



- (51) International Patent Classification:
C40B 30/02 (2006.01) *C12Q 1/18* (2006.01)
G06F 19/16 (2011.01)
- (21) International Application Number:
PCT/SG2013/000393
- (22) International Filing Date:
9 September 2013 (09.09.2013)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
201206670-0 7 September 2012 (07.09.2012) SG
- (71) Applicants: **AGENCY FOR SCIENCE, TECHNOLOGY AND RESEARCH** [SG/SG]; 1 Fusionopolis Way, #20-10 Connexis, Singapore 138632 (SG). **SINGAPORE HEALTH SERVICES PTE LTD** [SG/SG]; 11 Third Hospital Avenue #06-00, SNEC Building, Singapore 168751 (SG).
- (72) Inventors: **CHANDRA SHEKHAR, Verma**; 30 Biopolis Street, #07-01 Matrix, Singapore 138671 (SG). **LI, Jian-guo**; 11 Third Hospital Avenue, #06-00, SNEC Building, Singapore 168751 (SG). **RAJAMANI, Lakshminarayanan**; 11 Third Hospital Avenue #06-00, SNEC Building, Singapore 168751 (SG). **BEUERMAN, Roger Wilmer**; 11 Third Hospital Avenue #06-00, SNEC Building, Singapore 168751 (SG). **LIU, Shouping**; 11 Third Hospital Avenue #06-00, SNEC Building, Singapore 168751 (SG).
- (74) Agent: **SPRUSON & FERGUSON (ASIA) PTE LTD**; P. O. Box 1531, Robinson Road Post Office, Singapore 903031 (SG).
- (81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).
- Declarations under Rule 4.17:**
— of inventorship (Rule 4.17(iv))
- Published:**
— with international search report (Art. 21(3))

(54) Title: METHOD OF DESIGNING MOLECULES

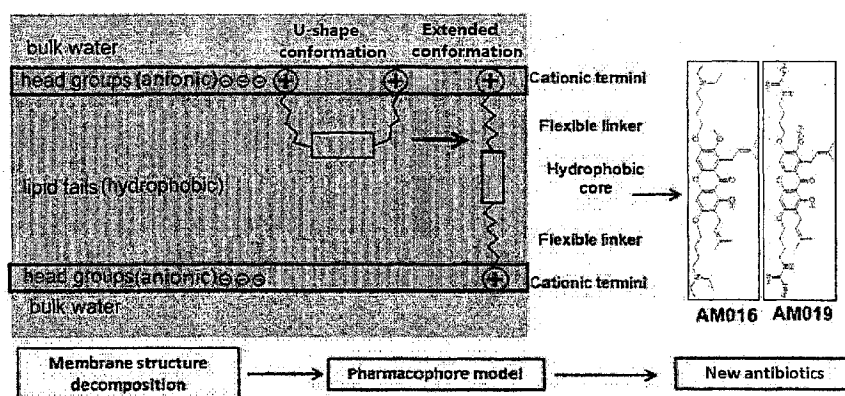


FIG. 1

(57) Abstract: The present disclosure relates to a method for identifying a compound that disrupts a membrane of a target cell, the method comprising the steps of: (a) identifying the structure of a test compound; (b) identifying the structure of a phospholipid membrane of the target cell; (c) using the identified structures of the test compound and the target cell to predict whether the test compound will disrupt the phospholipid membrane of the target cell.

METHOD OF DESIGNING MOLECULES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of SG provisional application No. 201206670-0, filed 7 September 2012, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates to a method of designing antimicrobial molecules.

BACKGROUND OF THE INVENTION

[0003] Bacterial resistance is a serious clinical problem resulting from the overuse of antibiotics. Membrane-active molecules, such as antimicrobial peptides (AMPs) and synthetic mimics of the antimicrobial peptides (SMAMPs), offer a potential solution to this problem. This is because such molecules are unlikely to induce resistance in bacteria since they act on the cell membrane in contrast to traditional antibiotics which target specific biosynthetic and enzymatic activities. It is postulated that it would be too costly for microbes in evolutionary terms to mutate the structure of its cell membrane in order to develop resistance against membrane-targeting antimicrobial molecules.

[0004] Fragment-based drug design (FBDD) has been able to provide atomic level information for ligand-receptor interactions, but FBDD is largely limited to the design of traditional antibiotics (e.g. protein inhibitors), with limited success with regard to the design of membrane-active antimicrobials. When it comes to designing membrane-active antimicrobial molecules, the FBDD approach is found to be deficient due to limited understanding of the actual action mechanisms, a lack of well-defined cavities in the cell membrane, and the lack of predictability of molecular interactions in part due to the fluid nature of the cell membrane as well as the diversity of binding modes between anti-microbial molecules and the membrane.

[0005] There is thus a need for the development for a method to design membrane active molecules or compounds such as synthetic mimics of antimicrobial peptides that can perturb or disrupt the membrane of bacteria cells in order to mediate lysis or cell-killing.

SUMMARY OF THE INVENTION

[0006] In a first aspect, there is provided a method for identifying a compound that disrupts a membrane of a target cell, the method comprising the steps of: (a) identifying the structure of a test compound; (b) identifying the structure of a phospholipid membrane of the target cell; (c) using the identified structures of the test compound and the target cell to predict whether the test compound will disrupt the phospholipid membrane of the target cell.

[0007] Advantageously, the disclosed method can be used to identify key features of the action mechanism and would further allow the identification of useful biophysical characteristics of the membrane active antimicrobials. In so doing, the disclosed method is capable of identifying potential new compounds that are capable of acting as anti-microbial agents by perturbing or disrupting the cell membrane of a target cell. Advantageously, anti-microbial agents developed using the disclosed method may be substantially less susceptible to the development of bacterial resistance due to the prohibitively high cost of mutating the chemical structure of the bacterial cell membrane.

[0008] In embodiments, the target cell may be a Gram-positive or Gram-negative bacterium, yeast cell, or fungi.

[0009] In embodiments, the prediction step (c) comprises predicting the motion of the test compound relative to the phospholipid membrane based on the theoretical thermodynamic forces existing between the test compound and the phospholipid membrane. In one embodiment, the prediction step is performed with one, or a combination of two, three, or four molecular simulation ("MD") computer programs. In one embodiment, the prediction step is performed with a molecular simulation ("MD") computer program suited to the type of test compound.

[0010] In embodiments, the test compound is considered to disrupt the phospholipid membrane if the test compound is predicted to be at least capable of embedding itself within the phospholipid membrane.

[0011] In other embodiments, the test compound is considered to disrupt the phospholipid membrane if the test compound is predicted to be at least capable of substantially perturbing the phospholipid membrane to cause lipid reorganization on the phospholipid membrane.

[0012] In some embodiments, the test compound is considered to disrupt the phospholipid membrane if the test compound is predicted to be capable of cleaving or inducing cleavage of said phospholipid membrane to thereby cause lysis of said target cell.

[0013] In embodiments, the test compound is considered to disrupt the phospholipid membrane if the test compound is predicted to be capable of causing a loss of membrane potential and/or also increasing water leaking into the target cell.

[0014] In one embodiment, there is provided a method for designing a compound, the method comprising the steps of: selecting at least a first test compound from a library of molecules exhibiting antimicrobial activity; simulating *in-silico* the interactions of the test compound with a phospholipid membrane, identify critical parameters that are correlated to antimicrobial activity; identify a second test compound comprising one or more of the identified parameters, synthesizing the new test compound; and validating the pharmacological activity of the synthesized compound using biophysical and biological experiments. In embodiments, the method comprises predicting the motion of the second test compound relative to the phospholipid membrane based on the theoretical thermodynamic forces existing between the second test compound and the phospholipid membrane.

[0015] In one embodiment, the disclosed method is capable of iterative application wherein one or more identified parameters is/are altered to a second test compound. The second test compound may be selected from an existing library of compounds known or expected to possess anti-microbial activity.

[0016] In one embodiment, the altering step involves modifying the second test compound to introduce one or more functional groups identified to be capable of causing membrane disruption onto the test compound. In another embodiment, the alteration step may further involve altering the molecular conformation of the compound, for instance, by introducing positively or negatively charged groups onto the molecule to modify its molecular properties in an aqueous or lipid environment.

[0017] In further embodiments, the altered compound is submitted to a simulation step to predict its ability to disrupt or perturb a membrane. The altered compound will be synthesized if the simulation results predict that the altered molecule exhibits sufficient membrane disruption/perturbation activity. In embodiments, the synthesized compound is validated with biocompatibility testing and susceptibility testing. In embodiments, the synthesized compound is further characterized by a battery of tests to identify new or verify already identified biophysical characteristics. Advantageously, the iterative nature of the disclosed method thus allows the identification of new, potentially useful biophysical

features, or the verification of the anti-microbial effectiveness of biophysical features already identified.

[0018] Advantageously, the disclosed method relates to a multidisciplinary approach towards molecular design. In particular, in one embodiment, the disclosed method complements the use of *in-silico* modeling with actual compound synthesis, biocompatibility testing, susceptibility testing and biophysical characterization. The inventors have found this multi-disciplinary approach to molecular design to be surprisingly effective in identifying new molecules having anti-microbial properties.

DEFINITIONS

[0019] Unless otherwise defined herein, scientific and technical terms used in this application shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclature used in connection with, and techniques of, chemistry, cell and tissue culture, molecular biology, cell and cancer biology, neurobiology, neurochemistry, virology, immunology, microbiology, pharmacology, genetics and protein and nucleic acid chemistry, described herein, are those well-known and commonly used in the art.

[0020] The following are some definitions that may be helpful in understanding the description of the present invention. These are intended as general definitions and should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

[0021] Unless the context requires otherwise or specifically stated to the contrary, integers, steps, or elements of the invention recited herein as singular integers, steps or elements clearly encompass both singular and plural forms of the recited integers, steps or elements.

[0022] The word "substantially" does not exclude "completely" e.g. a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

[0023] As used herein, the term "about", in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated

value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

[0024] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers, but not to the exclusion of any other step or element or integer or group of elements or integers. Thus, in the context of this specification, the term "comprising" means "including peripherally, but not necessarily solely".

[0025] When describing the compounds, compositions, methods and processes of the invention, the following terms have the following meanings unless otherwise indicated. Additionally, as used herein, the singular forms "a," "an" and "the" include the corresponding plural forms unless the context of use clearly dictates otherwise.

[0026] Certain embodiments may also be described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the embodiments with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[0027] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[0028] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

5 [0030] Fig. 1 depicts possible interaction between a phospholipid bilayer and a molecule designed based on the pharmacophore model disclosed herein. Exemplary molecules based on the model are also depicted.

[0031] Fig. 2A shows the results of molecular simulations performed on candidate molecule AM016 showing translocation across membrane.

10 [0032] Fig. 2B is a graph depicting the translocation free energy versus distance plot of two exemplary molecules AM016 and AM019 described herein.

[0033] Fig. 3A depicts simulation results of AM016 (a) and AM019 (b) clusters at high concentrations.

15 [0034] Fig. 3B is a graph depicting the lateral pressures of the bacterial membrane in the presence of AM016/AM019.

[0035] Fig. 4A is a graph depicting the percentage leakage of Calcein from large unilamellar vesicles (LUV) 30 minutes after the addition of AM016 / AM019 at different drug/lipid concentrations.

20 [0036] Fig. 4B is a graph depicting the fluorescence intensity induced by AM016 / AM019 using live bacteria cells and SYTOX green.

[0037] Fig. 5A shows the density profile of phosphate atoms at different AM016 / AM019 concentrations.

[0038] Fig. 5B shows the density profile of water molecules at different AM016 / AM019 concentrations.

25 [0039] Figs. 6A-6B depict simulation results showing high concentrations of AM016 (16 drug with 128 lipid molecules) at extended conformations.

[0040] Figs. 6C-6D depict simulation results showing high concentrations of AM019 (16 drug with 128 lipid molecules) at extended conformations

30 [0041] Fig. 7 is a graph showing that the area per lipid of the bacterial membrane increases significantly with AM016 concentration.

[0042] Fig. 8A contains graphs showing the Calcein Leakage experimental results for AM016.

[0043] Fig. 8B contains graphs showing the Calcein Leakage experimental results for AM019.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

5 [0044] In a first aspect, there is provided a method for identifying a compound that disrupts a membrane of a target cell, the method comprising the steps of: (a) identifying the structure of a test compound; (b) identifying the structure of a phospholipid membrane of the target cell; and (c) using the identified structures of the test compound and the target cell to predict whether the test compound will disrupt the phospholipid membrane of the target cell.

10 [0045] In embodiments, step (c) comprises identifying parameters that correlate to antimicrobial activity. In one embodiment, step (c) comprises identifying the critical parameters that are important in inducing membrane perturbations. These key parameters are believed to determine a compound's membrane activity.

[0046] In embodiments, prediction step (c) comprises predicting the interactions between
15 the test compound and a phospholipid membrane. In embodiments, the step (c) comprises simulating and predicting the interactions between the test compound and the phospholipid membrane in an aqueous environment. In embodiments, the modeled phospholipid membrane comprises at least two leaflets, each leaflet comprising at least one hydrophilic head section and at least one hydrophobic tail section, and wherein the leaflets are arranged such that the
20 hydrophobic tail sections are enclosed within an envelope defined by the hydrophilic head sections, i.e., a phospholipid bilayer. The hydrophilic head sections will be exposed to the aqueous environment. In embodiments, the membrane is modeled to be a bacterial membrane containing a higher percentage of negatively charged lipid groups relative to mammalian membranes.

25 [0047] In embodiments, the disclosed method is directed to designing a compound that can perturb both leaflets of the bacterial membrane, preferably a compound that can perturb both leaflets of the membrane simultaneously. In embodiments, the above method is directed to designing compound having particular chemical affinity with the negatively charged head groups of the lipids.

30 [0048] In embodiments, the above method is drawn to a pharmacophore model wherein the compound to be designed comprises five fragments, including one hydrophobic core, two

cationic terminal groups and two linker groups. Fig. 1 depicts how such a molecule may be predicted to interact with a phospholipid bilayer membrane.

[0049] In particular, it can be seen from Fig. 1 that the two cationic groups are expected to interact strongly with the anionic head groups of the bacterial membrane through electrostatic attractions, while the hydrophobic core penetrates into and perturbs the lipid tail region of the bacterial membrane. To rationally optimize each fragment and assemble them into a membrane active antimicrobial, it is required to adopt a methodical, step-wise approach. The presently disclosed method provides such an approach. In one embodiment, the above pharmacophore model is capable of providing compounds capable of penetrating into the hydrophobic core of the phospholipid bilayer membrane to cause partitioning or pore formation. This pharmacophore model captures the general feature of the action mechanism and can provide practical guidance for the design of powerful membrane active antimicrobials.

[0050] In embodiments, the phospholipid membrane identified in step (b) comprises at least one or more lipids selected from the group consisting of: cholesterol, ergosterol, [(2*R*)-3-hexadecanoyloxy-2-[(*Z*)-octadec-9-enoyl]oxypropyl] 2-(trimethylazaniumyl)ethyl phosphate ("POPC"), 2-oleoyl-sn-glycero-3-phosphoserine, phosphatidylcholine, phosphatidic acid, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, lysophosphatidylcholine, and mixtures thereof. In one embodiment, the phospholipid membrane comprises a mixture of at least two lipids selected from the group above. In other embodiments, the phospholipid membrane comprises a mixture of at least three lipids selected from the group above. In other embodiments, the phospholipid membrane comprises a mixture of at least four lipids selected from the group above. In embodiments, the phospholipid may comprise a mixture selected from the group consisting of: phosphatidylcholine and phosphatidic acid, phosphatidylcholine and phosphatidylethanolamine, phosphatidylcholine and phosphatidylglycerol, phosphatidylcholine and phosphatidylserine, phosphatidylcholine and lysophosphatidylcholine, phosphatidylethanolamine and phosphatidic acid, phosphatidylethanolamine and phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine, phosphatidylethanolamine and lysophosphatidylcholine.

[0051] In one embodiment, the phospholipid membrane comprises a mixture of phosphatidylethanolamine (POPE) and phosphatidylglycerol (POPG). The POPG and POPE

may be provided in ratios of 1:5 to 5:1. In embodiments, the molar ratio of POPG and POPE is selected from the group consisting of: 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1 and 5:1. In one embodiment, the molar ratio of POPE/POPG is 3:1. In one embodiment, the phospholipid membrane is modeled based on a mammalian model comprising [(2*R*)-3-hexadecanoyloxy-2-
5 [(*Z*)-octadec-9-enoyl]oxypropyl] 2-(trimethylazaniumyl)ethyl phosphate ("POPC") and cholesterol. In another embodiment, the phospholipid membrane mimics the cytoplasmic membrane of yeasts (e.g., consisting of 288 lipids with four typical lipids: POPC, POPE, 2-oleoyl-sn-glycero-3-phosphoserine ("POPS"), and ergosterol, at a ratio of 4:2:1:2)

[0052] In embodiments, the phospholipid membrane is identified as a cell membrane of a
10 non-mammalian cell. In other embodiments, the phospholipid membrane is identified as a cell membrane of Gram-positive bacteria, Gram-negative bacteria, fungi or yeast.

[0053] In embodiments, the prediction step (c) comprises predicting the motion of the test compound relative to the phospholipid membrane based on the theoretical thermodynamic forces existing between the test compound and the phospholipid membrane. In one
15 embodiment, the prediction step is performed with one, or a combination of two, three, or four molecular dynamics ("MD") simulation computer programs. The *in silico* prediction step provides fundamental understanding of the action mechanisms at an atomistic level and is complementary to the experiments. Advantageously, the combination of theoretical predictions and the experimental validations provides a rational way for the design of new
20 membrane active antimicrobial molecules.

[0054] In embodiments, the molecular dynamics (MD) simulation comprises using a publically available force field selected from the group consisting of: AMBER, CHARMM, OPLS, and GROMOS.

[0055] In one embodiment, the prediction step comprises the use of a Gromos53a6 force
25 field. The type of force field used is largely dependent on the type of molecule being simulated. For instance, where the molecule to be simulated is a small molecule, the GROMOS force field may yield more accurate simulations. In other instances, where the molecule is a peptide compound, the inventors have discovered that a CHARMM force field may yield more accurate results. The force fields discussed herein are for illustrative purposes
30 and do not serve to limit the scope of the invention.

[0056] In embodiments of the disclosed method, if the test compound has been assessed to be unable to perturb or disrupt the cell membrane, the method is reverted to step (a) to select a new test compound.

[0057] In embodiments, the molecular dynamics simulation identifies key parameters that correlate to the antimicrobial activity. These key parameters may include, but are not limited to, translocation free energy, pKa of cationic groups, amphiphilicity, and the propensity of hydrogen bond formation with phosphate groups. In another embodiment, the identified key parameters include lateral pressure and the number of water translocations across the membrane, which characterize the mechanical properties of the membrane and membrane permeability respectively.

[0058] The disclosed method may further comprise identifying a second test compound, wherein the second test compound comprises one or more of the above identified parameters. In embodiments, the second test compound is subjected to molecular simulation to predict its interaction with a phospholipid membrane described above. In particular, the method comprises a step of predicting the motion of the second test compound relative to the phospholipid membrane based on the theoretical thermodynamic forces existing between the second test compound and the phospholipid membrane.

[0059] In embodiments, the disclosed method further comprises a step of identifying a further test compound if the second test compound is not assessed to be able to substantially perturb or disrupt the phospholipid membrane. In other words, where antimicrobial activity is assessed to be absent in the second test compound, an iterative step is undertaken to identify a new test compound based using parameters identified in step (c).

[0060] In embodiments where the second test compound has been predicted to be capable of disrupting or perturbing the phospholipid membrane, the disclosed method further comprises a step (d) of synthesizing the second test compound for biophysical and biological validation. It is within the expertise of a person skilled in the art to arrive at an appropriate synthesis procedure and therefore the specific synthesis steps will not be discussed in detail in the present disclosure. By disrupting or perturbing a bacterial membrane, it is intended to mean that the candidate compound is capable of causing re-organization of the lipid molecules, or is capable of partially embedding or fully embedding itself across the cross-section of the phospholipid bilayer membrane, thereby disrupting the fluid mosaic layer. In embodiments, the ability of a test compound to perturb or disrupt the membrane can be co-

related with the translocation free energy of the test compound and/or number of water translocations in the hydrophobic section of the membrane during *in-silico* modeling. The actual membrane surface activity of the test compound can be confirmed through biophysical characterization steps, e.g., surface plasmon resonance, after compound synthesis.

5 [0061] In embodiments, the synthesized compound is further subjected to validation steps to ascertain its pharmacological activity. In embodiments, the disclosed method further comprises a step (e) of subjecting said synthesized compound to biocompatibility tests. In particular embodiments, the biocompatibility tests comprise toxicology testing to determine the effects of the synthesized compound on mammalian cells and living tissue. For example,
10 in embodiments, the haemolytic activity of the compounds against mammalian erythrocytes can be determined. In other embodiments, the biocompatibility testing comprises determining the release of lactose dehydrogenase (LDH) and ATP-release activity after challenging mammalian cells with the synthesized compounds.

[0062] In embodiments, the disclosed method further comprises a step (f) of subjecting
15 said synthesized compound to susceptibility testing to determine its anti-microbial property *in vivo* and/or *in vitro*. In particular, the step (f) may comprise subjecting the compound to one or more of microbiological assays to determine its minimum inhibitory concentration (MIC) of various bacteria strains. In particular embodiments, the susceptibility testing involves testing the compound's MIC with methicillin-resistant *Staphylococcus aureus* (MRSA). In
20 some embodiments, the susceptibility testing may further comprise Time-Kill kinetics and laboratory simulation of resistance development (if any) against the synthesized compounds.

[0063] In still further embodiments, the disclosed method further comprises a step (g) of biophysical characterization of the synthesized compound. In embodiments, biophysical characterization can be undertaken using one or more of the following: circular dichroism
25 (CD), nuclear magnetic resonance (NMR), fluorescence spectrometry, isothermal titration calorimetry, and surface plasmon resonance.

[0064] In further embodiments, where the susceptibility tests and biocompatibility tests did not validate the predictions on the compound's antimicrobial activity, the method is reverted to a step of identifying a new test compound based on one or more identified
30 parameters in step (c) in an iterative manner.

[0065] In embodiments of the disclosed method, the second test compound may be modified with one or more parameters identified from an earlier test compound. For instance,

using the disclosed method, the inventors have surprisingly identified specific cationic groups of a known antimicrobial peptide as being particularly effective in membrane perturbation due to interactions between the cationic groups and the negatively charged phosphate groups present on the external bacterial membrane. In particular, cationic arginine / guanidine groups were identified via molecular simulation of antimicrobial peptides. Advantageously, as will be shown by the Examples provided herein, the inventors have successfully designed at least two alpha-mangostin analogues by altering alpha-mangostin molecule with the identified cationic groups (see experimental section on AM016/AM019 below).

[0066] In embodiments, the disclosed method further comprises subjecting the test to *in-vivo* animal testing if the susceptibility tests validate the predictions made on antimicrobial activity.

[0067] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

EXPERIMENTAL SECTION

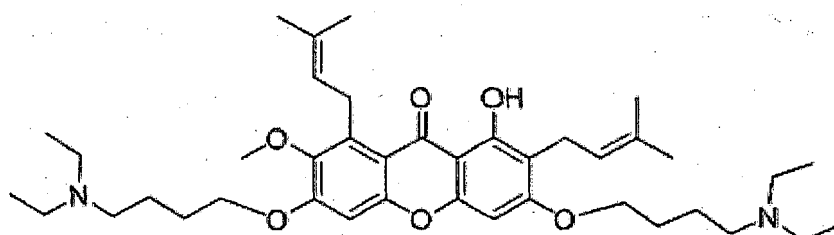
[0068] The following example shows the modeling and analysis of a molecule designed by the method disclosed herein. The examples and experimental results provided below are not intended to be limiting to the scope of the invention and merely serve to exemplify the design process.

[0069] Selection of candidate compound

[0070] Analogues of alpha-mangostin were selected as the starting anti-microbial molecule. Alpha-mangostin, being the precursor of the candidate compound, was selected

due to its unique heterocyclic structure with modifiable chemical side groups. Two structural properties, notably the planarity and the hydrophobicity of the core, favor penetration of the compound into the hydrophobic region of the bacterial membrane. Additionally, there are several oxygen atoms in the hydrophobic core, which provides the molecule with a certain degree of disruptive amphiphilicity, and which further favors membrane perturbation.

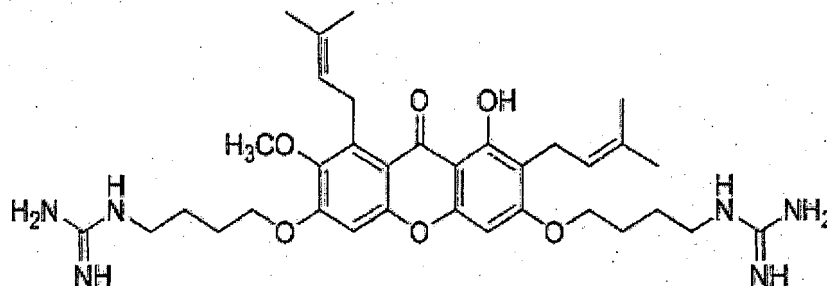
[0071] In one embodiment, the following compound is provided:



3,6-Di[4-(diethylamino)-butoxy]-1-hydroxy-7-methoxy-
2,8-bis(3-methylbut-2-enyl)-9Hxanthen-9-one

The compound above is hereafter denoted as **AM016** for brevity.

[0072] In yet another embodiment, the following compound is provided:



3,6-Di[4-(guanidino)-butoxy]-1-hydroxy-7-methoxy-
2,8-bis(3-methylbut-2-enyl)-9Hxanthen-9-one

The compound above is herein after denoted as “**AM019**” for brevity.

[0073] ***In-silico* modeling of AM016 and AM019**

[0074] The bacterial membrane was modeled using a mixture of POPE and POPG lipids with the ratio of POPE/POPG=3/1. The membrane patch contains 128 lipid molecules in conventional MD simulations and 72 lipid molecules in free energy calculations. The lipid molecules were modeled using the GROMOS53A6 force field. The topology of AM016 and AM019 were generated using the online server ATB with the AM1 method used for generating the atomic partial charges.

[0075] Translocation free energy

To examine the thermodynamics of AM016/AM019 when penetrating into the bacterial membrane, translocation free energy landscape was calculated using umbrella sampling and weighted histogram analysis method (WHAM). The cationic groups of the molecule need to overcome a free energy barrier when crossing the hydrophobic region of the membrane, leading a conformational change. The conformational change may involve the molecule changing from a U-shaped structure to an extended structure as shown in Fig. 1. This conformational change deforms the outer leaflet and the inner leaflet of the membrane. However, the extent of deformation of the outer leaflet and the inner leaflet are different. As the umbrella sampling requires sampling along the slowest reaction coordinate, which is the extent of membrane deformation, pulling the center of mass leads to hysteresis between the forward and reverse direction. To overcome this, umbrella sampling was performed along the distance between one of the cationic groups and the bilayer center. Long trajectories are needed to get converged results in translocation free energy calculations. In the simulations, we run at least 300 ns for each umbrella window and use the last 200 ns to calculate the free energies (See Figs. 2A and 2B).

[0076] Conventional MD simulations

To study the interactions of AM016/AM019 with a model bacterial membrane, molecular dynamics simulations were carried out at various concentrations (See Figs. 3A, 6A – 6D). All simulations were run for at least 1 micro-second as it was found that aggregation of AM016/AM019 occurs at the time scale of several hundredths of nano-seconds. Different number of AM016 molecules (4, 9, 16), corresponding to different concentrations were used. To compare the membrane perturbation of AM016 and AM019 at high concentrations, an additional simulation was performed using 16 AM019 molecules. As the extended conformation of AM016 interacted with both leaflets, it induced larger membrane perturbations than from the U-shape conformation. For each system, the required number of AM016 molecules with extended conformations was first pulled to the bilayer centre and then the system was relaxed and subject to at least 1 micron-second simulation. In order to study the aggregation of AM016 on the mechanical properties of the membrane, the pressure field of the bacterial membrane was calculated with 16 AM016 molecules using a customised version of GROMACS.

[0077] The lateral pressure is defined using the diagonal elements of the local pressure tensor $P(r)$ (eq. 1).

$$\pi(z) = \frac{P_{xx} + P_{yy}}{2} - P_{zz}$$

[0078] The local pressure tensor can further be decomposed into a kinetic term and a configurational term:

$$P(r) = \sum m_i \mathbf{v}_i \otimes \mathbf{v}_i + \frac{1}{V} \sum_{i < j} \mathbf{F}_{ij} \otimes \mathbf{r}_{ij}$$

5

[0079] For the purpose of lateral pressure calculation, the simulation with 16 AM016/AM019 molecules was extended to 1.6 micron seconds. The lateral pressure of the bacterial membrane was calculated using a slice of 0.1 nm in z direction and averaged using the last 0.6 micro second of the simulation.

10 [0080] Simulation details

Some of the simulation details are shown in Tables 1 & 2 below.

[0081] **Table 1**

Simulation	Drug	No. lipids	Temperature	No. Windows	Size of each umbrella window	Simulation time of each window
1	AM016	72	310	46	0.15 nm	400 ns
2	AM019	72	310	46	0.15 nm	300 ns

[0082] **Table 2**

Simulation	No. of drug	No. lipids	time	Initial condition
1	0 AM016	128	400 ns	
2	4 AM016	128	1 us	Extended conformation
3	9 AM016	128	1 us	Extended conformation
4	16 AM016	128	1.6 us	Extended conformation
5	16 AM019	128	1.3 us	Extended conformation

15

[0083] During the MD simulations, the LINCS algorithm was used to constrain the covalent bonds involving hydrogen atoms, which allows a time step of 2 fs to be used. Lennard-Jones potentials were cut at a distance of 1.4 nm. The real-space electrostatic

interactions were cut at a distance of 0.9 nm, while the long-range electrostatic interactions in reciprocal space were calculated using the particle-mesh Ewald algorithm. The temperature was maintained at 310 K by coupling the system to a Nose-Hoover heat bath, and the pressure was maintained at 1 atm using the semi-isotropic Parrinello-Rahman method.

[0084] The lateral pressure was calculated using a post-trajectory analysis using the rerun option of the mdrun of GROMACS. The lateral pressure of the bacterial membrane was calculated using a slice of 0.1 nm in the z direction and averaged using the last 0.6 micron of the simulation. Due to the difficulty in decomposing the reciprocal-space electrostatics into pairwise virial contributions, a long cut-off of 1.8 nm was used for the electrostatic interactions¹¹. In addition, SHAKES were used for bonds involving hydrogen atoms as LINCS does not decompose the force into pair wise interactions directly. The above simulations were carried out in supercomputing clusters in A*STAR computational resource centre (ACRC) and in bioinformatics institute. Lateral pressure calculations are presented in Fig. 3B.

[0085] Chemical Synthesis

Preparation of 3,6-bis(4-bromobutoxy)-1-hydroxy-7-methoxy-2,8-bis(3-methylbut-2-en-1-yl)-9H-xanthen-9-one (AM-0005, a precursor compound to AM016/AM019).

[0086] To α -mangostin (1.013 g, 2.47 mmol) and K_2CO_3 (1.6926 g, 12.2 mmol), 15 ml acetone and 1,4-dibromobutane (4.34 ml, 36.6 mmol) were added. The mixture was kept under reflux at 62 °C for 24 h. Acetone was removed via rotary vaporization. Product was diluted with EtoAc and washed with 3 times with 50 ml of saturated NaCl solution. The organic phase was dried over anhydrous sodium sulfate (Na_2SO_4) overnight and the solvent removed via rotary vaporization giving a yellow liquid. Purified by silica gel column chromatography (PE/EtoAc, 12/1, V/V) to give compound AM-0005 as a yellow solid; Yield 75.6 %; APCI-MS (m/z): 681.2 $[M+H]^+$; calculated for $C_{32}H_{40}Br_2O_6$ 680.5; ^1H NMR (400MHz, $CDCl_3$) δ = 13.48 (s, 1H, OH), 6.71 (s, 1H, Ar-H), 6.28 (s, 1H, Ar-H), 5.26-5.19 (m, 2H, $2\times CH$), 4.14-4.07 (m, 6H, $3\times CH_2$), 3.80 (s, 3H, OCH₃), 3.54-3.49 (m, 4H, $2\times CH_2$), 3.35 (d, $J=7.2$ Hz, 2H, CH₂), 2.15-2.00 (m, 8H, $4\times CH_2$), 1.85 (s, 3H, CH₃), 1.79 (s, 3H, CH₃), 1.68 (s, 6H, $2\times CH_3$); ^{13}C NMR (400MHz, $CDCl_3$): δ 181.98, 162.54, 159.92, 157.20, 155.24, 155.12, 144.06, 137.38, 131.76, 131.47, 123.22, 122.46, 112.14, 111.55, 104.00, 98.76, 89.19, 67.81, 67.29, 60.91, 33.19, 29.39, 25.80, 21.45, 18.17, 17.86.

[0087] **Preparation of 2,2'-(((1-hydroxy-7-methoxy-2,8-bis(3-methylbut-2-en-1-yl)-9-oxo-9H-xanthene-3,6-diyl)bis(oxy))bis(butane-4,1-diyl))bis(isoindoline-1,3-dione) (AM-0014)**

[0088] A mixture of (AM-0005) (148.7 mg, 0.218 mmol) and potassium phthalimide (98 mg, 0.529 mmol) in N,N-dimethylformamide (DMF) (7 ml) were stirred at 90 °C. After reaction came to an end (monitored by TLC, eluent, EtoAc/PE, 2/5, V/V), the product mixture was processed similar to AM-0005 above. The resulting residue was purified by silica gel column chromatography (EtoAc/ PE, 3/5, V/V) to give compound AM-0014 as a yellow solid Yield 70 %; APCI-MS (m/z): 815 [M+H]⁺; Calculated C₄₈H₄₈N₂O₁₀ 812.90; ¹H NMR (400MHz, CDCl₃) δ 13.46 (s, 1H, OH), 7.86-7.72 (m, 4H, Ar-H), 7.71-7.69 (m, 4H, Ar-H), 6.71 (s, 1H, Ar-H), 6.28 (s, 1H, Ar-H), 5.23-5.20 (t, J=1.6Hz, 2H, 2xCH), 4.13-4.07 (m, 6H, 3xCH₂), 3.80 (m, 7H, OCH₃, 2xCH₂), 3.33 (d, J=6.8Hz, 2H, CH₂), 1.96-1.61 (m, 24H, 4xCH₃, 6xCH₂); ¹³C NMR (400MHz, CDCl₃): δ 181.96, 168.41, 162.53, 159.84, 157.19, 155.21, 155.07, 144.01, 137.24, 133.99, 133.93, 132.04, 131.67, 131.36, 123.24, 123.20, 122.43, 112.03, 111.52, 103.92, 98.77, 89.25, 67.94, 67.53, 60.92, 37.49, 37.43, 26.43, 26.30, 26.15, 25.88, 25.74, 25.24, 25.21, 21.43, 18.16, 17.78.

[0089] **AM014** (100 mg, 0.123 mmol) was dissolved in DMSO (4 mL), and methylamine (4 mL) was added. The reaction mixture was stirred at 70 °C for 7 h. The mixture was then diluted with DCM and washed thrice with aqueous NaHCO₃. The organic phase was dried over anhydrous Na₂SO₄ and concentrated under vacuum. The residual crude oil was purified by preparative RP-HPLC with isocratic elution in 52 % CH₃OH/48% H₂O (0.1% formic acid), to give **AM018** as a light yellow solid (68.0 mg, 58%). ¹H NMR (400 MHz, MeOD) δ, 6.89 (s, 1H, Ar-H), 6.44 (s, 1H, Ar-H), 5.24-5.18 (m, 3H, 2xCH), 4.19 (t, 2H, CH₂), 4.14 (t, 2H, CH₂), 4.10 (d, 2H, CH₂), 3.79 (s, 3H, CH₃), 3.33 (2H, d, CH₂), 3.09-3.02 (4H, m, 2xCH₂), 2.02-1.88 (8H, m, 4xCH₂)-1.84 (s, 3H, CH₃), 1.79 (s, 3H, CH₃), 1.68 (s, 3H, CH₃), 1.67 (s, 3H, CH₃). ¹³C NMR (101 MHz, MeOD) δ 183.26, 170.29, 164.09, 160.67, 158.85, 156.76, 156.59, 145.48, 138.08, 132.06, 132.03, 124.81, 123.69, 112.93, 112.54, 104.72, 100.30, 90.69, 69.50, 69.00, 61.46, 40.57, 40.55, 27.33, 27.17, 26.99, 26.02, 25.95, 25.88, 25.74, 22.31, 18.34, 18.07. ESI-MS [M+1] found, 553.2, calculated for C₃₂H₄₄N₂O₆, 552.7. HPLC tR = 12.5 min.

[0090] To a solution of compound **AM018** (46.4 mg, 0.084 mmol) in 3 ml of anhydrous DMF was added 1H-Pyrazole-1-carboxamidine hydrochloride (32.0 mg, 0.218 mmol) and

followed by N,N-diisopropylethylamine (DIEA, 40 μ l). The reaction was stirred at room temperature overnight. Diethyl ether was added to the mixture after the reaction was complete. The resulting sticky suspension was centrifuged and yellow solid was collected. The solid was washed with diethyl ether and dried in vacuo. The crude product was purified by preparative RP-HPLC with isocratic elution in 52 % CH₃OH/48% H₂O (0.1% formic acid) to give pure product **AM019** as a light yellow solid (39.8 mg, 74%). ¹H NMR (400 MHz, DMSO-d₆) δ = 13.52 (1H, s, OH), 8.43 (br, 2H, NH), 7.84 (br, 6H, 2 $\times$ NH, 2 $\times$ NH₂), 7.03 (1H, s, Ar-H), 6.54 (1H, s, Ar-H), 5.12-5.18 (m, 2H, 2CH), 5.14, 4.18-4.12 (m, 4H, 2 $\times$ CH₂), 4.01 (2H, d, J = 7.2, 6.4 Hz, CH₂), 3.72 (s, 3H, OCH₃), 3.23 (d, J = Hz, 2H, CH₂), 3.17-3.15 (m, 4H, 2 $\times$ CH₂), 1.83-1.62 (m, 20H, 6 $\times$ CH₃, 4 $\times$ CH₂). ¹³C NMR (101 MHz, DMSO-d₆) δ = 181.47, 162.55, 158.80, 157.49, 156.99 (2 $\times$ C=NH), 154.84, 154.70, 143.80, 135.72, 130.70, 130.57, 123.35, 122.08, 110.72, 110.57, 102.84, 99.53, 89.94, 68.31, 67.93, 60.37, 40.28, 40.24, 25.72, 25.57 (2 $\times$ CH₂), 25.53, 25.48, 25.26, 25.20, 20.99, 17.97, 17.65. ESI-MS [M+1]⁺ found, 637.3, calculated for C₃₄H₄₈N₆O₆, 636.8. HPLC t_R = 11.5 min

[0091] Determination of the Minimum Inhibitory Concentration (MIC)

MIC determinations were performed in Mueller Hinton Broth (MHB) using the broth macro-dilution method following CLSI guidelines. AM016/019 were first dissolved in N,N-dimethylformamide (DMF) to make up the stock solutions of 1000 μ g/mL. Colonies were isolated for 18-20 hours on Tryptic Soy Agar (TSA) plates. Inoculums were prepared by direct suspension of the isolated colonies with MHB and adjusted to 5 $\times$ 10⁵ Colony Forming Units (CFU)/mL. Then, the inoculums were incubated at 35 °C for 22 hours. The strains used in the susceptibility testing are listed in Table 3.

Table 3. *In vitro* antibacterial activity of AM016 and AM019 (μ g/ml)

Compound	MRSA 9808R	SA DM 4001R
AM016	1.56	1.56
AM019	6.25	3.125

[0092] SYTOX green membrane permeabilization assay.

Overnight cultures of clinical isolate *S. aureus* DM4001 was harvested at exponential phase. Bacteria were then washed at least 3 times and suspended in 20 mM PBS until OD₆₂₀ (optical density at 620 nm) of 0.09 was obtained. The bacteria suspension was then incubated with 3 μ M SYTOX Green (Invitrogen) in dark condition for 5 minutes. Then, the mixture was transferred to a stirring cuvette and the fluorescence emission was monitored using an

excitation wavelength of 504 nm and emission wavelength of 523 nm until the signal was stabilized. Desired concentrations of AM016 and AM019 were then added and the fluorescence change was measured and recorded. Both compounds were dissolved in DMF and the final % of DMF in the culture was < 0.1 %. 0.1% DMF had negligible effect to the SYTOX Green fluorescence intensity.

[0093] **Calcein-loaded LUVs leakage study**

The release of calcein from the large unilamellar vesicles (LUVs) is studied. In brief, the lipids (DOPE/DOPG= 75/25) were dissolved in methanol/chloroform (1:2, by volume) and was dried gently using a constant stream of nitrogen gas until a thin lipid film was formed.

The film was completely dried by placing the film under vacuum for at least 2 hours. Then, the dried lipid film was hydrated using calcein solution (80 mM calcein, 50 mM HEPES, 100 mM NaCl, 0.3 mM EDTA, pH 7.4) to obtain a final lipid concentration of 30 mM. The hydrated lipids were freeze-thawed in liquid nitrogen and warm water for 7 cycles. Then, 100 nm homogenous LUVs were then prepared by using an extruder with a polycarbonate membrane (Whatman, pore size 100 nm). The extrusion was done for at least 10 cycles. Sephadex G-50 was used to purify the calcein-loaded LUVs from the free calcein. The concentration of calcein loaded LUVs were determined using total phosphorus determination assay. An aliquot of the LUV suspension was added into a stirred cuvette at various concentrations of AM016 and AM019 solution in DMF to obtain the desired compounds to lipid ratios of 1/2, 1/4 and 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256. The final concentration of lipid was 50 μ M and the final percentage of DMF is < 0.2%. Complete leakage was assumed by adding 2% Triton X-100. Control experiment using 0.2% of DMF showed that leakages of calcein from the LUVs were negligible. The calcein released was monitored using a Photon Technology International Model 814 fluorescence spectrophotometer at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Percentage of leakage (%L) was calculated with $\%L = [(I_t - I_o)/(I_\infty - I_o)] * 100$, where I_o and I_t denote respectively intensity before and after addition of AM016 or AM019 respectively and I_∞ is intensity after addition of 2% triton X-100.

[0094] **Results**

[0095] Fig. 1 is the schematic view of the pharmacophore model, which contains five fragments: one hydrophobic core, two cationic terminal groups and two flexible linkers. Initially the molecule gets adsorbed with the U-shape conformations, at high surface

concentrations, a conformational transition occurs, resulting in an extended conformation, which induces larger membrane perturbations than the U-shape conformation.

[0096] Fig. 2 shows the translocation free energies of AM016 and AM019 across a model bacterial membrane. During penetration into the bacterial membrane, both AM016 and AM019 were found to undergo a conformational change from U-shape conformation to the extended conformation (Fig 2A), consistent with the predictions of our pharmacophore model in Fig 1.

[0097] Fig. 3A shows both AM016 and AM019 aggregates when taking the extended conformation, resulting in the formation of cationic AM016/AM019 clusters surrounded by the anionic lipids. The reorganization of the lipids induced significant perturbations of the bacterial membrane, including membrane deformation, water translocation across the membrane, membrane expansion, etc. In addition, it also affected the mechanical properties of the bacterial membrane, as shown by the lateral pressure of the bacterial membrane (Fig. 3B).

[0098] Fig. 4 shows the fluorescence intensity induced by AM016 / AM019 in a dye-leakage experiment using either calcein with LUV (Fig. 4A) or SYTOX green with living bacterial cells (Fig. 4B). Both Fig. 4A and Fig. 4B show significant dye-leakage, indicating the membrane activity of AM016/AM019. Figs. 5A and 5B show the density profiles of phosphate atoms and water molecules at different AM016 concentrations. Fig. 5A shows that as AM016 concentration increases, the two peaks characterizing the phosphate atoms of the two leaflets become wide and less sharp, indicating significant membrane deformations. In addition, at high concentrations, AM019 is shown to perturb the bacterial membrane more significantly than AM016. The water density profiles (Fig 5B) show a similar trend. As AM016 concentration increases, the water density at the bilayer centre increases, indicating increased membrane permeability. Moreover, at high concentrations, AM019 was found to cause more water translocations relative to AM016, as seen from the higher water density in the bilayer center.

[0099] Figs. 6A-6D are snapshots of simulation results showing high concentrations of AM016 and AM019 (16 drug with 128 lipid molecules) at extended conformations. It can be seen that AM019 induces larger membrane deformation than AM016. In the case of 16 AM019 with 128 lipid molecules, the membrane is significantly distorted. Also seen is the aggregation of AM016 and AM019 when embedded into the bacterial membrane.

[00100] Fig. 7 shows that the area per lipid of the bacterial membrane increases significantly with AM016 concentration. At the highest AM016 concentration (16 AM016 molecules and 128 lipids), the area per lipid increased approximately 40% with respect to the pure bacterial membrane. The expansion of the bilayer area is expected to significantly affect the elastic properties of the membrane. However, in the case of 16 AM019 molecules and 128 lipid molecules, the area per lipid only has a slightly increase, which is because the membrane is significantly distorted, as seen in Fig 6C-6D.

[00101] Figs. 8A and 8B show the Calcein Leakage experimental results at different drug:lipid ratios. The percentage of leakage was correlated to the florescence and was found to increase with the drug/lipid ratio. Moreover, at the same concentration, AM019 induces more leakage than AM016, indicating that AM019 perturbs the bacterial inner membrane more significantly than AM016.

Claims

1. A method for identifying a compound that disrupts a membrane of a target cell, the method comprising the steps of:
 - (a) identifying the structure of a test compound;
 - 5 (b) identifying the structure of a phospholipid membrane of the target cell;
 - (c) using the identified structures of the test compound and the target cell to predict whether the test compound will disrupt the phospholipid membrane of the target cell.
2. The method according to claim 1, wherein the test compound is considered to disrupt
10 the phospholipid membrane if the test compound is predicted to be at least capable of embedding itself within the phospholipid membrane.
3. The method according to claims 1 or 2, wherein the test compound is considered to disrupt the phospholipid membrane if the test compound is predicted to be at least capable of
15 substantially perturbing the phospholipid membrane to cause lipid reorganization of the phospholipid membrane.
4. The method according to any one of the preceding claims, wherein said test compound is considered to disrupt the phospholipid membrane if the test compound is
20 predicted to be capable of cleaving said phospholipid membrane to cause lysis of said target cell.
5. The method according to any one of claims 1 to 4, wherein said phospholipid membrane identified in step (b) comprises at least one or more lipids selected from the group
25 consisting of: cholesterol, ergosterol, [(2*R*)-3-hexadecanoyloxy-2-[(*Z*)-octadec-9-enoyl]oxypropyl] 2-(trimethylazaniumyl)ethyl phosphate, 2-oleoyl-sn-glycero-3-phosphoserine, phosphatidylcholine, phosphatidic acid, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine, lysophosphatidylcholine, and mixtures thereof.
- 30 6. The method according to claim 5, wherein said phospholipid membrane identified in step (b) comprises a mixture of phosphatidylethanolamine and phosphatidylglycerol.

7. The method according to claim 6, wherein the mixture of phosphatidylethanolamine and phosphatidylglycerol is in a molar ratio of 1:5 to 5:1.

8. The method according to any one of claims 1 to 7, wherein the target cell is selected from the group consisting of: Gram-positive bacteria, Gram-negative bacteria, fungi, and yeast.

9. The method according to any one of the preceding claims, wherein the prediction step (c) involves predicting the motion of the test compound relative to the phospholipid membrane based on the theoretical thermodynamic forces existing between the test compound and the phospholipid membrane.

10. The method according to any one of the preceding claims, wherein step (c) further comprises a step of identifying key parameters of the test compound that correlate to antimicrobial activity.

11. The method according to claim 10, wherein said key parameters to be identified are selected from the group consisting of: translocation free energy, pKa of cationic groups, amphiphilicity, propensity for hydrogen bond formation with phosphate groups, lateral pressure of said membrane and number of water translocations across said membrane.

12. The method according to claim 11, further comprising identifying a second test compound having one or more parameters identified in step (c).

13. The method according to claim 12, further comprising a step of predicting the motion of the second test compound relative to the phospholipid membrane based on the theoretical thermodynamic forces existing between the second test compound and the phospholipid membrane.

14. The method according to claim 13, further comprising a step (d) of synthesizing the second test compound.

15. The method according to claim 14, further comprising a step (e) of subjecting the synthesized compound to biocompatibility tests.

16. The method according to claim 15, further comprising a step (f) of subjecting the
5 synthesized compound to susceptibility tests to determine its antimicrobial property.

17. The method according to claim 16, further comprising a step (g) of biophysical characterization of said synthesized compound.

1/8

FIGURES

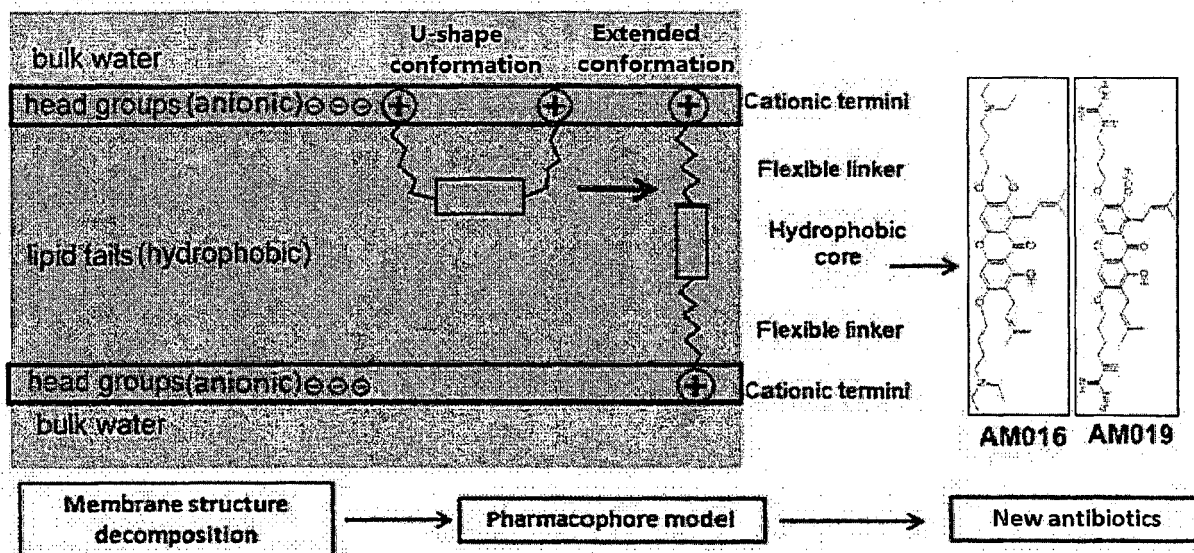


FIG. 1

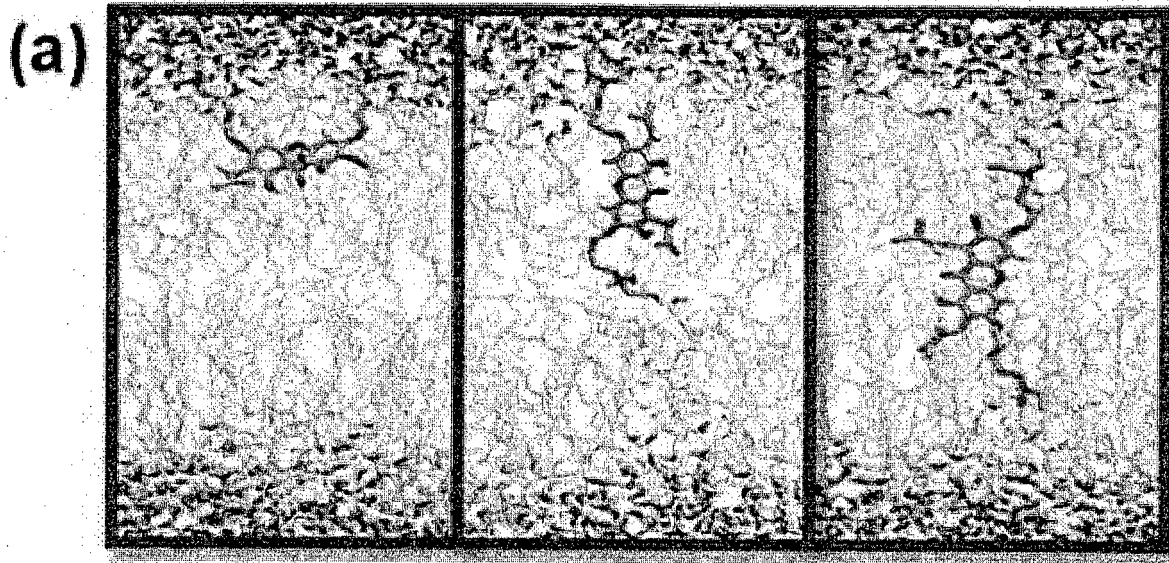


FIG. 2A

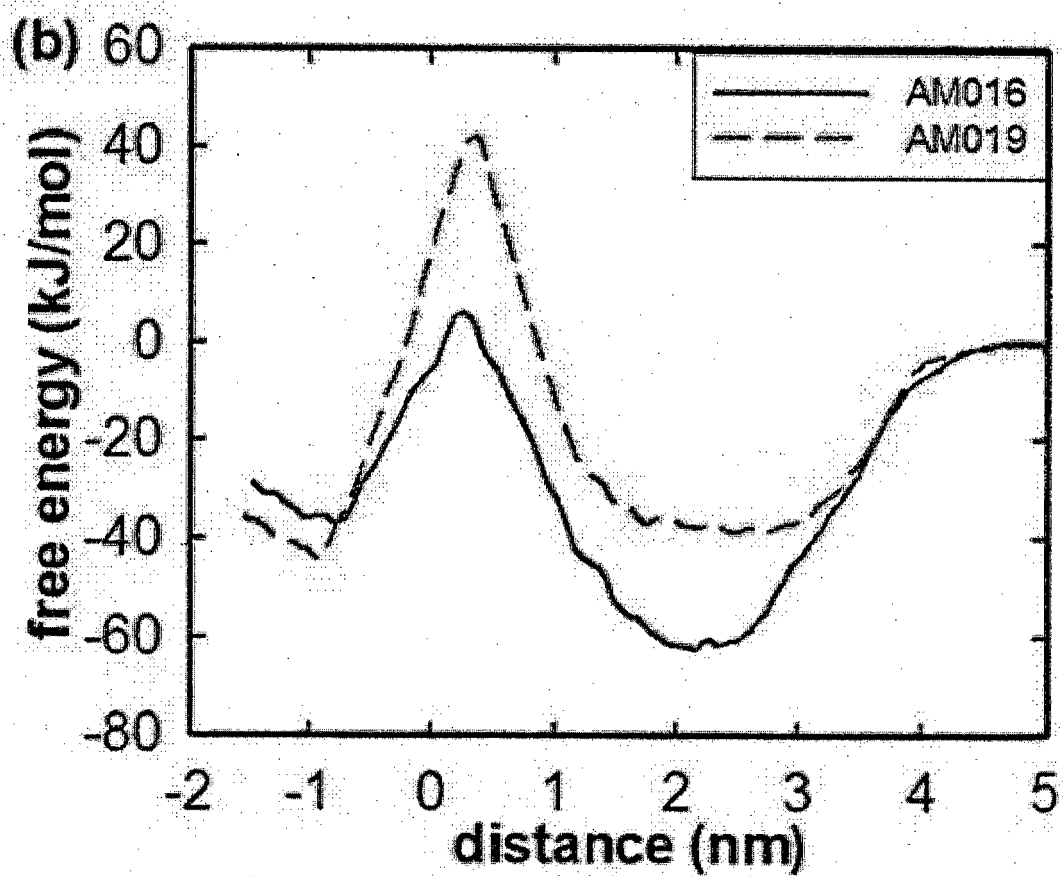


FIG. 2B

3/8

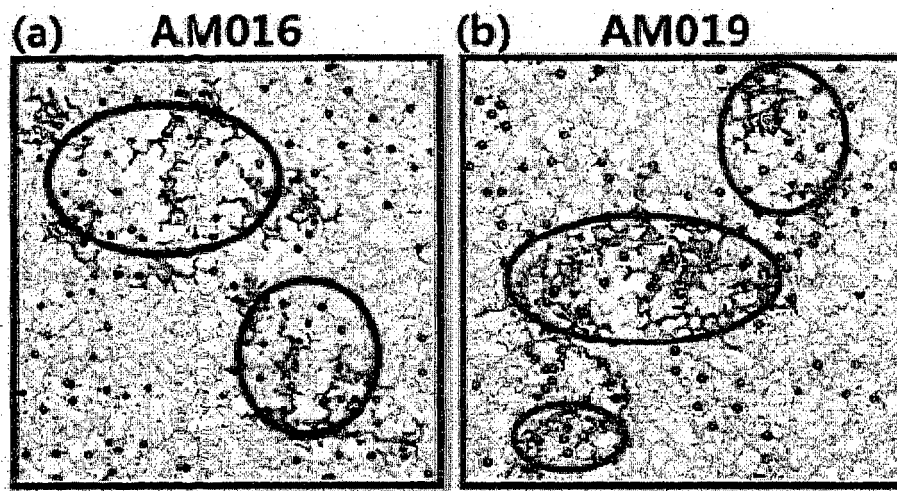


FIG. 3A

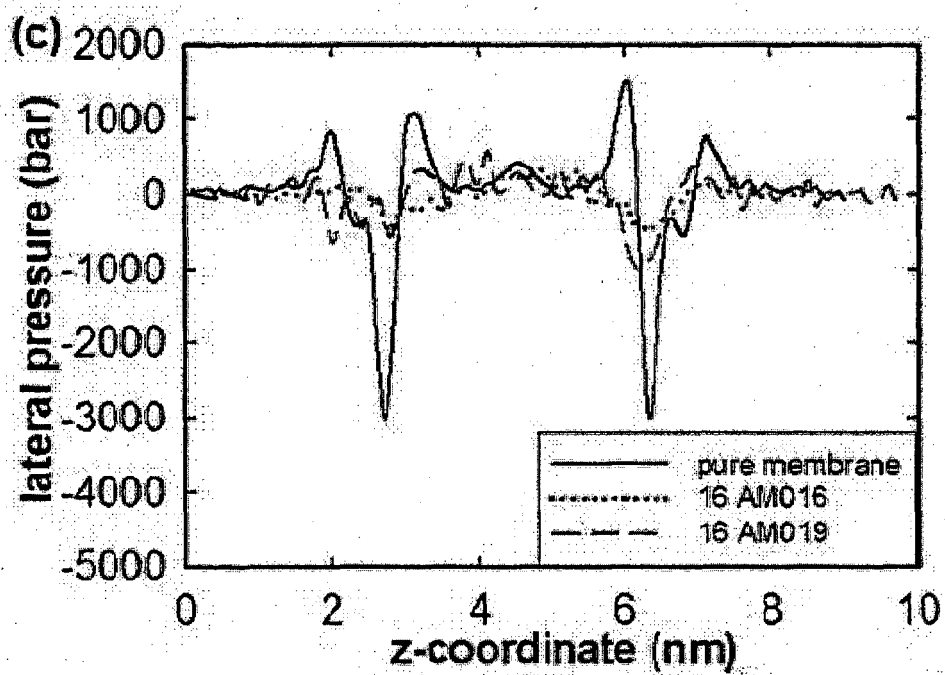


FIG. 3B

4/8

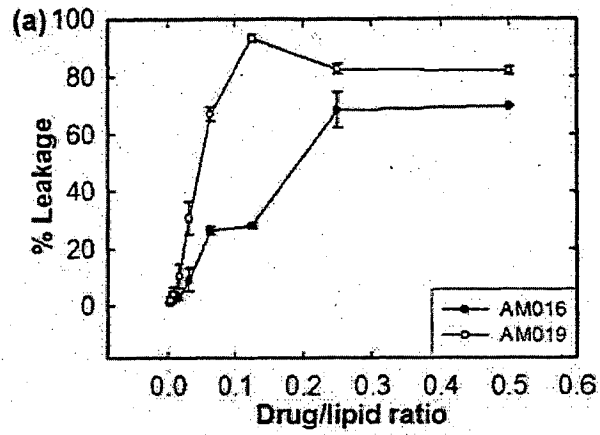


FIG. 4A

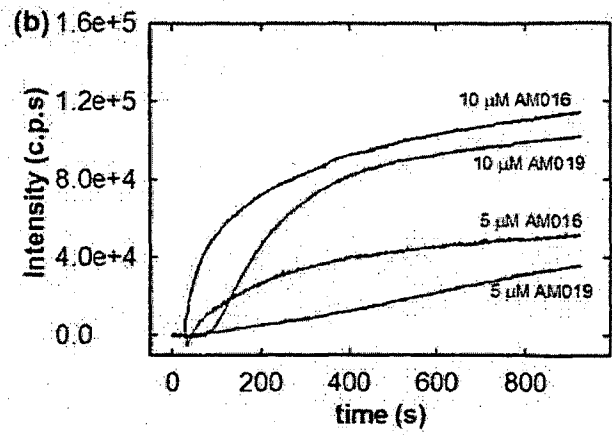


FIG. 4B

5/8

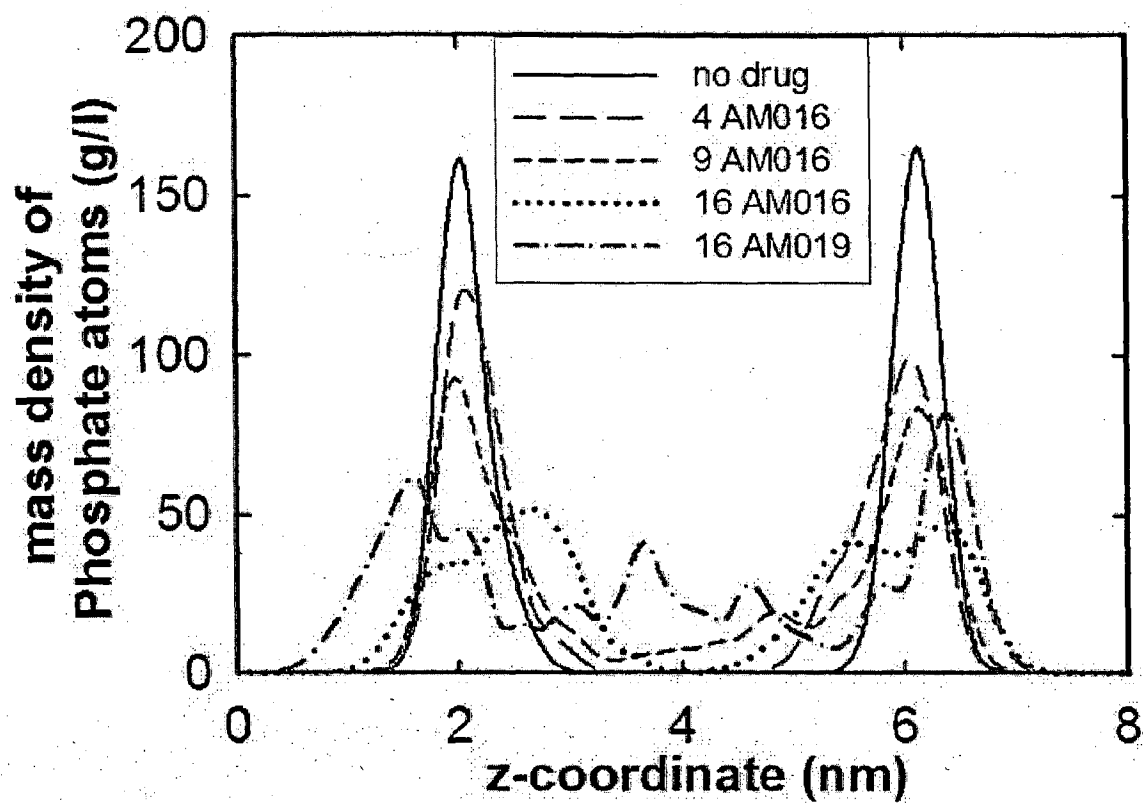


FIG. 5A

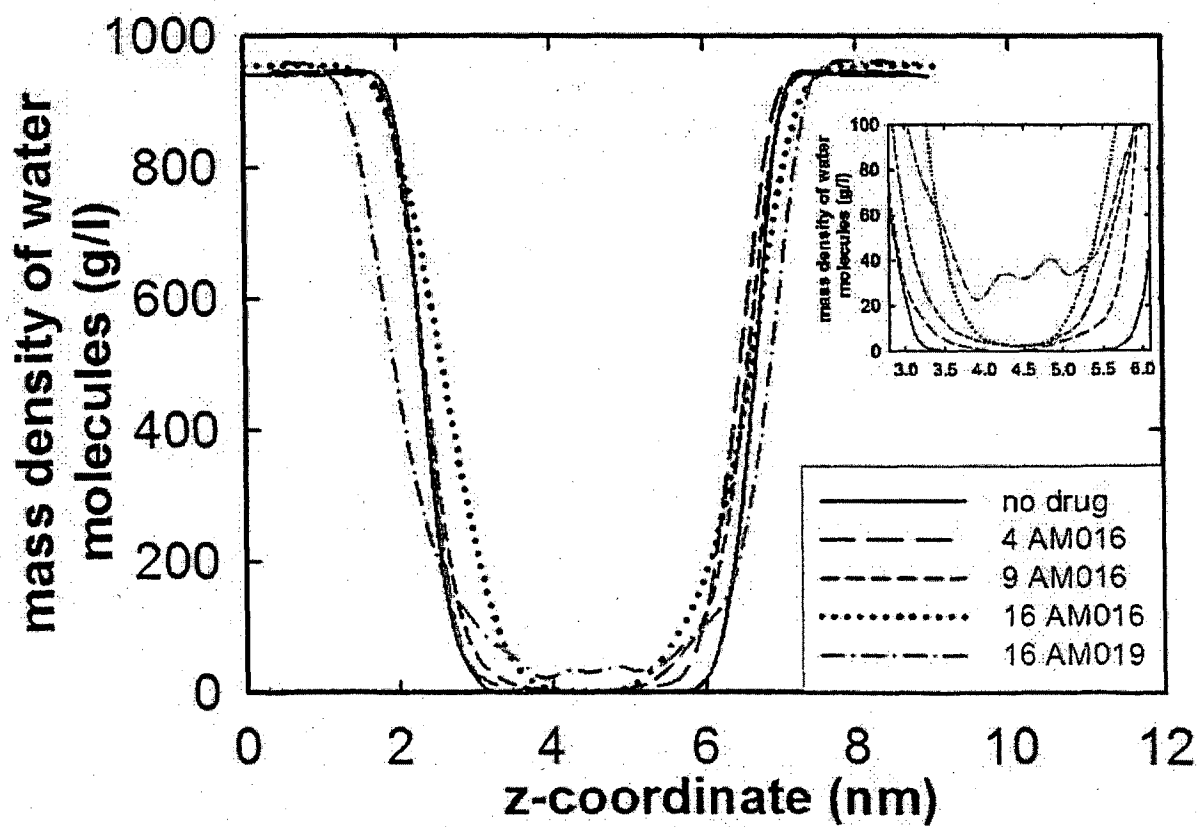


FIG. 5B

6/8

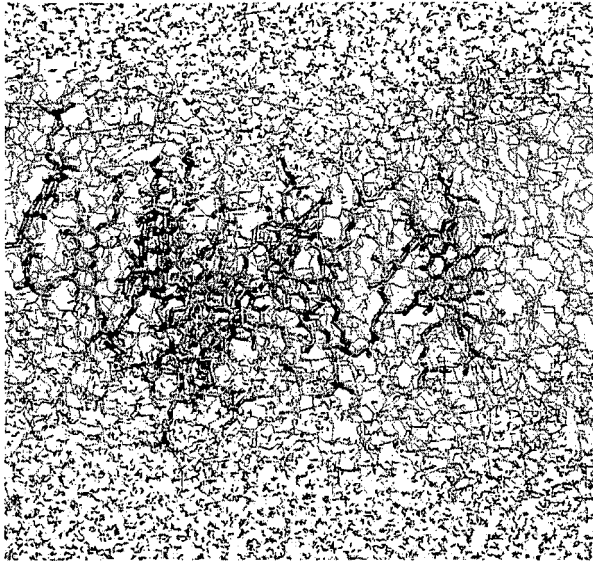


FIG. 6A

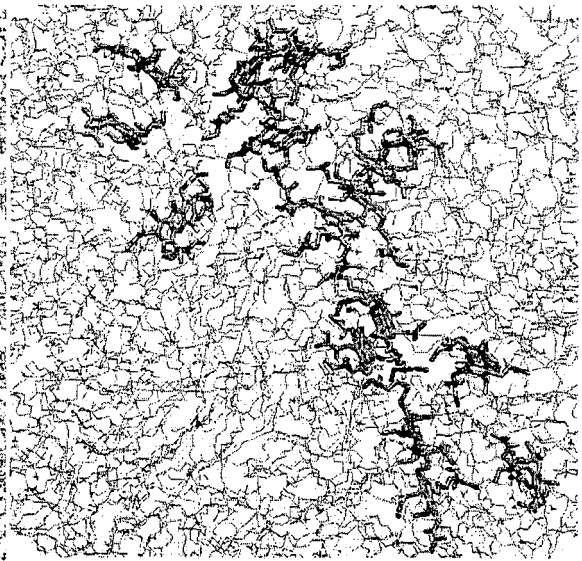


FIG. 6B

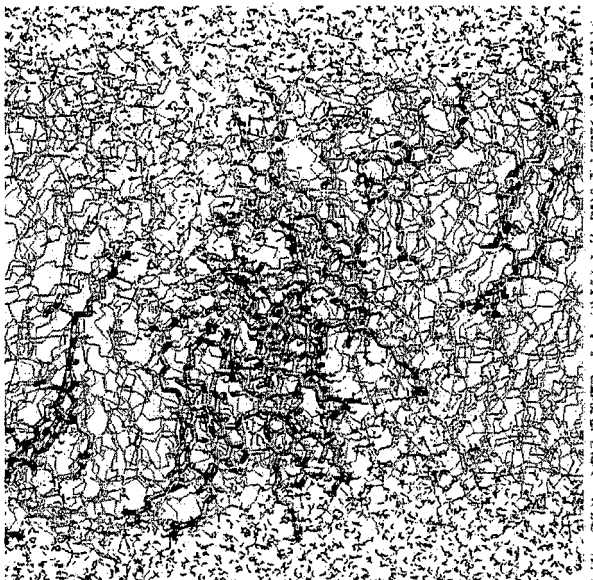


FIG. 6C

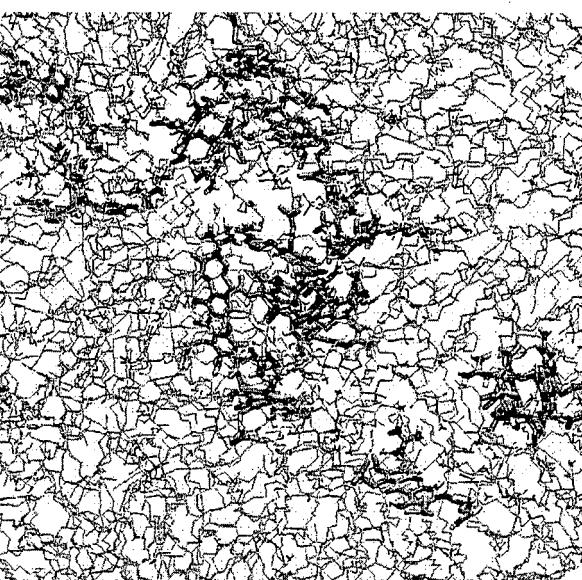


FIG. 6D

5

7/8

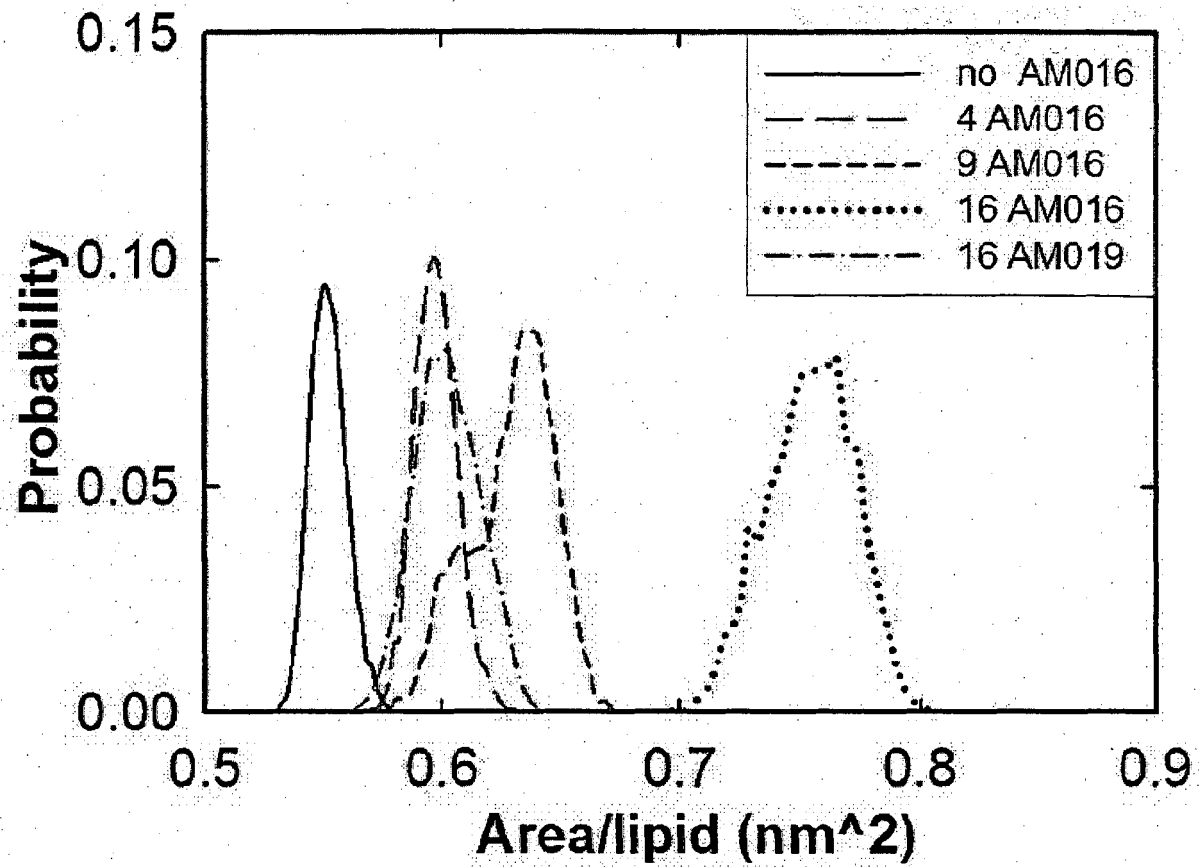


FIG. 7

8/8

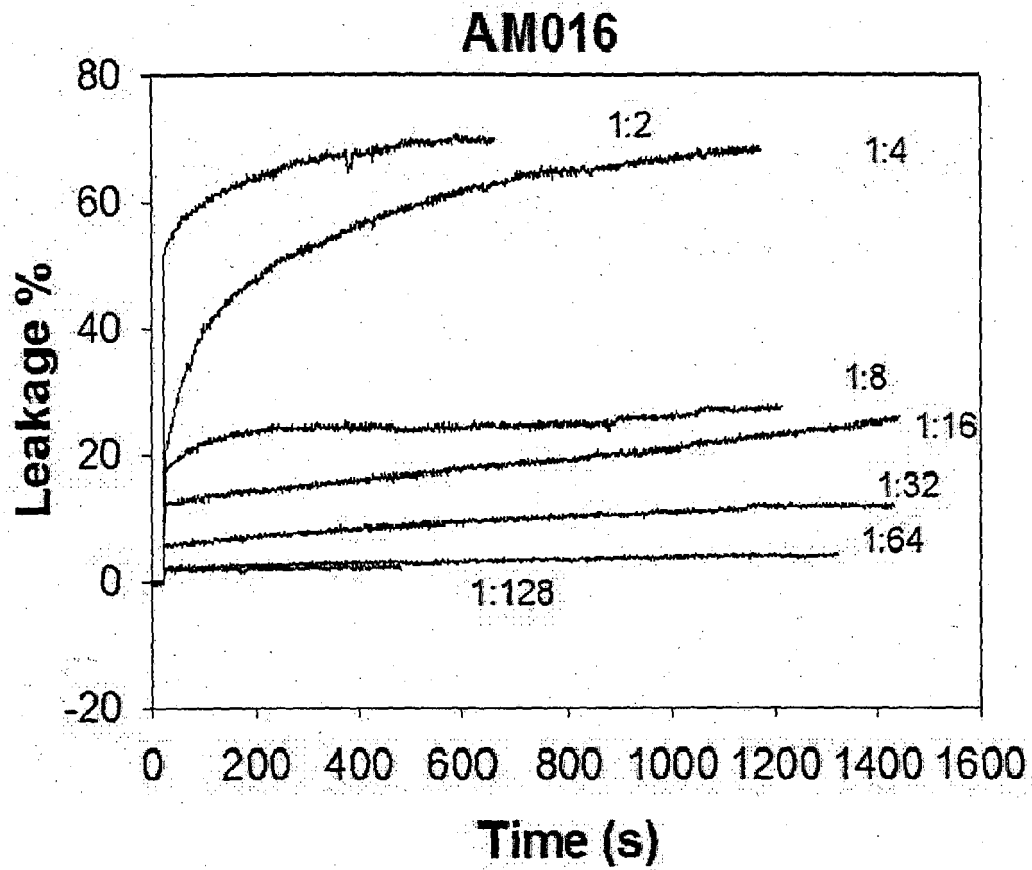


FIG. 8A

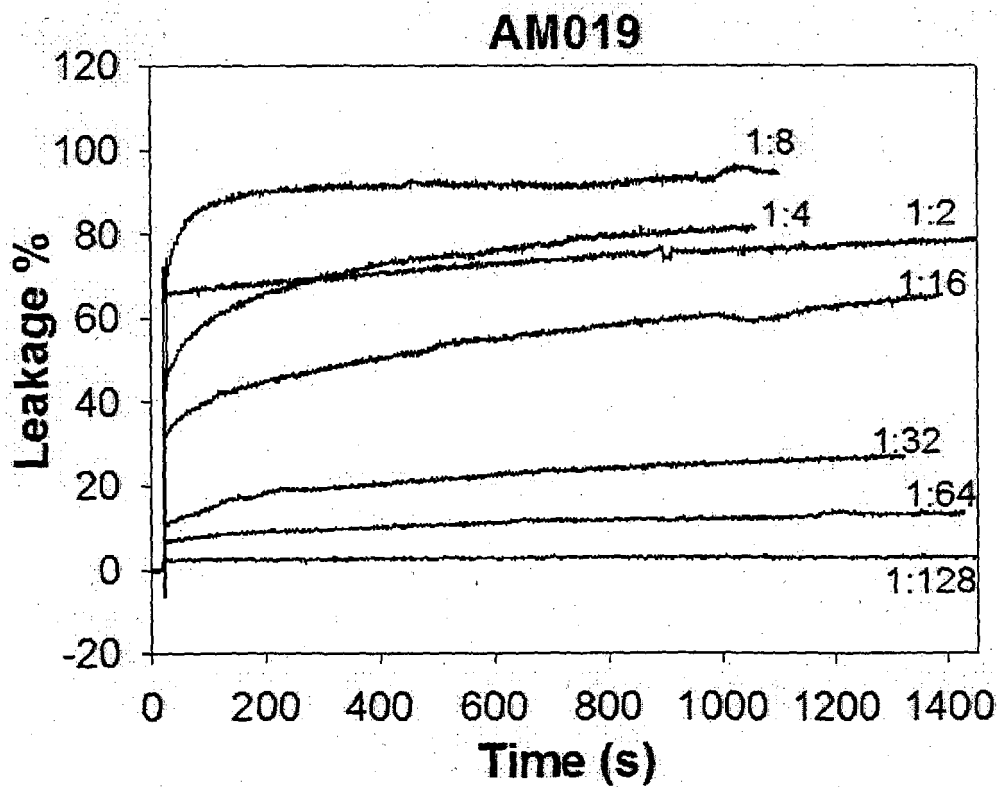


FIG. 8B

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/SG2013/000393

A. CLASSIFICATION OF SUBJECT MATTER

C40B 30/02 (2006.01) G06F 19/16 (2011.01) C12Q 1/18 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, MEDLINE, HCAPLUS, BIOSIS & keywords: membrane, phospholipid, disrupt, lyse, molecular modelling, simulation, antimicrobial, screen, compound and like terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 4 December 2013	Date of mailing of the international search report 04 December 2013
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au Facsimile No.: +61 2 6283 7999	Authorised officer Richard Filmer AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 6283 2735

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/SG2013/000393
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TSAI, C.-W. et al., "Coupling Molecular Dynamics Simulations with Experiments for the Rational Design of Indolicidin-Analogous Antimicrobial Peptides", Journal of Molecular Biology, 2009, vol. 392, pages 837-854 Whole Document, particularly the Abstract	1-17
X	BAI, Y. et al., "Progressive Structuring of a Branched Antimicrobial Peptide on the Path to the Inner Membrane Target", The Journal of Biological Chemistry, 3 August 2012, vol. 287, no. 32, pages 26606-26617 Abstract; Experimental Procedures; Table 1	1-17
X	RUSSELL, A. L. et al., "The effect of the length and flexibility of the side chain of basic amino acids on the binding of antimicrobial peptides to zwitterionic and anionic membrane model systems", Bioorganic & Medicinal Chemistry, 1 March 2012, vol. 20, pages 1723-1739 Abstract; Materials and Methods; Table 1	1-17

Form PCT/ISA/210 (fifth sheet) (July 2009)