2 mg/L
Growth control

Fig 3

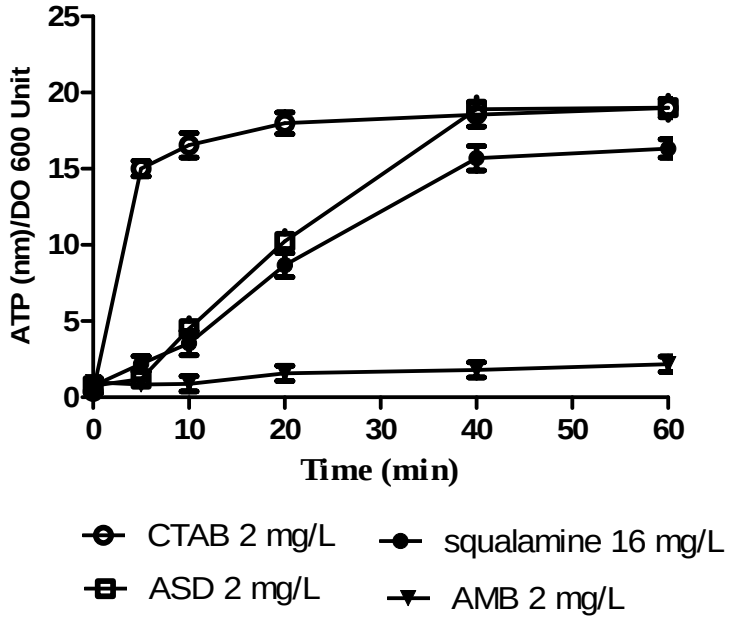

ATP (nm)/DO 600 Unit
25
20
15
10
5
0
0
10
20
30
40
50
60
Time (min)
CTAB 2 mg/L
squalamine 16 mg/L
ASD 2 mg/L
AMB 2 mg/L

Fig 4

Part 2

- **Analyse du mécanisme d’action antibactérienne de la squalamine**

Article 5

New Insights on the Antibacterial Mechanism of Action of Squalamine

Kamel Alhanout, Soazig Malesinki, Nicolas Vidal, Vincent Peyrot, Jean Marc Rolain and Jean Michel Brunel*

* corresponding author, e-mail : bruneljm@yahoo.fr.

Accepté in **Journal of Antimicrobial Chemotherapy**

Résumé de l'article

Les résultas obtenus précédemment suggèrent que les composés aminostéroïdiens possèdent deux mécanismes distincts d'action à l'égard des bactéries à Gram-positif et à Gram-négatif. L'interaction avec les groupements phosphates a été nécessaire pour que la squalamine ainsi que la colistine produisent leurs effets ce qui a été démontré par l'action inhibitrice des cations divalents sur l'activité des deux composés. Ainsi, l'addition de cations divalents de type Mg^{2+} ou Ca^{2+} à une concentration finale de 10 mM conduit à l'augmentation de la CMI de la squalamine et de la colistine vis-à-vis de *P. aeruginosa* et *E. coli* d'un facteur 4. En outre, la CMI de la squalamine, de la colistine restent inchangées par l'ajout de Mg^{2+} ou Ca^{2+} dans le cas de *S. aureus*. La squalamine a montré un effet bactéricide complet plus rapide que celui de la colistine contre les souches de *P. aeruginosa et E. coli*. Par ailleurs, l'effet bactéricide de la squalamine a été plus rapide vis-à-vis des bactéries à Gram positif que les bactéries à Gram-négatif. Les changements morphologiques adoptés par les bactéries lorsqu'elles sont traitées par la squalamine et la colistine, ont été évalués en utilisant l'imagerie par microscopie électronique à transmission (MET). Pour *P. aeruginosa*, un mélange de cellules remplies avec des membranes présentant des projections en forme de « blebs » et des cellules complètement vides ont été observées lors du traitement avec la squalamine tandis que l'utilisation de la colistine a donné lieu à des projections en forme de rayonnement provenant du compartiment cytoplasmique traversant

la membrane externe ce qui est en accord avec des études précédentes. D'autre part, le traitement de *S. aureus* avec la squalamine a causé une rupture importante de la membrane cytoplasmique bactérienne avec un écoulement du contenu cellulaire alors que la colistine ne modifie en rien la morphologie générale de la bactérie. Les effets de la squalamine sur l'intégrité des membranes bactériennes ont été étudiés par la mesure de la cinétique d'efflux ATP intracellulaire pendant 20 min. La squalamine a induit une libération rapide d'ATP à partir des souches de *S. aureus* et *S. pneumoniae* pour atteindre 100% de l'efflux maximal après 3 minutes. Inversement, pour les souches de *P. aeruginosa* et *E. coli* un efflux d'ATP dépendant du temps a été observé avec moins de 35% d'efflux maximal enregistré après 20 minutes. Dans ce contexte, le traitement par la colistine a entraîné un efflux léger, mais significatif, d'ATP ($p < 0,0001$) dans le cas de *P. aeruginosa* et d'*E. coli*, conduisant à 4-5% de l'efflux maximale après 20 minutes tandis qu'aucun effet n'a été constaté dans le cas de *S. aureus* ou de *S. pneumoniae* tout au long de la durée du test ($p < 0,0001$). Enfin, la squalamine a produit une dépolarisation flagrante des membranes des bactéries de *S. aureus* et de *S. pneumoniae*. A l'inverse, aucun effet dépolarisant n'a été remarqué dans le cas de *P. aeruginosa* ou *E. coli* traitées par la squalamine.

L'utilisation des cinétiques de l'efflux ATP intracellulaire comme indicateur de lésions membranaires, suggère que les lésions induites par la squalamine sont nettement plus importantes que celles causées par la colistine. D'une manière

intéressante, cela a été démontré récemment dans une étude analysant l'interaction de la squalamine et de la colistine avec des bicouches lipidiques des bactéries et les conséquences de cette interaction sur les propriétés électriques des membranes. Les auteurs ont indiqué que la squalamine et la colistine agissent toutes les deux par la création de lésions électriquement actives qui diffèrent toutefois dans leurs tailles de diamètre (33,3 ± 5 versus 9,1 ± 1 nm respectivement pour la squalamine et la colistine). Par conséquent, l'action de la squalamine contre les bactéries à Gram négatif peut être simulée par le modèle de tapis (Carpet model) précédemment proposé pour les antibiotiques cationiques peptidiques. Ce modèle suggère qu'après l'interaction avec les lipopolysaccharides (LPSs), l'antibiotique cause une vaste rupture de la membrane bactérienne due à un effet de type détergent qui pourrait conduire à la formation de micelle. Toutefois, ce modèle n'est pas valide pour les bactéries à Gram positif qui sont dépourvues de LPSs. Ainsi, il n'est pas surprenant que la présence des cations divalents n'ait eu aucun effet sur les activités de la squalamine ou de la colistine dans le cas des études menées sur les bactéries à Gram positif. D'une manière intéressante, la squalamine a montré un effet bactéricide plus rapide vis-à-vis des bactéries à Gram positif que celui noté contre les bactéries à Gram négatif. Comme la montre les images de microscopie électronique à transmission, la différence entre l'effet de la squalamine et celui de la colistine est flagrante et montre que la première conduit à une rupture de la membrane bactérienne alors que la deuxième n'est

pas active. En outre, la squalamine a produit un efflux d'ATP intracellulaire très important et instantané dans le cas des bactéries à Gram positif indiquant l'existence d'un phénomène rapide. Ainsi, la squalamine conduit à une forte dépolarisation des membranes des bactéries à Gram positif alors qu'aucune dépolarisation n'a été observée pour les bactéries à Gram négatif. La daptomycine, un antibiotique lipopeptide, et la valinomycine sont les seules molécules connues à l'heure actuelle pour agir par dépolarisation de la membrane des bactéries à Gram positif. Ces molécules ne sont pas actives contre les bactéries à Gram négatif probablement en raison de leur taille et qui restent bloquées par la membrane externe de ces bactéries. En outre, la résistance à la daptomycine a été signalée notamment avec les bactéries de *S. aureus* qui sont résistantes à la vancomycine. Ainsi, l'effet "mécanique" induit par ces les aminostérols sur la membrane bactérienne pourrait potentiellement réduire la possibilité d'apparition de résistance contre l'activité de la squalamine et de ces molécules.

J Antimicrob Chemother
doi:10.1093/jac/dkq213

Journal of Antimicrobial Chemotherapy

New insights into the antibacterial mechanism of action of squalamine

Q1 Kamel Alhanout[1], Soazig Malesinki[2], Nicolas Vidal[3], Vincent Peyrot[2], Jean Marc Rolain[1] and Jean Michel Brunel[1]*

[1]Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes URMITE UMR 6236 CNRS, Faculté de Pharmacie et de Médecine, Université de la Méditerranée, 27 boulevard Jean Moulin, 13385 Marseille 05, France; [2]CRO2, U911 INSERM, Faculté de Pharmacie, Université de la Méditerranée, 27 boulevard Jean Moulin, 13385 Marseille 05, France; [3]UPCAM iSm2, Case 342, Université Paul Cézanne, Av. Escadrille Normandie Niémen, 13397 Marseille cedex 13, France

*Corresponding author. Tel: +33-4-86-13-68-30; Fax: +33-4-91-38-77-72; E-mail: bruneljm@yahoo.fr

Received 14 March 2010; returned 6 April 2010; revised 1 May 2010; accepted 7 May 2010

Objectives: Antimicrobial resistance is an increasingly life-threatening problem that emphasizes the need to develop new antibacterial agents. The *in vitro* antibacterial activity of squalamine, a natural aminosterol, has been previously demonstrated against multidrug-resistant bacteria and moulds. Although the antibacterial activity of squalamine was found to correlate with that of other drugs, such as colistin, against Gram-negative bacteria, the former was active against Gram-positive bacteria, which are resistant to colistin. In this work, we provide new insights into squalamine's antibacterial mechanism of action compared with other known antibiotics.

Methods: We evaluated squalamine's antibacterial mechanism of action using the broth microdilution method for MIC determination and time–kill assays, transmission electron microscopy for morphological change studies, bioluminescence for ATP release measurements, and fluorescence methods for membrane depolarization assays.

Results: Concerning Gram-negative bacteria, squalamine, similar to colistin, required interaction with the negatively charged phosphate groups in the bacterial outer membrane as the first step in a sequence of different events ultimately leading to the disruption of the membrane. Conversely, squalamine exhibited a depolarizing effect on Gram-positive bacteria, which resulted in rapid cell death.

Conclusions: The new insights into the mechanism of action of squalamine highlight the importance of aminosterols in the design of a new class of antibacterial compounds that could be used as disinfectants and detergents.

Keywords: aminosterols, detergent-like mechanism of action, depolarization

Introduction

Microbial resistance is a life-threatening danger that is also increasingly considered an economic problem.[1] Among the different mechanisms of resistance in bacteria, the most prevalent are those found in methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, β-lactamase-producing Enterobacteriaceae, and multidrug-resistant (MDR) *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Burkholderia cepacia*.[2] Thus, even though a rationalized use of antibiotics is highly recommended to limit the emergence of such resistance, research into new classes of antimicrobial agents represents a necessary and potent strategy to overcome this problem.[2] Squalamine **1** (Figure 1) is a natural aminosterol isolated from the dogfish shark *Squalus acanthias* that possesses a steroid skeleton with a *trans*-AB ring junction, a cholestane-related sulphated side chain and a flexible polyamino-hydrophilic spermidine group linked to the hydrophobic unit at the C-3 position.[3,4] Squalamine has numerous therapeutic virtues, including antimicrobial and antiangiogenic properties.[5] Although numerous clinical studies dealing with the antiangiogenic potential of squalamine have been performed,[5,6] the clinical usefulness of this compound as an antimicrobial agent remains less investigated. We have recently demonstrated, however, that both squalamine and a synthetic squalamine-related aminosterol possess interesting *in vitro* antibacterial effects against various MDR Gram-negative and Gram-positive bacteria isolated from the sputum of cystic fibrosis (CF) patients.[7] Though a significant correlation was observed between the activity of both squalamine and colistin against Gram-negative isolates, squalamine showed notably higher activity against Gram-positive isolates, which raises the question

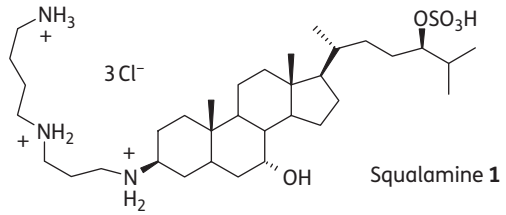


Figure 1. Structure of squalamine **1**.

of whether this compound exhibits different mechanisms of action against both groups of bacteria.[7] Because squalamine's mode of action against Gram-positive bacteria has not been previously investigated, this study focused on a deeper analysis of squalamine's antibacterial mechanism of action against both Gram-negative and Gram-positive bacteria. This analysis reveals a new mechanism of action for this class of compound.

Materials and methods

Squalamine stock solutions were prepared in water at 2 g/L. The stock solutions were subsequently diluted to working concentrations of 250 mg/L. The antibiotic controls used in this study were colistin (Sanofi Aventis, Paris, France) and tobramycin (Chiron, Suresnes, France). The reference bacterial strains used were *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. *Streptococcus pneumoniae* is a clinical isolate that was recovered from the sputum of a CF patient. In all cases, the results shown are the means of three different assays.

Susceptibility testing

MICs were determined in duplicate using the broth microdilution method according to a previously reported protocol under BSAC guidelines.[8]

Time–kill assays

Time–kill assays were conducted with concentrations corresponding to the MIC values of squalamine and colistin for reference strains of *P. eruginosa* ATCC 27853, *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. Squalamine or colistin was added to a bacterial suspension of $\sim 5\times10^5$ cfu/mL of each of the tested bacteria. Then, 2 mL of the tested suspension was sampled at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 h for viable cell counting that was conducted by spiral plating on Trypticase Soy Agar medium (bioMérieux, Craponne, France) followed by incubation at 37°C for 24 h.

Measurement of ATP release

Squalamine solutions were prepared in doubly distilled water at different concentrations. A suspension of growing bacteria in Mueller–Hinton (MH) broth was prepared and incubated at 37°C. Then, 900 μL of this suspension was added to 100 μL of squalamine solution for a final concentration of squalamine of 4× MIC value for each of the tested bacteria. An aliquot of 100 μL of this mixture was sampled at time intervals of 0, 5, 10, 15, 20, 120 and 240 min, and subsequently vortexed for 1 s. Next, 50 μL of luciferin–luciferase reagent (Yelen, France) was immediately added and the luminescent signal was quantified using a Lucy luminometer (Yelen, France) for 5 s. The ATP concentration was quantified by internal sample addition. The maximum ATP release was considered to be obtained with a concentration of squalamine up to 100 mg/L.

Effect of divalent salt solutions on squalamine activity and ATP release

The effect of divalent cations on the MIC values of squalamine, colistin and tobramycin was analysed using the broth microdilution method after addition of the cation to a final concentration of 10 mM. Then, ATP release was measured using the protocol described above. The results are expressed as the percentage inhibition of ATP release relative to the salt-free squalamine solution.

Transmission electron microscopy (TEM)

Two reference strains, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923, were incubated overnight at 37°C in MH broth containing either 2 mg/L colistin or 8 mg/L squalamine for *P. aeruginosa* and either 32 mg/L colistin or 2 mg/L squalamine for *S. aureus*. The bacteria were fixed in 2.5% glutaraldehyde EMS in 0.1 M phosphate buffer for 4 h for TEM studies. After post-fixation in 1% osmium tetroxide EMS for 1 h followed by dehydration in an ascending series of ethanol, the samples were embedded in Epon 812 resin EMS. Sections of 70 nm were stained with 4% uranyl acetate and lead citrate before examination using a transmission electron microscope (Philips Morgagni 268D at 80 kV). Statistical analyses were performed using a linear regression test using the SPSS software for Windows (version 16).

Membrane depolarization assays

Bacteria were grown in MH broth for 24 h at 37°C and then centrifuged at 10000 rpm at 20°C. The supernatant was discarded, and the bacteria were washed twice with buffered sucrose solution (250 μM) and magnesium sulphate solution (5 μM). The fluorescent dye 3,3-diethylthiodicarbodyanine iodide was added to a final concentration of 3 μM and left to penetrate into bacterial membranes during a 1 h incubation at 37°C. Squalamine was then added at the MIC for each of the tested bacteria. Fluorescence measurements were performed using a Jobin Yvon Fluoromax 3 spectrofluorometer with slit widths of 5/5 nm. The relative corrected fluorescence (RCF) was recorded at time intervals of 0, 5, 10, 15, 20, 120 and 240 min. The maximum RCF was considered to be that recorded with a pure solution of the fluorescent dye in the buffer used (3 μM).

Statistical methods

Statistical analyses were performed using the two-sample *t*-test option in the Prism 5 for Windows GraphPad software program.

Results

As shown in Table 1, the addition of 10 mM Mg^{2+} or Ca^{2+} increased the MIC of squalamine and colistin for *P. aeruginosa* and *E. coli* by at least 4-fold. The MIC values of tobramycin were not affected. Additionally, the MIC values of squalamine, colistin and tobramycin remained unchanged by Mg^{2+} or Ca^{2+} supplementation in the case of *S. aureus*. Squalamine exhibited complete killing of the *P. aeruginosa* and *E. coli* reference strains in 2 h, whereas colistin required 4 h (Figure 2a and b). However, squalamine showed a direct bactericidal effect against the *S. aureus* reference strain, reflected by a nearly 1.5 log drop in the counts of this strain by 0.25 h, with complete killing achieved in 1 h (Figure 2c). The morphological changes of bacteria when treated with squalamine and, for comparison purposes, colistin were assessed using TEM imaging (Figure 3). For *P. aeruginosa*, a mixture of filled cells with membranes

Table 1. Effect of divalent cation salt solutions on the bactericidal activity of squalamine, colistin and tobramycin against Gram-negative and Gram-positive reference strains

	MIC (mg/L)					
	without Mg^{2+} or Ca^{2+}			with 10 mM Mg^{2+} or Ca^{2+}		
Strain	squalamine	colistin	tobramycin	squalamine	colistin	tobramycin
P. aeruginosa ATCC 27853	8	1	1	64	8	1
E. coli ATCC 25922	4	0.5	2	64	16	1
S. aureus ATCC 25923	2	>128	1	2	>128	2

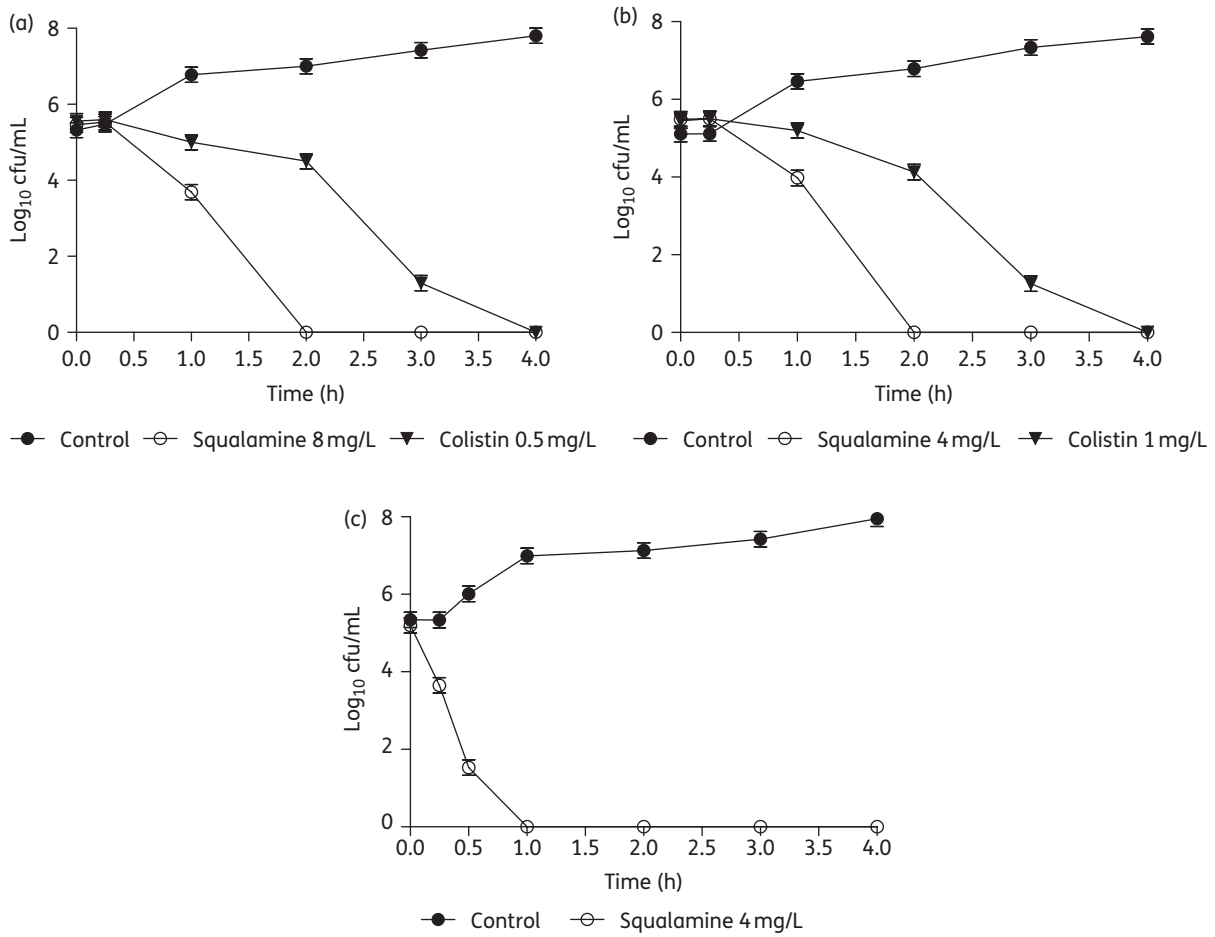


Figure 2. Time–kill curves of squalamine and colistin at the MIC over a 4 h period against *P. aeruginosa* ATCC 27853 (a), *E. coli* ATCC 25922 (b) and *S. aureus* ATCC 25923 (c) strains.

exhibiting bleb-like projections and completely emptied cells was observed in response to treatment with squalamine. Treatment with colistin resulted in radiating projections originating from the cytoplasmic compartment and crossing the outer membrane (Figure 3). On the other hand, treatment of *S. aureus* with squalamine resulted in a dramatic disruption of the bacterial membrane, with drained cytoplasmic content, and no morphological changes were observed in colistin-treated S. *aureus* cells compared with the control (Figure 3). The effects of squalamine on bacterial membrane integrity were investigated by measuring intracellular ATP release kinetics for 20 min (Figure 4). For *P. aeruginosa* and *E. coli*, a time-dependent ATP release was observed, with <35% of maximal efflux recorded after 20 min (Figure 4a). Conversely, squalamine induced a rapid ATP release from *S. aureus* and *S. pneumoniae* that reached 100% of maximal efflux after only 3 min (Figure 4b). In this context,

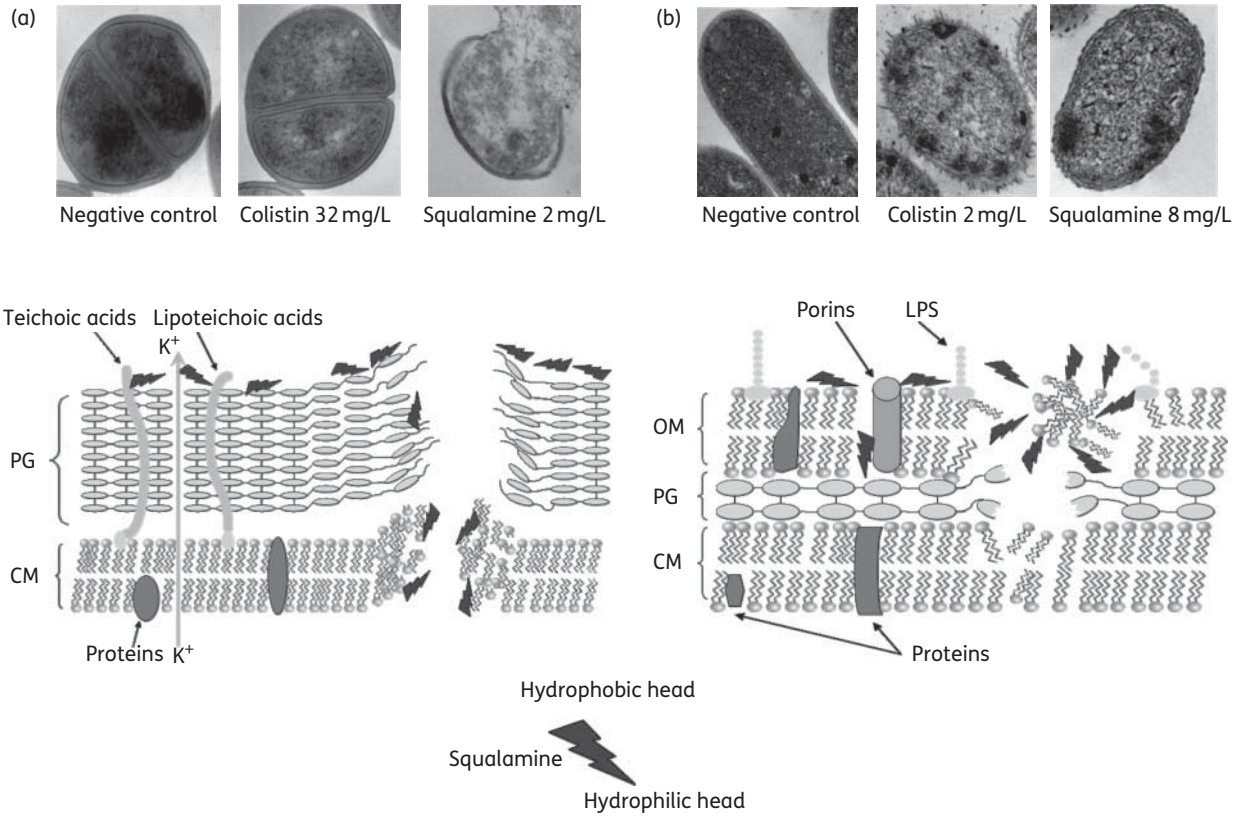


Figure 3. Morphological changes of the *S. aureus* ATCC 25923 (a) and *P. aeruginosa* ATCC 27853 (b) reference strains after overnight exposure to colistin or squalamine. A schematic of the antibacterial action of squalamine includes depolarization of the membranes of Gram-positive bacteria **SQ2** leading to intracellular ion efflux (e.g. potassium cations) and disruption of the membranes of Gram-negative bacteria.

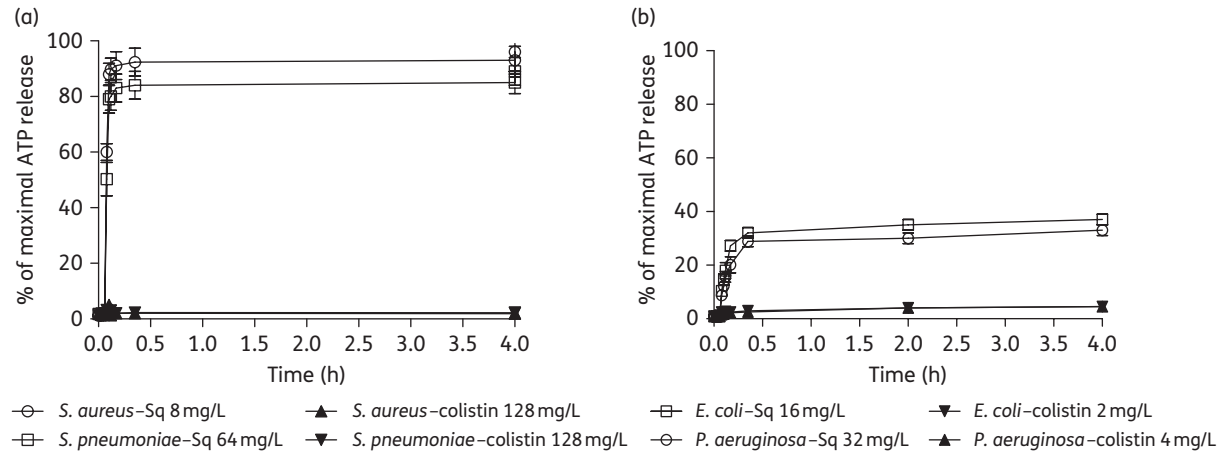


Figure 4. Effect of squalamine (Sq) and colistin on ATP release kinetics for Gram-positive bacteria (a) and Gram-negative bacteria (b). The maximum ATP release was considered to be obtained with a concentration of squalamine up to 100 mg/L.

treatment with colistin resulted in a slight but significant ATP release ($P<0.0001$) in *P. aeruginosa* and *E. coli*, leading to 4%–5% of maximal efflux after 20 min. No significant effect was found in *S. aureus* or *S. pneumoniae* during the test time ($P<0.0001$; Figure 4). Finally, no depolarizing effect was observed in squalamine-treated *P. aeruginosa* or *E. coli*, whereas depolarization of *S. aureus* and *S. pneumoniae* bacterial membranes was observed. This depolarization was shown by a

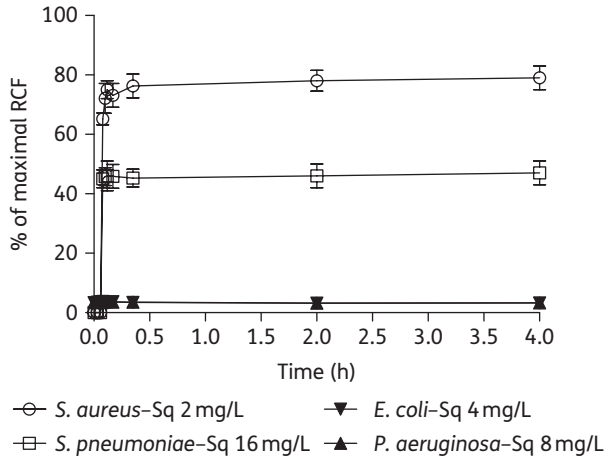


Figure 5. Depolarization of the bacterial membranes of various Gram-negative and Gram-positive bacteria in the presence of squalamine (Sq). The maximum RCF was considered to be that recorded with a pure solution of the fluorescent dye in the buffer used (3 μM).

rapid and strong increase in the RCF values, which reached 80% and 50% of the maximal RCF for each strain, respectively, within 1 min (Figure 5).

Discussion

Salmi *et al.*[9] provided the first report on the mechanism of action of squalamine against Gram-negative bacteria. They concluded that squalamine acts as a membrane-active molecule that targets bacterial membrane integrity through interactions of its positively charged amino groups with the negatively charged phosphate groups in the lipopolysaccharide (LPS) structure. Such a mechanism had been previously described with the polymyxin antibiotic colistin, which, by using positively charged amine groups, interacts with the negatively charged phosphate groups of LPS and displaces divalent cations, such as Ca^{2+} and Mg^{2+}.[10,11] It is known that the activity of colistin can be antagonized by increased concentrations of these divalent cations, which further inhibits the binding of this polycationic antibiotic to LPS.[10,11] Accordingly, an inhibitory effect of Ca^{2+} and Mg^{2+} on colistin and squalamine activities against *P. aeruginosa* and *E. coli* was found. This result indicates that the interaction with the negatively charged phosphate groups in the LPS structure is mandatory for both agents to be active. Additionally, tobramycin, an aminoglycoside antibiotic that acts by inhibiting protein synthesis, was not affected by divalent cation supplementation (Table 1). Interestingly, squalamine showed a markedly faster killing rate than colistin against Gram-negative bacteria, which suggests that both compounds might interact differently with bacterial membranes. Thus, TEM imaging was used to reveal the morphological changes that squalamine and colistin generate on the bacterial membrane. Squalamine-treated *P. aeruginosa* bacteria showed different membrane shapes from those treated with colistin. The radiating projections originating from the cytoplasm through the bacterial membranes in the case of colistin-treated *P. aeruginosa* were consistent with previous reports, reflecting the membrane-perforator effect of this compound.[12] In this latter case, the bactericidal mechanism is not yet fully understood, but has been proposed to involve the formation of molecular contacts between the inner and outer lipid layer of the outer membrane, causing the induction of lipid exchange. This results in a loss of the compositional specificity of the membrane and osmotic instability.[13–15] For squalamine-treated *P. aeruginosa*, however, different effects on the bacterial membrane were noted and represented by wrinkled membrane structures with emptied cells. Accordingly, though the availability of negatively charged phosphate groups represents a common requirement for the activity of squalamine and colistin, both agents probably go through a different series of actions after interaction with LPS. Indeed, treatment of *P. aeruginosa* and *E. coli* with squalamine resulted in significantly higher and faster ATP release as compared with colistin, using intracellular ATP release as an indicator of membrane lesions. These results suggest that LPS damage induced by squalamine is clearly greater and faster than that caused by colistin, and this has recently been demonstrated in a study on the interaction of squalamine and colistin with the bacterial lipid bilayer and the consequences of such interactions on the electrical properties of these membranes.[16] The authors indicated that squalamine and colistin act similarly in creating electrically active lesions that differ in their diameter (33.3 ± 5 versus 9.1 ± 1 nm for squalamine and colistin, respectively).[16] Accordingly, the activity of squalamine against Gram-negative bacteria may be simulated by the carpet model previously proposed for describing cationic peptide antibiotics, which lead to a large disruption of the bacterial membrane due to a detergent-like effect of micelle formation.[17] However, this model would not be valid for Gram-positive bacteria, which are devoid of LPS. It was not surprising, therefore, that the divalent cations had no effect on squalamine, colistin or tobramycin activities against the Gram-positive bacterium *S. aureus*. Remarkably, squalamine demonstrated a faster killing rate against *S. aureus* than that noted with Gram-negative bacteria, which signifies that this compound may possess a rapid and direct bactericidal effect against Gram-positive bacteria. As shown in TEM images, treatment of *S. aureus* with squalamine resulted in a dramatic disruption of the bacterial membrane, with drained cytoplasmic material. Colistin caused no morphological change, a reflection of its inactivity against Gram-positive bacteria. Moreover, squalamine also produced an instantaneous intracellular ATP release in *S. aureus* and *S. pneumoniae*, an indication that a rapid phenomenon might be involved in squalamine's mode of action against Gram-positive bacteria. Indeed, squalamine led to strong depolarization of the *S. aureus* and *S. pneumoniae* membranes, while no depolarization was observed for the Gram-negative bacteria. The lipopeptide antibiotics daptomycin and valinomycin are the only antibiotics known to act via depolarizing the bacterial membrane of Gram-positive bacteria without disruption.[18,19] These compounds have no effect against Gram-negative bacteria, probably due to them being blocked by the outer membranes of these bacteria because of their high molecular mass.[18–20] Resistance to daptomycin has previously been reported, especially in vancomycin-resistant *S. aureus* bacteria.[18] Thus, squalamine and other related aminosterols have a particular 'mechanical' mode of action mediated by bacterial membrane disruption that would reduce the possibility of resistance.

Collectively, and without excluding other intra- or extracellular targets of squalamine, our results indicate that squalamine acts by disrupting the outer membranes of Gram-negative bacteria in a detergent-like mechanism of action and by depolarizing the bacterial membranes of Gram-positive bacteria. Such a unique mechanism of action may be interesting for further development of this compound for use as a disinfectant or detergent.

Acknowledgements

We acknowledge Professor M. Zasloff from Georgetown University (Washington, DC, USA) for providing us with a sample of squalamine. We are thankful to Mr B. Campana for his assistance in electron microscopy imaging.

We would also like to acknowledge American Journal Experts, who assisted with language editing.

Funding

This work was supported by the Centre National de la Recherche Scientifique (France).

Transparency declarations

None to declare.

References

1 Kaier K, Wilson C, Chalkley M *et al*. Health and economic impacts of antibiotic resistance in European hospitals—outlook on the BURDEN project. *Infection* 2008; **36**: 492–4.

2 Hawkey PM, Jones AM. The changing epidemiology of resistance. *J Antimicrob Chemother* 2009; **64** Suppl 1: i3–10.

3 Moore KS, Wehrli S, Roder H *et al*. Squalamine: an aminosterol antibiotic from the shark. *Proc Natl Acad Sci USA* 1993; **90**: 1354–8.

4 Wehrli SL, Moore KS, Roder H *et al*. Structure of the novel steroidal antibiotic squalamine determined by two-dimensional NMR spectroscopy. *Steroids* 1993; **58**: 370–8.

5 Brunel JM, Salmi C, Loncle C *et al*. Squalamine: a polyvalent drug of the future? *Curr Cancer Drug Targets* 2005; **5**: 267–72.

6 Salmi C, Brunel JM. Therapeutic potential of cationic steroid antibacterials. *Expert Opin Investig Drugs* 2007; **16**: 1143–57.

7 Alhanout K, Brunel JM, Raoult D *et al*. *In vitro* antibacterial activity of aminosterols against multidrug-resistant bacteria from patients with cystic fibrosis. *J Antimicrob Chemother* 2009; **64**: 810–4.

8 Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 2001; **48** Suppl 1: 5–16.

9 Salmi C, Loncle C, Vidal N *et al*. Squalamine: an appropriate strategy against the emergence of multidrug resistant Gram-negative bacteria? *PLoS ONE* 2008; **3**: e2765.

10 Zavascki AP, Goldani LZ, Li J *et al*. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J Antimicrob Chemother* 2007; **60**: 1206–15.

11 Li J, Nation RL, Milne RW *et al*. Evaluation of colistin as an agent against multi-resistant Gram-negative bacteria. *Int J Antimicrob Agents* 2005; **25**: 11–25.

12 Koike M, Iida K, Matsuo T. Electron microscopic studies on mode of action of polymyxin. *J Bacteriol* 1969; **97**: 448–52.

13 Oh JT, Cajal Y, Skowronska EM *et al*. Cationic peptide antimicrobials induce selective transcription of micF and osmY in *Escherichia coli*. *Biochim Biophys Acta Biomembr* 2000; **1463**: 43–54.

14 Oh JT, Van Dyk TK, Cajal Y *et al*. Osmotic stress in viable *Escherichia coli* as the basis for the antibiotic response by polymyxin B. *Biochem Biophys Res Commun* 1998; **246**: 619–23.

15 Mortensen NP, Fowlkes J, Sullivan CJ *et al*. Effects of colistin on surface ultrastructure and nanomechanics of *Pseudomonas aeruginosa* cells. *Langmuir* 2009; **25**: 3728–33.

16 Di PE, Salmi-Smail C, Brunel JM *et al*. Biophysical studies of the interaction of squalamine and other cationic amphiphilic molecules with bacterial and eukaryotic membranes: importance of the distribution coefficient in membrane selectivity. *Chem Phys Lipids* 2010; **163**: 131–40.

17 Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. *Clin Microbiol Rev* 2006; **19**: 491–511.

18 Hawkey PM. Pre-clinical experience with daptomycin. *J Antimicrob Chemother* 2008; **62** Suppl 3: iii7–14.

19 Lars R, Ata J. The effect of the ionophore valinomycin on biomimetic solid supported lipid DPPTE/EPC membranes. *Bioelectrochem* 2007; **70**: 387–93.

20 Baltz RH. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering. *Curr Opin Chem Biol* 2009; **13**: 144–51.

Conclusions Générales et perspectives

Dans ce travail, l'activité antimicrobienne des dérivés aminostéroïdiens a été, pour la première fois, déterminée contre des souches cliniques de bactéries et de champignons isolés chez des patients atteints de mucoviscidose. Pour les bactéries à Gram négatif, nous avons constaté que l'hétérogénéité d'activité des DASs était clairement associée à la résistance à la colistine et au phénotype mucoïde et la présence de capsule ce qui indique que ce groupe de composés agit principalement sur la membrane bactérienne.

Il convient de noter que les DASs possèdent généralement une activité antimicrobienne plus importante contre les bactéries à Gram positif que les bactéries à Gram-négatif ce qui est probablement lié à la présence d'une membrane externe chez ces dernières (Article **1-2**). [22, 30] Ainsi, les futures études de synthèse de nouvelles molécules aminostéroïdiennes pourraient prendre en compte ce point afin d'essayer de moduler l'activité de ces molécules en modifiant leurs structures.

De la même manière, nous avons démontré que les DASs possèdaient une activité antifongique intéressante contre un panel de champignons filamenteux et de levures contenant des souches multi résistantes aux agents antifongiques. Ceci indique probablement que le mécanisme d'action antifongique de ces molécules est distinct des modes d'action des antifongiques classiques. Il convient de noter qu'à l'heure actuelle, aucun élément concernant le mécanisme d'action antifongique des composés aminostéroïdiens n'a été déterminé et à ce jour

aucune souche de champignon ne s'est montrée résistante aux DASs.

Concernant le mécanisme d'action antibactérien de la squalamine, nos résultats indiquent que la squalamine possède un mécanisme d'action original et agit en perturbant la membrane externe des bactéries à Gram négatif avec un mécanisme comparable à celui d'un détergent et en dépolarisant la membrane des bactéries à Gram positif. D'une manière comparable, les données préliminaires que nous avons obtenues en analysant le mécanisme d'action de la squalamine contre les levures montrent que cette molécule agit également en perturbant l'intégrité de la membrane fongique. Bien que la sécurité d'emploi de la squalamine ait été démontrée dans des études cliniques, aucune étude *in vivo* ou *in vitro* n'est disponible concernant la toxicité pour les composés aminostéroïdiens synthétiques (Article **1**). [25, 31]

Toutefois, l'intérêt de ce type de mécanisme réside dans le fait que la possibilité de développer une résistance à un tel effet « physique » est improbable. Pour cette raison nous avons insisté dans ce travail sur le fait que ces molécules pouraient être développées pour une utilisation par voie locale comme par exemple la voie pulmonaire. Nous avons commencé dans le cadre de ce travail à mettre en forme une solution aqueuse contenant le composé DAS1 afin d'évaluer la convenance de ces molécules à une administration pulmonaire locale. Pour cette raison, les caractères aérodynamiques de l'aérosol nébulisé en utilisant deux types de nébuliseurs ont été déterminés. Ainsi, nous avons pu mettre en évidence *in vitro* la faisabilité d'utiliser ces molécules sous forme d'aérosol (Annexe, Article 7).

Par ailleurs, un projet a été entamé au sein de notre unité de recherche pour évaluer l'activité *in vivo* de ces molécules dans un modèle animal d'infection pulmonaire à *P. aeruginosa.* En conclusion, le large spectre d'activité antimicrobien des DASs pourait permettre d'envisager l'utilisation comme désinfectants ou comme détergents dans de nombreux domaines dans les années à venir.

Reference

1. Chen LF, Chopra T, Kaye KS. Pathogens resistant to antibacterial agents. *Infect Dis Clin North Am* 2009; **23**: 817-45, vii.

2. Klepser ME. Antifungal resistance among Candida species. *Pharmacotherapy* 2001; **21**: 124S-32S.

3. Pournaras S, Iosifidis E, Roilides E. Advances in antibacterial therapy against emerging bacterial pathogens. *Semin Hematol* 2009; **46**: 198-211.

4. Kaier K, Wilson C, Chalkley M, et al. Health and economic impacts of antibiotic resistance in European hospitals--outlook on the BURDEN project. *Infection* 2008; **36**: 492-4.

5. Bittar F, Richet H, Dubus JC, et al. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *PLoS ONE* 2008; **3**: e2908.

6. Bittar F, Leydier A, Bosdure E, et al. Inquilinus limosus and cystic fibrosis. *Emerg Infect Dis* 2008; **14**: 993-5.

7. Bittar F, Reynaud-Gaubert M, Thomas P, et al. Acetobacter indonesiensis pneumonia after lung transplant. *Emerg Infect Dis* 2008; **14**: 997-8.

8. Menuet M, Bittar F, Stremler N, et al. First isolation of two colistin-resistant emerging pathogens, Brevundimonas diminuta and Ochrobactrum anthropi, in a woman with cystic fibrosis: a case report. *J Med Case Reports* 2008; **2**: 373.

9. Rolain JM, Francois P, Hernandez D, et al. Genomic analysis of an emerging multiresistant Staphylococcus aureus strain rapidly spreading in cystic fibrosis patients revealed the presence of an antibiotic inducible bacteriophage. *Biol Direct* 2009; **4**: 1.

10. Pihet M, Carrere J, Cimon B, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis - a review. *Med Mycol* 2008: 1-11.

11. Campbell PW, III, Saiman L. Use of aerosolized antibiotics in patients with cystic fibrosis. *Chest* 1999; **116**: 775-88.

12. Casey P, Garrett J, Eaton T. Allergic bronchopulmonary aspergillosis in a lung transplant patient successfully treated with nebulized amphotericin. *J Heart Lung Transplant* 2002; **21**: 1237-41.

13. Dudley MN, Loutit J, Griffith DC. Aerosol antibiotics: considerations in pharmacological and clinical evaluation. *Curr Opin Biotechnol* 2008; **19**: 637-43.

14. Geller DE. Aerosol antibiotics in cystic fibrosis. *Respir Care* 2009; **54**: 658-70.

15. Langton Hewer SC, Smyth AR. Antibiotic strategies for eradicating Pseudomonas aeruginosa in people with cystic fibrosis. *Cochrane Database Syst Rev* 2009: CD004197.

16. Davies JC. Pseudomonas aeruginosa in cystic fibrosis: pathogenesis and persistence. *Paediatr Respir Rev* 2002; **3**: 128-34.

17. Davies JC, Bilton D. Bugs, biofilms, and resistance in cystic fibrosis. *Respir Care* 2009; **54**: 628-40.

18. Manno G, Cruciani M, Romano L, et al. Antimicrobial use and Pseudomonas aeruginosa susceptibility profile in a cystic fibrosis centre. *Int J Antimicrob Agents* 2005; **25**: 193-7.

19. Pritt B, O'Brien L, Winn W. Mucoid Pseudomonas in cystic fibrosis. *Am J Clin Pathol* 2007; **128**: 32-4.

20. Stone A, Saiman L. Update on the epidemiology and management of Staphylococcus aureus, including methicillin-resistant Staphylococcus aureus, in patients with cystic fibrosis. *Curr Opin Pulm Med* 2007; **13**: 515-21.

21. Brunel JM, Salmi C, Loncle C, et al. Squalamine: a polyvalent drug of the future? *Curr Cancer Drug Targets* 2005; **5**: 267-72.

22. Salmi C, Brunel JM. Therapeutic potential of cationic steroid antibacterials. *Expert Opin Investig Drugs* 2007; **16**: 1143-57.

23. Moore KS, Wehrli S, Roder H, et al. Squalamine: an aminosterol antibiotic from the shark. *Proc Natl Acad Sci U S A* 1993; **90**: 1354-8.

24. Bhargava P, Marshall JL, Dahut W, et al. A phase I and pharmacokinetic study of squalamine, a novel antiangiogenic agent, in patients with advanced cancers. *Clin Cancer Res* 2001; **7**: 3912-9.

25. Herbst RS, Hammond LA, Carbone DP, et al. A phase I/IIA trial of continuous five-day infusion of squalamine lactate (MSI-1256F) plus carboplatin and paclitaxel in patients with advanced non-small cell lung cancer. *Clin Cancer Res* 2003; **9**: 4108-15.

26. Li D, Williams JI, Pietras RJ. Squalamine and cisplatin block angiogenesis and growth of human ovarian cancer cells with or without HER-2 gene overexpression. *Oncogene* 2002; **21**: 2805-14.

27. Cho J, Kim Y. Sharks: a potential source of antiangiogenic factors and tumor treatments. *Mar Biotechnol (NY)* 2002; **4**: 521-5.

28. Ciardiello F, Bianco R, Damiano V, et al. Antiangiogenic and antitumor activity of anti-epidermal growth factor receptor C225 monoclonal antibody in combination with vascular endothelial growth factor antisense oligonucleotide in human GEO colon cancer cells. *Clin Cancer Res* 2000; **6**: 3739-47.

29. Salmi C, Loncle C, Vidal N, et al. New stereoselective titanium reductive amination synthesis of 3-amino and polyaminosterol derivatives possessing antimicrobial activities. *Eur J Med Chem* 2008; **43**: 540-7.

30. Salmi C, Loncle C, Vidal N, et al. Squalamine: an appropriate strategy against the emergence of multidrug resistant gram-negative bacteria? *PLoS ONE* 2008; **3**: e2765.

31. Hao D, Hammond LA, Eckhardt SG, et al. A Phase I and pharmacokinetic study of squalamine, an aminosterol angiogenesis inhibitor. *Clin Cancer Res* 2003; **9**: 2465-71.

Annexes

Article 6

Burden of emerging filamentous fungi in cystic fibrosis

Kamel Alhanout, Fadi Bittar, Didier Raoult, Jean Marc Rolain* and Stéphane Ranque*

URMITE UMR 6236, CNRS-IRD, Faculté de Médecine et de Pharmacie, 27 Boulevard Jean Moulin, 13385 Marseille cedex 05, France.

1) Dr Stéphane RANQUE

Email: stephane.ranque@ap-hm.fr

Phone: (33) 4 91 38 60 90 Fax: (33) 4 91 38 49 58

2) Pr Jean-Marc ROLAIN

Email: jean-marc.rolain@univmed.fr

Phone: (33) 4 91 32 43 75 Fax: (33) 4 91 38 77 72

Abstract word count = 48

Text word count: 1292

To be submitted to **Emerging Infectious Diseases**

Summary

Fungal pulmonary infections are frequently encountered in cystic fibrosis (CF) patients. We report that 23 out of 47 (48.9%) filamentous fungi recovered from sputa of CF patients, including 18 isolates reported for the first time in this context, could be identified only by the use of molecular tools.

Cystic fibrosis (CF) remains the main genetic disorder in the European Caucasian population and is due to mutations in the gene CFTR. Although many organs can be affected, mortality and morbidity is mainly due to chronic respiratory infections including bacteria and filamentous fungi [1]. Major bacterial infections in CF are caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus* and are characterized by recurrent exacerbations that need intensive antibiotic therapy resulting in a fragile pulmonary environment that allows airway infection and /or colonization by various mould species [2]. New combinations of antibiotics for the treatment of bacterial infection has led to an increase life expectancy in CF patients but has inadvertently created a niche for the colonization and proliferation of fungi in the CF lung [3]. The most common pathogen moulds recovered from CF patients are *Aspergillus fumigatus, Aspergillus terreus,* and *Scedosporium apiospermum* [4]. Although many new or emerging bacteria have been detected and identified over the past years, only few new and/or emerging moulds have been reported in CF patients including *Penicillium emersonii, Acrophialophora fusispora* and *Exophiala dermatitidis* [5].

Such discrepancy is believed to be due to i) phenotype variation of microorganisms during chronic colonization and iterative therapies ii) the lack of routine use of molecular tools for correct identification of filamentous fungi in most clinical microbiology laboratories and iii) inhibition of fungal growth and overgrowth by rapidly growing bacteria, especially *P. aeruginosa* [6, 7]. In the context of CF, it is well known that many bacteria can be misidentified or unidentified due to phenotypic variation or absence of phenotypic characteristics and these bacteria could be identified at the species level only with molecular tools [8]. Unfortunately, studies evaluating molecular identification techniques for filamentous fungi in the context of CF are scarce [9] whilst data emphasizing the need for accurate identification methods to be developed are increasingly reported [10, 11]. Indeed, correct identification of these fungi at the species level may be critical for the management of patients especially for the choice of antifungal therapy since some species harbored intrinsic and/or acquired antifungal resistance and for epidemiological purposes. Thus, the aim of this work was to compare phenotypic to molecular identification techniques in a panel of 47 filamentous fungi recovered from sputa of CF patients.

The study

A panel of 47 filamentous fungi recovered from the sputa of CF patients from December 2007 to November 2008 and identified by morphological and biochemical analyses used in clinical laboratories were included in the study [12, 13]. Briefly, initial identification was based on colonial morphology of isolates

grown on Sabouraud Dextrose agar plates (BioMérieux , France), inhibitory mold agar, mycobiotic agar, and/or brain heart infusion agar with sheep blood with or without chloramphenicol (50 g/ml), gentamicin (40 g/ml), and cycloheximide (500 g/ml) and microscopic morphology of lactophenol aniline blue-stained preparations. Molecular identification was based on internal transcribed spacer (ITS) PCR amplification and sequencing procedure using previously reported protocol [14]. Nucleotide sequences from both forward and reverse primers were assembled using Sequencher 4.8. The assembled DNA sequences of partial ITS were used as query to do BLAST search in GenBank for species identification. Identification at the species level was considered if sequences had $\geq$ 99.0% of identity with sequences retrieved from GenBank. All sequences have been deposited in GenBank under accession numbers from GU594733 to GU594779 (Figure **1**). The ITS sequences of our panel of isolates as well as reference sequences retrieved from GenBank were aligned using multiple sequence alignment software (ClustalX). A cladogram was constructed using the neighbor-joining clustering method implemented using MEGA software - version 4 (http://www.megasoftware.net/).

The results of phenotypic and molecular identification are summarized in Figure 1. Only 26 out of 47 (55.3%) of tested isolates could be identified at the species level using phenotypic criteria including *A. fumigatus* (n=10), *A. terreus* (n=5), *A. niger* (n=2), *A. flavus* (n=8), and *A. nidulans* (n=1). The remaining 21 isolates were identified at the genus level only (Figure 1). Conversely, using ITS-PCR

amplification and sequencing procedure, all isolates were eventually identified at the species level with percentages of homology for all sequences >99% with sequences available in GenBank. Among the 26 isolates identified at the species level by phenotypic method, one isolate initially identified as *A. nidulans* was identified as *Emericella quadrilineata* using the molecular method (Figure 1). Among the remaining 21 isolates identified at the species level by the molecular tool only, 13 different species were found including 1 isolate of *Aspergillus ustus*, 4 isolates of *Penicillium griseofulvum*, 3 isolates of *Cladosporium cucumerinum,* 2 isolates of *Alternaria triticina,* 1 isolate of *Alternaria alternata***,** 1 isolate *Alternaria longipes,* 1 isolate of *Fusarium verticillioides*, 1 isolate of *Fusarium proliferatum*, 1 isolate of *Scedosporium prolificans,* 1 isolate of *Scedosporium apiospermum*, 2 isolates of *Pseudallescheria boydii,* 1 isolate of *Rhizopus oryzae* and 2 isolates of *Rhizomucor* identified as *R. pusillus* and *R. tauricus* at identical scores (100%).

Conclusions

The fact that phenotypic methods failed to identify about 48 % of tested isolates strongly indicates the importance of an accurate identification method especially for moulds of the genus *Aspergillus, Penicillium, Cladosporium, Alternaria, Fusarium* or *Rhizomucor* in CF patients. Among a panel of 47 isolates, a burden of 18 isolates (38.2%) belonging to 11 different species of filamentous fungi is reported for the first time in the context of CF patients including *E. quadrilineata, A. ustus, P. griseofulvum, C. cucumerinum, A. triticina, A.*

alternata, A. longipes, F. verticillioides, F. proliferatum, R. oryzae, and *R. pusillus/tauricus*. Similarly, Nagano *et al* have detected and identified many other fungal species in adult CF patients from Northern Ireland using sequence analysis of ITS region [15]. Interestingly, the fact that the isolate of *Aspergillus nidulans* (the anamorphous form of *Emericella nidulans)* was identified as *E. quadrilineata* by molecular method may probably indicate that this fungus might be overlooked or misidentified by routine phenotypic methods. To the best of our knowledge this fungus has never been reported in a CF patient [16]. This may be clinically relevant since *E. quadrilineata* is believed to be resistant to caspofungin whereas *E. nidulans* is less susceptible to amphotericin B [17]. Likewise, the isolate of *Aspergillus* sp. that was identified as *A. ustus* using molecular methods indicates that the absence of this fungus from CF literature may be due to misidentification such as described with phenotypic methods [18]. However, this fungus is reported to be associated with high mortality in transplant recipients and with clear resistant to azoles [16, 19]. Finally, for some isolates, molecular identification at the species level with partial ITS sequence was not enough discriminating between two different species suggesting that other more variable target genes such as beta tubulin should be developed in the future [20].

Our work demonstrates that in order to accurately detect and identify new and/or emerging mould species in the context of CF, new and reliable methods should be developed as exemplified with the use of universal 16S rRNA gene PCR

amplification and sequencing for bacterial identification that increased the burden of known valid bacterial names described over the past decade [21]. This concept is now well established for bacteria in CF where many CF specific bacterial species were discovered after developing accurate identification tools [8]. Finally, our study reemphasizes the need for simple, rapid and reliable tools for correct identification of filamentous fungi also in the context of CF. Among the new technologies used in clinical microbiology laboratories for rapid identification of microorganisms, whole cell mass spectrometry is now considered as the most powerful technique that is currently a revolution for bacterial identification including bacterial identification of CF isolates [22]. Few works have started to evaluate mass spectrometry for identification of fungal species such as *Fusarium* species outside the context of CF [23, 24]. We believe that this technique should be developed in order to detect and identify new or emerging fungi in the context of CF.

Reference

1. Pihet M, Carrere J, Cimon B, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. *Med Mycol* 2009; **47**: 387-97.

2. Pihet M, Carrere J, Cimon B, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. *Med Mycol* 2009; **47**: 387-97.

3. Pihet M, Carrere J, Cimon B, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. *Med Mycol* 2009; **47**: 387-97.

4. Pihet M, Carrere J, Cimon B, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. *Med Mycol* 2009; **47**: 387-97.

5. Pihet M, Carrere J, Cimon B, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. *Med Mycol* 2009; **47**: 387-97.

6. Nagano Y, Millar BC, Goldsmith CE, et al. Development of selective media for the isolation of yeasts and filamentous fungi from the sputum of adult patients with cystic fibrosis (CF). *J Cyst Fibros* 2008; **7**: 566-72.

7. Nagano Y, Elborn JS, Millar BC, et al. Comparison of techniques to examine the diversity of fungi in adult patients with cystic fibrosis. *Med Mycol* 2009: 1-12.

8. Bittar F, Richet H, Dubus JC, et al. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *PLoS One* 2008; **3**: e2908.

9. Nagano Y, Elborn JS, Millar BC, et al. Comparison of techniques to examine the diversity of fungi in adult patients with cystic fibrosis. *Med Mycol* 2009: 1-12.

10. Pihet M, Carrere J, Cimon B, et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. *Med Mycol* 2009; **47**: 387-97.

11. Bakare N, Rickerts V, Bargon J, et al. Prevalence of Aspergillus fumigatus and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses* 2003; **46**: 19-23.

12. Ciardo DE, Schar G, Altwegg M, et al. Identification of moulds in the diagnostic laboratory--an algorithm implementing molecular and phenotypic methods. *Diagn Microbiol Infect Dis* 2007; **59**: 49-60.

13. Rakeman JL, Bui U, Lafe K, et al. Multilocus DNA sequence comparisons rapidly identify pathogenic molds. *J Clin Microbiol* 2005; **43**: 3324-33.

14. Pryce TM, Palladino S, Price DM, et al. Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT media using polymerase chain reaction and DNA sequencing of the internal transcribed spacer regions. *Diagn Microbiol Infect Dis* 2006; **54**: 289-97.

15. Nagano Y, Elborn JS, Millar BC, et al. Comparison of techniques to examine the diversity of fungi in adult patients with cystic fibrosis. *Med Mycol* 2009: 1-12.

16. Balajee SA, Houbraken J, Verweij PE, et al. Aspergillus species identification in the clinical setting. *Stud Mycol* 2007; **59**: 39-46.

17. Verweij PE, Varga J, Houbraken J, et al. Emericella quadrilineata as cause of invasive aspergillosis. *Emerg Infect Dis* 2008; **14**: 566-72.

18. Ciardo DE, Schar G, Altwegg M, et al. Identification of moulds in the diagnostic laboratory--an algorithm implementing molecular and phenotypic methods. *Diagn Microbiol Infect Dis* 2007; **59**: 49-60.

19. Panackal AA, Imhof A, Hanley EW, et al. Aspergillus ustus infections among transplant recipients. *Emerg Infect Dis* 2006; **12**: 403-8.

20. cazar-Fuoli L, Mellado E, astruey-Izquierdo A, et al. Species identification and antifungal susceptibility patterns of species belonging to Aspergillus section Nigri. *Antimicrob Agents Chemother* 2009; **53**: 4514-7.

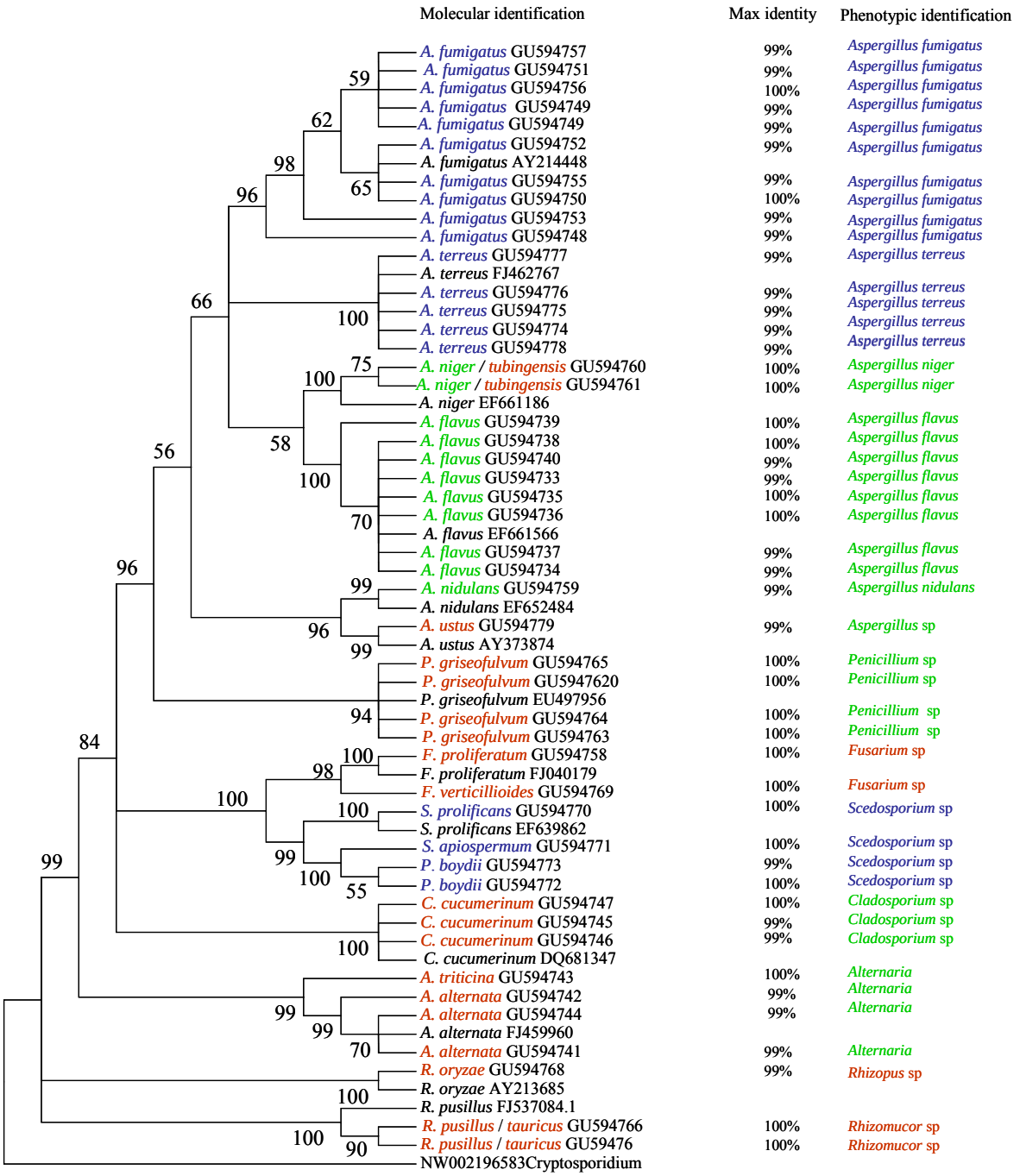
21. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol* 2007; **45**: 2761-4.

22. Seng P, Drancourt M, Gouriet F, et al. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Infect Dis* 2009; **49**: 543-51.

23. Marinach-Patrice C, Lethuillier A, Marly A, et al. Use of mass spectrometry to identify clinical Fusarium isolates. *Clin Microbiol Infect* 2009; **15**: 634-42.

24. Hettick JM, Green BJ, Buskirk AD, et al. Discrimination of Aspergillus isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. *Anal Biochem* 2008; **380**: 276-81.

Figure 1: phylogenic tree displaying tested mold strains identified by molecular and phenotypic tools.



Blue color: species that were reported in CF with confirmed pathogenic role.

Green color: species that were reported in CF without confirmed pathogenic role.

Red color: species that have not been previously reported in CF.

Sequences retrieved from GenBank were: : *Aspergillus fumigatus* AY214448, *Aspergillus terrus* FJ462767, *Aspergillus niger* EF661186, *Aspergillus flavus* EF661566, *Aspergillus nidulans* EF652484, *Aspergillus ustus* AY373874, *Penicillium griseofulvum* EU497956, *Cladosporium cucumerinum* DQ681347, *Alternaria alternata* FJ459960, *Fusarium proliferatum* FJ040179, *Scedosporium prolificans* EF639862, *Rhizopus oryzae* AY21368, *Rhizomucor pusillus* EU293849 *and Cryptosporidium muris* AF381167.

Article 7

Suitability of a synthesized antimicrobial aminosterol derivative to be integrated in aerosol formulation

Kamel Alhanout, Jean Michel Brunel, Jean Marc Rolain and Véronique Andrieu *

* Corresponding author

Dr Véronique Andrieu

E. mail : veronique.andrieu@univmed.fr

Tel : (33) 04 91 83 55 48

Fax : (33) 04 91 78 75 75

Abstract word count**: 181**

Text word count**: 1374**

In progress

Abstract

Our previous reports demonstrated that aminosterols possess interesting antimicrobial activities against various bacterial and fungal pathogens involved in lung infections of cystic fibrosis (CF) patients. These compounds exhibited relatively high MIC values rendering their use in general mode of administration inconvenient and imposing the local route of administration such as aerosols that ensures sufficient MIC values with minimized systemic adverse effects. Thus, this work aimed to evaluate the suitability of an aminosterol derivative (ASD 1), having wide spectrum antimicrobial activity as demonstrated in our previous reports, to be integrated in an aerosol formulation (aqueous solution of ASD **1** at 10 mg/L, solution **A**) and nebulized via two different types of nebulizers. Our primary results indicate that this compound can be successfully nebulized using both nebulizers. Obtained aerodynamic parameters from both nebulizers were comparable and predict an adequate disposition of nebulized solution **A** in the respiratory tract. This work represents an introductory effort demonstrating the suitability of this group of compounds for local pulmonary administration. Further work is warranted in order to evaluate the antimicrobial activity of aminosterols using lung-infected animal model.

Introduction

We have recently demonstrated that the natural aminosterols, squalamine as well as synthetic aminosterol derivatives (ASDs) possess interesting antimicrobial activities against clinical bacterial and fungal isolates recovered from patients with cystic fibrosis (CF) including multidrug resistant species. [1] As reported in our previous works and though these compounds showed wide antimicrobial spectrum, their minimal inhibitory concentration (MICs) were relatively high. Thus, we emphasized that these compounds may be developed as aerosol for local administration in lungs rather than as systemic drugs Indeed, local pulmonary administration of antibiotics as aerosol represents a very important strategy to treat lung infections in patients with CF. [2] In this pathology, an important benefit of pulmonary route of administration over systemic route is that the antibiotic is sent directly to the target region in lungs ensuring sufficient local concentrations and minimizing the systemic adverse effects. [2] Aerosols for medical use are produced by devices called nebulizers that convert the drug in liquid form into vapour, aerosol, to be inhaled by the patient. These nebulizers are able to send a sufficiently elevated dose of the antibiotic to the lungs as aerosol formed of millions of micro-particles of various sizes. For an adult patient, only the micro-particles having size ranging from 3 to 5 microns are able to penetrate the targeted area in lungs situated between primary and terminal. Below 3 microns, particles are easily expired whereas above 5 microns, they are mainly deposited in the mouth. [2-4] The apparatus used to

measure the size of aerosol particles are the impactors that provide primarily measures quantifying the Mass Median Aerodynamic Diameter (MMAD) that should be between 3-5 microns and the Inhalable Fraction (IF), which by definition represents the fraction of particles that have a MMAD ranging between 3 and 5 microns. [5] In addition, medical formulation intended to be inhaled are subjected to physicochemical tests such as the osmolality and pH since they influence the reaction of to entering aerosols. [2, 5] Moreover, in the pathology of cystic fibrosis, the bronchial secretions contain compounds, such as mucins and degradation products of DNA that bind to, and inhibit the activity of antibiotics currently used in the management of pulmonary infection in CF patients such as tobramycin. [6, 7] We aimed in this work to evaluate the suitability of integrating a synthetic aminosterol derivative (ASD, Figure 1) possessing antibacterial and antifungal activities in an aerosol formulation by evaluating the aerodynamic and physicochemical properties of the prepared formulation.

Materials and Methods

Bacteria and Testing Activity

Reference strains used were *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923). MICs were determined by the standard method of broth dilution Muller Hinton broth (MH) as previously reported. [1] Effects of mucin on ASD1, colistin and tobramycin activities were studied as follows. Porcine mucin (Sigma Aldrich, France) was prepared in distilled water and purified by filtration and then added

to 96 well plates to final concentration of 1 or 10 mg/ml. Then, MICs of ASD1, tobramycin and colistin (Sanofi aventis, Paris, France) were determined as described above.

Aerosol testing

The pH (Radiometer, Copenhagen, Denmark) and osmolality (Osmometer Knauer 4050, Wilton, Etten Leur, The Netherlands) were determined. Form aerosol testing, a solution of 10 mg/mL of ASD [1] was prepared in water. Two nebulizers were used : PARI LC Sprint Baby (jet nebulizer) derived by the compressor PARI **BOY**®SX and **eFLOW**® (vibrating mesh-nebulizer both from PulmoMed- France). The system of aerosol aerodynamic testing was configured according to previous study. [5] Briefly, the tested nebulizer was filled with 5 ml of a solution of connected to the tip of the impactor; next generation impactor (Applied Physics, Inc., USA) representing the throat. At the other end of the impactor, the nozzle was attached to a volume-controlled ventilator (Harvard Apparatus, Les Ulis, France) and parameters were set to mimic the normal human respiration (breathing rate 15 L/min, tidal volume: 500 ml and respiratory rate of 30 breaths / min with 50/50 % inhalation/exhalation). Nebulization was continued until the total automatic cessation of the nebulizer. Tests were repeated 6 times for 6 different solutions and results are presented on average ± standard deviation.

Quantification of ASD1

Quantities of ASD emitted by the nebulizer and distributed in impactor's cups

were recovered and quantified using spectrophotometric method (SPECTRO, France) at a wavelength of 224 nm. The method was linear within concentrations ranging from 1 to 100 µg/ml. The repeatability and reproducibility of the method were tested and demonstrated.

Statistical methods

Statistical analysis was performed in the software Prism 5 for Windows (GraphPad Software), options: linear regression or Student t test

Results

As showed in Table 1, MICs of ASD 1 against all tested bacteria ranged from 2 to 8 mg/L with or without mucin. For tobramycin, MICs against all bacteria without mucin were varied from 0.5 to 1 mg/ml while after the addition of mucin at 1 and10 mg/ml, MICs ranged from 1 to 2 mg and form 8 to 16 mg/ml, respectively. MICs of 0.5 mg/ml were noted for colistin without mucin against the Gram-negative bacteria. After addition of mucin at 1 mg /ml, MICs of colistin ranged from 2 to 4 mg/ml and whilst MICs of 32 mg/ml were noted when mucin at 10 mg /ml was added. The pH of ASD solution prepared at 10 mg/ml was 6.8 (± 0.1) and its osmolality of 330 mOsm (± 5). As shown in Table 2, nebulization time of PARI BOY®SX and eFLOW® were 5 and 20 minutes, respectively. MMAD and IF of particles produced by PARI BOY®SX was 4.10 µ (± 0.1) and 62 % (±1), respectively while those generated by eFLOW® had MMAD of 4.36 µ (± 0.1) and IF of 59% (± 2). Individual analysis of the size of particles emitted by each device showed that PARI BOY®SX generated around

28 % (±1) of particles having size above 3 µ while eFLOW® engendered 25 % (±1) of particles in this category. Moreover, 20% (±1) and 18% (±1) of particles generated with size inferior to 5µ were produced by PARI BOY®SX and eFLOW®, respectively. The dead volume (the volume remaining at the bottom of the nebulizer after automatic stop of nebulization) was 0 and 0.4 ml for PARI BOY®SX and eFLOW®, respectively.

Discussion

MICs for ASD are consistent with our previous reports indicating that these compounds have interesting antibacterial activity with relative elevated MICs. As determined by MICs, the addition of mucin inhibited the effects of tobramycin in a concentration dependant as described in previous data [6] and, surprisingly, the activity of colistin which had not been reported before this work. This finding might be related to the structure of these two antibiotics containing highly charged groups that would facilitate their interaction and association with mucin. Unlike these two agents, ASD was not affected by the presence of mucin which may probably be explained by the presence of a steroidal hydrophobic core that plays an expeller role with respect to mucins. The pH and osmolality testing showed that the ASD might effectively be dissolved in water giving adequate physiochemical characteristics that are consistent with the recommended values for inhalable preparations. [2, 8] MMADs, IFs and the fractions of particles having sizes inferior to 3µ or superior to 5 µ produced by both nebulizers were convenient and statistically non-different (p =

0.07). Importantly, no dead volume was found in the case of PARI BOY®SX while eFLOW® reserved a part of initial liquid fill. Since both nebulizers produced statistically non-different aerodynamic parameters, the fact that the nebulizer PARI BOY®SX discharged its entire initial dose may indicate that the fraction of initial dose that would have been emitted in surrounding air is considerably important. It should be noted that no foaming was observed during the nebulization both devices. In reality, foaming is the problem of colistin affecting its pulmonary deposition after nebulization. [9] Overall, this work demonstrated that the integration of ASD in aerosol formulation for nebulization via two different types of nebulizers is possible. Thus, it will be promising in the future to test other molecules since these antimicrobial compounds are now easily synthesized with interesting antimicrobial properties. An *in vivo* study will be necessary in order to evaluate ASDs antimicrobial activity as aerosols in a lung-infected animal model.

References

1. Alhanout K, Brunel JM, Raoult D *et al.* In vitro antibacterial activity of aminosterols against multidrug-resistant bacteria from patients with cystic fibrosis. *J Antimicrob Chemother* 2009; **64**: 810-4.

2. Geller DE. Aerosol antibiotics in cystic fibrosis. *Respir Care* 2009; **54**: 658-70.

3. Kuni CC, Regelmann WE, duCret RP *et al.* Aerosol scintigraphy in the assessment of therapy for cystic fibrosis. *Clin Nucl Med* 1992; **17**: 90-3.

4. Schoni MH, Nikolaizik WH. Aerosol therapy in patients with cystic fibrosis. *Schweiz Med Wochenschr* 1997; **127**: 158-64.

5. Abdelrahim ME, Chrystyn H. Aerodynamic characteristics of nebulized terbutaline sulphate using the
Next Generation Impactor (NGI) and CEN method. *J Aerosol Med Pulm Drug Deliv* 2009; **22**: 19-28.

6. Hunt BE, Weber A, Berger A *et al.* Macromolecular mechanisms of sputum inhibition of tobramycin activity. *Antimicrob Agents Chemother* 1995; **39**: 34-9.

7. Weber A, Williams-Warren J, Ramsey B *et al.* Tobramycin Serum Concentrations After Aerosol and Oral Administration in Cystic Fibrosis. *Am J Ther* 1995; **2**: 81-7.

8. Littlewood JM, Smye SW, Cunliffe H. Aerosol antibiotic treatment in cystic fibrosis. *Arch Dis Child* 1993; **68**: 788-92.

9. Diot P, Dequin PF, Rivoire B *et al.* Aerosols and anti-infectious agents. *J Aerosol Med* 2001; **14**: 55-64.

Table 1 : effects of mucin addition on MICs of ASD, tobramycin (TOB) and colistin (COL).

Strains	MICs								
	Without mucin			with mucin 1 mg/ml			with mucin 10 mg/ml		
	DAS 1	TOB	COL	DAS	TOB	COL	DAS	TOB	COL
Escherichia coli ATCC 25922	4	0.5	1	4	1	4	2	8	32
Psedomonas aeruginosa ATCC 27853	8	1	0.5	8	2	4	8	16	32
Staphylococcus aureus ATCC 25923	2	1	< 0.5	1	2	>128	2	8	>128

Table 2 : aerodynamic parameters of the nebulized solutions of ASD (10 mg/ml)

.

Nebulizer	Nebulizer fill (mg/ml)	Nebulization time (min)	Inhalable fraction %	Dead volume ml	MMAD µm	Fraction of Particles <3µ and >5µ
PARI LC PLUS®	10mg/5ml	20	62 (±1)	0	4.1 (± 0.1)	28 and 20% (±1)
eFLOW®	10mg/3ml	5	59 (±1)	0.4	4.35 (± 0.1)	20 and 15 % (±1)

Présentation orales et posters

Présentation orale

La squalamine, a new antibacterial agent form shark. Faculté de médecine, Université de la Méditerranée, Aix- Marseille II, XVIème Colloque de l'Ecole Doctorale des sciences de la Vie et de la Santé, Faculté de Médecine, Université de la Méditerranée, Aix-Marseille II, les 5 et 6 Juin 2008.

Posters

Posters internationaux

1. K. Alhanout, D. Raoult, J. M. Brunel and J.M. Rolain. Large antibacterial activity spectrum of aminosterols derivatives towards multidrug-resistant Gram-negative and Gram-positive bacteria from patients with cystic fibrosis, ECCMID 16-19 mai 2009, Helsinki, Finlande.

2. K. Alhanout, D. Raoult, J. M. Brunel and J.M. Rolain.Squalamine, a new antibiotic extracted from marine environment, ECCMID 16-19 mai 2009, Helsinki, Finlande

Posters nationaux

1- K. Alhanout, J. M. Brunel and J.M. Rolain. La squalamine, a new antibacterial agent form shark, Faculté de médecine, Université de la Méditerranée, Aix- Marseille II, XVIème Colloque de l'Ecole Doctorale des sciences de la Vie et de la Santé, Faculté de Médecine, Université de la Méditerranée, Aix-Marseille II, les 5 et 6 Juin 2008.

2- K. Alhanout, D. Raoult, J. M. Brunel and J.M. Rolain. Large antibacterial activity spectrum of aminosterols derivatives towards multidrug-resistant Gram-negative and Gram-positive bacteria from patients with cystic fibrosis, Faculté de médecine, Université de la Méditerranée, Aix- Marseille II, XVIème Colloque de l'Ecole Doctorale des sciences de la Vie et de la Santé, Faculté de Médecine, Université de la Méditerranée, Aix-Marseille II, les 5 et 6 Juin 2008.

3- K. Alhanout, D. Raoult, J. M. Brunel and J.M. Rolain.Squalamine, a new antibiotic extracted from marine environment, Faculté de médecine, Université de la Méditerranée, Aix- Marseille II, XVIème Colloque de l'Ecole Doctorale des sciences de la Vie et de la Santé, Faculté de Médecine, Université de la Méditerranée, Aix-Marseille II, les 5 et 6 Juin 2008.

4- K. Alhanout, D. Raoult, J. M. Brunel and J.M. Rolain. Large antibacterial activity spectrum of aminosterols derivatives towards multidrug-resistant Gram-negative and Gram-positive bacteria from patients with cystic fibrosis, 10ème Colloque des Jeunes Chercheurs de la Mucoviscidose, Institut Pasteur, Paris, le 16 Mars 2009.