



PGLa-H tandem-repeat peptides active against multidrug resistant clinical bacterial isolates

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ABSTRACT

Antimicrobial peptides (AMPs) are promising candidates for new antibiotic classes but often display an unacceptably high toxicity towards human cells. A naturally produced C-terminal fragment of PGLa, named PGLa-H, has been reported to have a very low haemolytic activity while maintaining a moderate antibacterial activity. A sequential tandem repeat of this fragment, diPGLa-H, was designed, as well as an analogue with a Val to Gly substitution at a key position. These peptides showed markedly improved *in vitro* bacteriostatic and bactericidal activity against both reference strains and multidrug resistant clinical isolates of Gram-negative and Gram-positive pathogens, with generally low toxicity for human cells as assessed by haemolysis, cell viability, and DNA damage assays. The glycine substitution analogue, kiadin, had a slightly better antibacterial activity and reduced haemolytic activity, which may correlate with an increased flexibility of its helical structure, as deduced using molecular dynamics simulations. These peptides may serve as useful lead compounds for developing anti-infective agents against resistant Gram-negative and Gram-positive species.

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1. Introduction

The phenomenon of spreading bacterial resistance to approved antibiotics presents an increasingly serious problem. It is aggravated by accelerated evolution of multidrug resistant bacterial species due to mismanagement and over-usage of antibiotics. In nature, however, the coevolution of host defence effectors and microbes in multicellular species, through millions of years, has not resulted in the loss of their capacity to protect the host organisms against microbial infection. Among these, host defence peptides (HDPs) are an important component of innate immune system, but despite their

recognized potential as possible leads for therapeutic agents, molecules based on them have as yet to be used as commercial antimicrobial drugs [1–3].

HDPs provide a rapidly mobilized first line defence against infection and have been identified in effectively all multicellular organisms examined for their presence [4,5], including very primitive ones [6]. They have several advantages and some disadvantages when compared to conventional antibiotics [7]. The most common mechanism of action, namely perturbation of the bacterial membrane bilayer structure, is of so general a nature that bacterial species find it difficult to evolve permanent resistance [8,9]. While the different characteristics of bacterial and eukaryotic membranes allow for some selectivity in their action against microbial with respect to host cells, they are often toxic to the latter at concentrations not sufficiently higher than antibacterial ones to be useful for therapeutic use. A good example of a currently used drug with some of the structural and functional characteristics of membrane-active AMPs is the last-resort antibiotic colistin. While efficient and rapid in killing of Gram-negative pathogens, its use is limited by nephrotoxicity [10–12]. Given the huge diversity of membrane-active AMPs, it should however be possible to identify candidates with useful bacteriostatic and bactericidal activities against multiresistant pathogens that are less toxic for human cells.

Abbreviations: AO, Acridine orange; EtBr, ethidium bromide; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine, diC12; DMSO, dimethyl sulphoxide; dPG, diphosphatidyl glycerol; EDTA, ethylenediaminetetraacetic acid; ESBL, extended-spectrum β -lactamase; FBS, foetal bovine serum; HPBLs, human peripheral blood leukocytes; LUVs, large unilamellar vesicles; LMP, low melting point; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMP, normal melting point; PG, phosphatidylglycerol; PI, propidium iodide; SDS, sodium dodecyl sulphate; SPB, sodium phosphate buffer; TFA, trifluoroacetic acid; TFE, trifluoroethanol; HDP, host defence peptide.

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Small, linear HDPs are abundant and widely distributed and so are natural candidates for computational algorithms that can help in searching for potential antibiotics [13,14]. Anuran AMPs, in particular, are numerous and among the best explored [15,16]. A magainin-2 analogue (MSI-78 or pexiganan), with optimized antibacterial activity, is being clinically tested for topical use against diabetic foot ulcers [17], but it has a significantly increased toxicity with respect to the parent peptide [18], making it less suitable for systemic use. PGLa-H, a 10 residue C-terminal fragment of PGLa, reported to have a moderate *in vitro* activity against both Gram-negative and Gram-positive bacteria and negligible haemolytic activity, is one of the smallest natural AMPs [19, 20]. We speculated that doubling the size of this peptide, as a sequential tandem repeat, could increase activity, but could also affect its selectivity. However, an algorithm that can quite accurately predict the selectivity index (SI, in terms of HC_{50}/MIC for *E. coli*) of anuran peptides [13,14, 21,22] suggested *di*PGLa-H should still have an acceptable SI value. The same algorithm allowed for fine tuning of the peptide sequence and suggested a possible substitution of Val¹⁵ in *di*PGLa-H with Gly to further improve selectivity. This peptide was named kiadin based on its first three residues, and molecular dynamics simulations indicated a somewhat reduced stability of the helix with respect to *di*PGLa-H.

*Di*PGLa-H and kiadin were synthesized and tested for *in vitro* activity against *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *S. aureus*, including multi-drug resistant clinical isolates, as well as for cytogenotoxic effects on human blood cells and fibroblasts. Results were encouraging, and indicated a potent, broad-spectrum activity with good measured SI values. These two peptides are therefore good candidates for further development, and confirm the usefulness of our algorithm for predicting SI and suggesting substitutions that might improve it.

2. Materials and methods

2.1. Materials

Acridine orange, ethidium bromide, histopaque, low and normal melting point agaroses, phosphatidylglycerol, propidium iodide, RPMI-1640 medium and colistin sulphate salt were from Sigma (St Louis, Missouri, USA); heparinized vacutainer tubes were from Becton Dickinson (Franklin Lakes, USA); diphosphatidylglycerol was from Avanti Polar Lipids (Alabaster, Alabama, USA); foetal bovine serum (FBS), Pen Strep, L-glutamine and trypsin-EDTA were from Gibco (Thermo Fisher Scientific, Massachusetts, USA). All other reagents used were laboratory-grade chemicals from Kemika (Zagreb, Croatia).

2.2. Peptides

Peptides were C-terminally amidated and obtained from GenicBio Limited (Shanghai, China) at >98% purity as confirmed by RP-HPLC and mass-spectrometry (see Table 1). Chromatographic separation was achieved on a reversed-phase Phenomenex Gemini-NX column (C18, 5 μ m, 110 Å, 4.6 \times 250 mm) using a 25–50% acetonitrile/0.1% TFA gradient in 25 min at a 1 ml/min flow rate, so that the K^* values were 2.9 and 3.8 [23] for kiadin and *di*PGLa-H respectively. Stock

solutions were prepared by dissolving accurately weighed aliquots of peptide in doubly distilled water, and the concentrations further verified by using the extinction coefficients at 214 nm, calculated as described by Kuipers and Gruppen [24].

2.3. Preparation of liposomes

LUVs (large unilamellar vesicles) were prepared as described by Morgera et al. [25]. Briefly, dry phosphatidylglycerol and diphosphatidylglycerol (PG:dPG 95:5 w/w) were dissolved in chloroform/methanol (2:1) solution, then evaporated using a dry nitrogen stream and vacuum-dried for 24 h. The liposome cake was resuspended in 1 ml of SPB to a concentration of 5 mM phospholipid and spun for 1 h at 40 °C. The vesicles were then subjected to several freeze–thaw cycles before passing through a mini-extruder (Avanti Polar Lipids, Alabaster, Alabama, USA) through successive polycarbonate filters with 1 μ m, 0.4 μ m and 0.1 μ m pores and resuspended to a final phospholipid concentration of 0.4 mM. Based on the bilayer membrane surface area of a ~100 nm liposome, and area of a phospholipid head group (~0.7–1 nm²) [26], the concentration of liposomes is about 5 nM.

2.4. Circular dichroism

CD spectra were obtained on a J-710 spectropolarimeter (Jasco, Tokyo, Japan) in SPB, sodium dodecyl sulphate micelles (10 mM SDS in SPB), increasing proportions of TFE in SPB (up to 50%) and in the presence of anionic LUVs (PG:dPG 95:5) in SPB. The % helix content was determined as $\alpha = [\theta]^{meas} / [\theta]^\alpha$, where $[\theta]^{meas}$ is the measured molar per/residue ellipticity at 222 nm under any given condition and $[\theta]^\alpha$ is the molar ellipticity for perfectly formed alpha helix of the same length, estimated by the method of Chen et al. [27].

2.5. Bacterial strains and antibiograms

Standard laboratory strains used in assays were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 13883, and *S. aureus* ATCC 29213. Clinical isolates of these five bacteria were obtained from University Hospital Centre Split, Croatia. Antibiograms indicate that they are variously resistant to several classes of antibiotics (see Table S1 in Supplementary material).

2.6. Antimicrobial activity

The minimal inhibitory concentration (MIC) was visually determined as the lowest concentration of peptide that completely inhibited bacterial growth in a serial dilution assay, carried out according to EUCAST guidelines (see Table S1 in Supplementary material), and was the consensus value from at least two independent experiments, each performed in triplicate. All bacteria were cultured in MH medium to the mid-logarithmic phase. 50 μ l of a bacterial suspension of 10⁶ CFU/ml were added to 50 μ l of serial dilutions of synthetic peptides

Table 1
Sequence and physico-chemical characteristics of peptides.

Peptide	Sequence	^a MW ^{calc}	^b MW ^{meas}	Charge	^c H	^d μ H ^{rel}	^e SI ^{calc}
PGLa-H	KIAKVALKAL-NH ₂	1053.4	1052.8	+4	−0.08	0.6	93
<i>Di</i> PGLa-H	KIAKVALKALKIAKVALKAL-NH ₂	2089.8	2089.8	+7	−0.08	0.46	93
Kiadin	KIAKVALKALKIAKGALKAL-NH ₂	2047.7	2047.7	+7	−0.4	0.51	94
Melittin	GIGAVKLVLTGLPALISWIKRKRQQ-NH ₂	2846.4	—	+6	—	—	17

^a Calculated using Peptide Companion, CoshiSoft.

^b Determined using Waters ZQ 2000.

^c Calculated using the CCS consensus hydrophobicity scale [45].

^d Hydrophobic moment relative to a perfectly amphipathic helical peptide of 18 residues.

^e Predicted selectivity index by using the tool <http://split4.pmfst.hr/mutator/> on a 1–95 scale.

to a final load of $5 \cdot 10^5$ CFU/ml per well, and incubated 18 h at 37 °C before visual inspection of wells.

For MBC determination, 4 μ l bacterial suspensions (corresponding to about 2000 CFU from initial inoculum) were taken from wells corresponding to the MIC, $2 \times$ MIC and $4 \times$ MIC and plated on solid MH medium. Agar plates were incubated for 18 h at 37 °C to allow the viable colony counts. MBC was taken as the concentration from the wells causing ~99.9%–99.99% killing (4–10 colonies per agar plate).

2.7. Cyto/genotoxicity assays on circulating blood cells

Studies were approved by the Ethics Committee and observed the ethical principles of the Declaration of Helsinki. Informed consent was obtained to participate in this study. Peripheral blood samples were drawn from young healthy male donors into vacutainers containing anticoagulant under aseptic conditions. Leukocytes and erythrocytes were then isolated for different assays. Each experiment was repeated at least two times.

For haemolytic activity, 50 μ l aliquots of whole blood were diluted in PBS to a concentration of 1% v/v and then 100 μ l aliquots were added to an equal volume of peptide in PBS to a final concentration of 0.5% erythrocytes and 25–500 μ M peptide. The haemolytic activity was assessed by monitoring the release of haemoglobin at 450 nm after incubating at 37 °C for 60 min. Total lysis (100% haemolysis) was determined by the addition of 1% (v/v) Triton X-100. The HC_{50} value was taken as the mean concentration of peptide producing 50% haemolysis. All evaluations were carried out in triplicate.

Cell viability was determined by differential staining of human peripheral blood leukocytes (HPBLs) with AO and EtBr, by fluorescence microscopy [28]. Whole blood (900 μ l) was exposed to increasing concentrations of peptides (0, 1.5, 3, 6, 12, 25, 50 and 100 μ M) or colistin (up to 200 μ M) for 4 and 24 h periods at 37 °C. After the treatment, whole blood (300 μ l) was added onto an equal volume of histopaque and the buffy coats were collected after density gradient centrifugation. The slides were prepared by adding AO/EtBr mixture (diluted in PBS) to HPBL suspension in final concentration of 100 μ g/ml (1:1; v/v). A total of 100 HPBLs per repetition were examined with an epifluorescence microscope (Olympus BX51, Tokyo, Japan). Quantitative assessments were made by determining the percentage of live and dead cells. The nuclei of live cells emitted a green fluorescence and of dead cells red fluorescence.

Finally, genotoxicity was assessed via the alkaline comet assay, carried out as described by Singh et al. [29] with minor modifications [30]. Whole blood (900 μ l) was exposed to increasing concentrations of peptides or colistin (0, 1.5, 3, 6, 12, 25, 50 and 100 μ M) for 4 and 24 h periods at 37 °C, and after the treatment 5 μ l of whole blood was mixed with 100 μ l of 0.5% LMP agarose and added to fully frosted slides pre-coated with 0.6% NMP agarose. After solidifying, the slides were covered with 0.5% LMP agarose, and the cells were lysed (2.5 M NaCl, 100 mM EDTA Na₂, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulphoxide, pH 10) overnight at 4 °C. The slides were then placed into alkaline solution (300 mM NaOH, 1 mM EDTANa₂, pH 13) for 20 min at 4 °C to allow DNA unwinding and subsequently electrophoresed for 20 min at 1 V/cm. Finally, they were neutralized in 0.4 M Tris buffer (pH 7.5) for 5 min 3 times, stained with EtBr (10 μ g/ml) and analysed at 250 \times magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). One hundred randomly captured comets from each slide were examined. The percent of tail DNA was used to measure the level of DNA damage.

2.8. Cytotoxicity assay on non-circulating cells

Fibroblasts were isolated from a young healthy male donor and stored as described previously in Keira et al. [31]. Cells were cultured

in RPMI 1640 medium, supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1% Pen Strep (v/v) and maintained in a humidified incubator with 5% CO₂ at 37 °C until they reached confluency. They were detached using trypsin-EDTA solution, subcultured in 96 well plates in 200 μ l medium (2.4×10^4 cells/well) and incubated overnight. The toxicity of selected peptides was assessed by a standard MTT assay [32].

After overnight incubation, medium was removed and 100 μ l of fresh medium was added to fibroblasts cell culture with increasing concentrations of peptides and incubated for 24 h at 37 °C with 5% CO₂. Medium was then removed and 200 μ l of fresh medium supplemented with 20 μ l of MTT (5 mg/ml) solution was added and the suspension incubated for 4 h. Medium was removed and 200 μ l of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 595 nm with an EnSight Multimode Plate Reader (Perkin Elmer, Inc.). All evaluations were performed twice in triplicate.

2.9. Membrane integrity assay

The effect of diPGLa-H and kiadin on bacterial membrane permeabilization was studied by measuring the percentage of propidium iodide (PI) positive cells using a Cytomics FC 5000 flow cytometer (Beckman-Coulter, Inc., Fullerton, CA). Measurements were carried out on *E. coli* ATCC 25922 cells which were cultured in MH medium to the mid-logarithmic phase. After incubation, PI was added to the bacterial suspension (1×10^6 CFU/ml) at a final concentration of 15 μ M (10 μ g/ml). Peptides were then added in different concentrations just before the beginning of the analysis and the measurement was taken at 15, 30 and 60 min. Cells incubated with 5 μ M melittin were used as positive control, while cells in MH medium without peptides were used as negative control. Analyses were carried out as described previously [33].

2.10. Molecular modelling

Molecular dynamics simulations of diPGLa-H and kiadin were carried out in three different environments: water, TFE/water mixture, and in neutral solvated DLPC membrane. In the case of the TFE/water mixture, two conditions for N-termini were used, neutral and positively charged, where the latter showed a significant reduction in fluctuations of the N-terminal segment (data not shown), while the former allowed to access other possible secondary structures for the peptides. C-terminals were amidated (neutral) in all simulations. Starting peptide atomic coordinates were obtained using the QUARK template-free protein structure predictor [34]. The GROMOS (53a6) force field [35,36], and SPC/E model for water [37] were then used in simulations carried out on the Gromacs 4.6.5 package [38]. The QUARK predictor provided as output a number of structures in PDB format for each peptide, all of which corresponded to a helical conformation. The structure showing the most extensive such conformation was immersed in: a) a cubic box of 5.6 nm side with either ~5700 water molecules, or 380 TFE together with ~3800 water molecules (this 10% molar solution corresponds to about 30% TFE/H₂O v/v); b) in the central part of the equilibrated lipid bilayer (comprising about 110 lipids in each leaflet immersed in ~11,000 water molecules) using a similar procedure as explained in [39].

Counterions were then added to neutralize the system and it was subjected to: a) 50,000 steps of energy minimization using the steepest descent algorithm followed by 100 ps equalization runs in the NVT ensemble and 100 ps runs in the NpT ensemble; b) the warming up procedure starting from 50 K and increasing the temperature for 50 K more every 30 ps simulation step, up to the last 30 ps step where the annealing temperature of 300 K was increased up to the reference temperature of 310 K. During equilibration the position restrain algorithm was used to restrain atoms of the peptides at the fixed reference positions, in order to preserve the initial α -helical structure. Production runs were then performed over 120 ns.

Isothermal isobaric (constant NpT) ensemble conditions were used ($T = 300$ K, $P = 1$ ATM, Parrinello–Rahman barostat, 0.1 ps time constant for the temperature, and 2.0 ps for the pressure) [40,41]. The compressibility was set at 4.5×10^{-5} bar. The leap-frog integrator time step was fixed at 2 fs. Electrostatic interactions were modelled using particle-mesh-Ewald method, and the van der Waals cutoff was set to 1.0 nm. Bonds were treated using the LINCS option [42]. After the molecular dynamics runs, the obtained secondary structures for both peptides were analysed with the DSSP program [43], and the fraction of residues adopting α - or π -helical conformation estimated (not including the C-terminal —NH₂ group). The % of α -helicity per residue as a function of time was calculated using the *gmx helix* gromacs utility [38].

2.11. Statistical analysis

Basic statistical parameters were obtained using descriptive statistics. Results were evaluated using the Statistica 12 program package (StaSoft, Tulsa, USA) and GraphPad Software (La Jolla, USA). The difference in the HPBLs viability between control and exposed samples was done by χ^2 -test, while for fibroblast's viability Student's *t*-test was used. In order to normalize the distribution and equalize the variances of the comet assay data, a logarithmic transformation was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post-hoc analyses of differences were done by the Scheffé test. Results were presented as means \pm SD (standard deviation of the mean), except for the comet assay data, which were presented as means \pm SE (standard error of the mean). Data were considered statistically significant at $P < 0.05$.

3. Results

3.1. Peptide design

Naturally occurring AMPs present a good template for the design of novel antimicrobial peptides, particularly if a relatively short length and improved antimicrobial potency and selectivity can be achieved. A search of the DADP database [15] identified PGLa-H, a PGLa fragment containing C-terminal residues corresponding to the sequence KIAKVALKAL-NH₂. It was reported to have negligible haemolytic activity while being moderately active against both Gram-negative and Gram-positive bacteria [19]. When synthesized and tested for bacteriostatic activity against *E. coli* and *S. aureus*, MIC values were however found to be higher than 64 μ M (data not shown).

The peptide size was likely insufficient for a useful antimicrobial activity under our conditions, but we speculated that a tandem repeat of the sequence should provide a more stable helical conformation, favouring interaction with bacterial membranes and therefore increasing activity (see Table 1). A previously developed and tested algorithm [14,21] suggested that diPGLa-H should retain a good selectivity for bacterial cells with respect to erythrocytes. To further improve selectivity, we reduced global hydrophobicity and increased the peptide's flexibility with a single substitution (Val¹⁵ \rightarrow Gly). Running this sequence through HeliQuest [44] suggested that this substitution improves the amphipathicity profile, so it should not decrease the antimicrobial activity. DiPGLa-H and this analogue, called kiadin, both have an increased charge (+7) with respect to PGLa-H, which should favour activity, and both have predicted SI indices with respect to erythrocytes close to the upper limit for the algorithm's output, which is 95, indicating low toxicity towards these circulating cells. Relative hydrophobic moments are also similar (see Table 1).

3.2. Peptide structure

The conformations of diPGLa-H and kiadin under different conditions were evaluated using CD spectroscopy: a) aqueous buffer (SPB); b) increasing proportions of TFE; c) SDS micelles (10 mM SDS in SPB);

d) anionic LUVs (PG:dPG 95:5 in SPB) (see Fig. 1). The spectra in aqueous buffer are typical of a random coil structure for both peptides, whereas show the typical shape of an α -helix in the presence of 50% TFE or SDS micelles. The shape of the spectra, and in particular the $\theta^{208}/\theta^{222}$ ratio > 1 indicates that they are lone helices under these conditions [46], with an estimated helix content of $\sim 60\%$ in both cases. Taking into account end effects for the three N-terminal and three C-terminal residues, this indicates that most of the peptides' sequence is involved in helix formation. This behaviour is typical of helical AMPs, and is consistent with the fact that both peptides are quite rich in alanine (30% for diPGLa-H, see Table 1), which is helix promoting. The similar helicity of diPGLa-H and kiadin indicates that the introduction of a flexible glycine residue at position 15 does not significantly affect helix stability in the presence of helix stabilizing conditions. This is also indicated by similar % helix content at increasing proportions of TFE (see insets to Fig. 1).

It is interesting to note that both peptides have a quite different spectrum in the presence of anionic LUVs. In this case $\theta^{208}/\theta^{222} < 1$, possibly indicates that peptides aggregate on the surface of LUVs. In this respect, kiadin shows a more marked effect than diPGLa-H. In any case these changes indicate a strong propensity of the peptides for interacting with anionic membranes, which affects their conformation.

3.3. Antimicrobial activity

The bacteriostatic (MIC) and bactericidal (MBC) activities of diPGLa-H and kiadin were determined against a Gram-positive bacterial species and a panel of Gram-negative ones, including reference ATCC strains and drug/resistant clinical isolates (see Table 2). With respect to PGLa-H (MIC > 64 μ M for reference ATCC *E. coli* and *S. aureus* strains, data not shown) the tandem repeat peptide showed a markedly improved activity. As reported for the parent peptide [19], diPGLa-H was active against Gram-positive *S. aureus*, and maintained a comparable activity against the ATCC strain and MRSA clinical isolate (see Table S1 in Supplementary material for antibiogram). Furthermore, the MBC values were similar to the MIC, indicating the peptides are bactericidal rather than bacteriostatic, as expected for membranolytic helical AMPs. Kiadin performed similarly.

DiPGLa-H and its kiadin analogue were tested against a panel of Gram-negative species, using both ATCC strains and clinical isolates. It was active against all four tested ATCC strains, with *P. aeruginosa* being the least susceptible, although with a respectable MIC of 6 μ M. In this case also, the MBC values were generally similar to the MIC, and both peptides maintained activity against drug resistant clinical isolates for *A. baumannii* and *P. aeruginosa*, whereas activity was somewhat decreased against c.i. of *E. coli* and *K. pneumoniae*. Kiadin had a comparable or slightly better activity than the parent peptide, so that the Val \rightarrow Gly substitution was acceptable in this respect. It is interesting to note that both peptides have antimicrobial activities that compare favourably to those of melittin, a longer and much more toxic helical peptide (see below).

3.4. Haemolytic activity

DiPGLa-H showed a limited haemolytic activity (see Fig. 2), with an estimated HC₅₀ value of 270 ± 30 μ M. Kiadin, as predicted, showed a slight increase in HC₅₀ to 340 ± 30 μ M. Considering the MIC value range the SI varies from about 20 to 450 (see Table 2), with values typically > 90 and > 115 respectively for diPGLa-H and kiadin, consistent with the calculated SI values of over 90 (see Table 1). In any case, considering that MIC values are generally < 12 μ M, the % haemolysis at or below this concentration is $< 5\%$. This compares with a reported HC₅₀ value of ~ 1 μ M for the helical peptide melittin under similar conditions, which is below its reported MIC values.

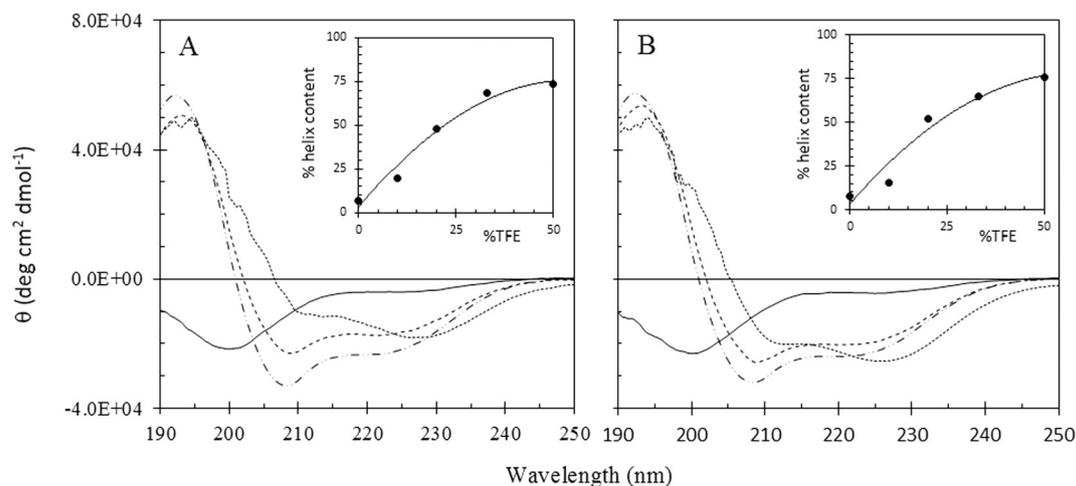


Fig. 1. CD spectra of diPGLa-H (A) and kiadin (B) under different conditions. Spectra are the accumulation of three scans carried out with 20 μM peptide in SPB (—), 10 mM SDS in SPB (---), anionic LUVs in SPB (PG:dPG 95:5, 0.4 mM phospholipid, (.....), 50% TFE (- · - · -). The insets show the % helix content at different proportions of TFE (calculated according to Chen [27], considering the θ value at 222 nm).

3.5. Cytogenotoxicity

After exposure of whole blood to peptides for 4 or 24 h the cytotoxicity towards HPBLs was assessed as being quite weak (see Fig. 3). No statistically significant effect was observed up to a concentration of 25 μM . After 24 h exposure to 100 μM peptide, viability was $88 \pm 5\%$ for both peptides, whereas a similar result was observed for 6 μM of the peptide antibiotic colistin (see Fig. 3). From these results, it is not possible to determine an IC_{50} , which is likely to be well above that of colistin, determined as 160 μM . These results are consistent with haemolysis experiments, and were considered to be in an acceptable range for conducting further genotoxic analysis [54]. Genotoxicity on HPBLs, as determined using the alkaline comet assay, showed no statistically significant difference in the amount of DNA strand breaks for both peptides, compared to the corresponding control samples, regardless of concentration and exposure time used, as shown in Fig. 3. In contrast with the literature [55] we did not find a significant genotoxicity for colistin either, under our conditions.

3.6. Viability of primary fibroblasts in culture

The cytotoxicity of both diPGLa-H and kiadin as well as for colistin on human fibroblasts was investigated using MTT assay. For all three peptides we observed a dose dependent cytotoxicity that was in this case less prominent after colistin treatment (see Fig. 4). The IC_{50} values were about 10 μM for diPGLa-H and kiadin and $>100 \mu\text{M}$ colistin, based on MTT results. Results thus showed differences in sensitivities to the three tested peptides with respect to different cell types and the observed effects were cell type-dependent. However, it should be noted that the haemolytic activity was assessed by spectrophotometric monitoring of haemoglobin release from erythrocytes, while cytotoxicity towards HPBLs was determined by differential staining with AO and EtBr, using fluorescent microscopy, which enables distinction between live and dead cells based on their appearance. On the other hand, the MTT assay used for determining cytotoxicity towards fibroblasts is based on measuring the activity of mitochondrial succinate dehydrogenase, thus reflecting the number of living cells. Another methodological

Table 2
MIC and MBC values (μM) and measured selectivity indices (SI) for peptides.

	DiPGLa-H			Kiadin			Melittin	
	MIC	MBC	SI	MIC	MBC	SI	MIC ^b	SI
<i>Gram-negatives</i>								
<i>E. coli</i> ATCC 25922	1.5	1.5	180 ± 20	0.75	0.75	450 ± 40	5–15	<1
<i>E. coli</i> c.i. ^a	6	12	45 ± 5	12	24	30 ± 5	3	<1
<i>K. pneumoniae</i> ATCC 13883	3	3	90 ± 10	3	3	115 ± 10	–	–
<i>K. pneumoniae</i> c.i. ^a	12	24	22.5 ± 2.5	12	24	30 ± 5	11	<1
<i>A. baumannii</i> ATCC 19606	1.5	1.5–3	180 ± 20	1.5	1.5	225 ± 20	–	–
<i>A. baumannii</i> c.i. ^a	1.5–3	3	135 ± 50	1.5	1.5	225 ± 20	6	<1
<i>P. aeruginosa</i> ATCC 27853	6	12	45 ± 5	6	12	60 ± 5	30	<1
<i>P. aeruginosa</i> c.i. ^a	6	12	45 ± 5	3	6	115 ± 10	>22	<1
<i>Gram-positive</i>								
<i>S. aureus</i> ATCC 29213	0.75	1.5	360 ± 40	0.5–1	1.5	450 ± 40	4	≤ 1
<i>S. aureus</i> c.i. (MRSA)	1.5	1.5	180 ± 20	3	3	115 ± 10	8	<1
Host cells	HC ₅₀	IC ₅₀	HC ₅₀	IC ₅₀	HC ₅₀ ^c	IC ₅₀ ^d		
RBC	270 ± 30	–	340 ± 30	–	0.50 ± 0.25	–		
HPBLs	–	>100	–	>100	–	2.9		

^a See Table S1 for ESBL of clinical isolates (c.i.).

^b From [47–51], conditions used were MH broth according to CLSI standards (42,45,46), unspecified (43) and LB medium (44).

^c From [47]; using 6% v/v final erythrocyte the HC₅₀ is estimated at 5 μM [52].

^d From [53].

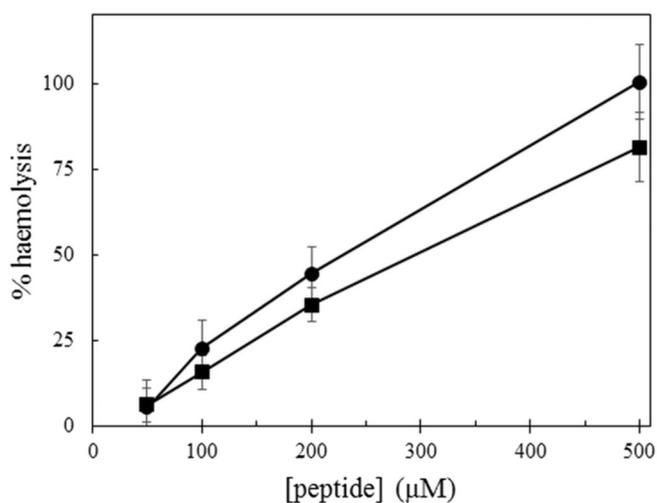


Fig. 2. Haemolytic assay on human erythrocytes. (●) *diPGLa-H*, (■) *kiadin*. Data are expressed as mean values \pm SD.

difference is available cytoplasmatic membrane surface, as presumed target for helical AMPs incubated with HPBLs or fibroblasts, which was >10 times higher for HPBLs due to their much higher number ($\sim 10^7$) in comparison with larger but fewer fibroblasts (2.4×10^4). It follows that surface concentration of designed peptides has been >10 times higher for fibroblasts.

3.7. Effect of *diPGLa-H* and *kiadin* on bacterial membrane permeability

Flow cytometry was used to monitor the capacity of the peptides to permeabilize the inner bacterial membrane of individual *E. coli* cells, as the fluorescent probe PI incorporates and stains nucleic acid only in cells with damaged membranes (see Fig. 5). Treatment with *diPGLa-H* caused high level of permeabilization ($>90\%$ PI+ cells) at concentrations corresponding to the MIC value (1–2 μM) irrespective of exposure time (15–60 min). At a sub-MIC concentration of 0.5 μM an appreciable permeabilization ($\sim 80\%$ PI+ cells) was observed already at 15 min, increasing to over 90% at 30 min. Treatment of *E. coli* ATCC 25922 with

kiadin had a similar effect. At sub-MIC concentration (0.5 μM) however it was somewhat lower ($\sim 40\%$ after 15 min, increasing to $\sim 80\%$ after 30 min, and $>90\%$ after 1 h) (see Fig. 4). These results are typical for membranolytic peptides, supporting a similar mode of action for both peptides.

3.8. MD simulations

An α -helical structure with strong amphiphilic character is a feature often correlated with AMP activity [26,56]. AMPs are generally cationic, which enables binding to the anionic head groups of bacterial phospholipids, while the hydrophobic sector that forms on adoption of the amphiphilic helical conformation allows insertion of the peptide into the hydrophobic core of the bilayer. MD simulations suggest a high content of helical conformation for *diPGLa-H* and *kiadin* embedded in a lipid bilayer, as well as for these peptides in 30% TFE aqueous solution. It is somewhat higher for *diPGLa-H* that also preserves a small amount of α -helical structuring in pure water, while *kiadin* loses it completely (see Fig. 6). These results are in substantial agreement with CD measurements (Section 3.2), which suggest helical contents of 60–70% for *diPGLa-H* and *kiadin* under these conditions (see inset to Fig. 1), while both peptides present a comparable helical content of $\sim 10\%$ in aqueous solution (SPB).

A more detailed analysis of the secondary structure as a function of time in MD runs shows unfolding of the helical structure in water for both peptides, but with a faster rate and extent for *kiadin* (see Fig. S1 in Supplementary material). In the presence of TFE (30% v/v), *diPGLa-H* retains the helical structure for all 120 ns of the run, with approximately 70% of residues in the α -helical conformation, in good agreement with CD spectra. In the case of *kiadin*, the conformation seems to interchange between an α -helix and a π -helix, with on average each being present 40% of the time during the 120 ns simulation. When embedded in the hydrophobic core of the phosphatidylcholine membrane, *kiadin* adopts a turn structure for amino acids 11–16 at the 15 ns simulation time, which remains stable until the end of the run retaining about 70% α -helical conformation. Under the same condition, *diPGLa-H* preserves a linear α -helical structure with only a small degree of unfolding at the C-terminus (residues 17–21) and an average of 75% α -helical conformation during the simulation run. These simulations also suggest that the π -helix conformation and/or formation of the

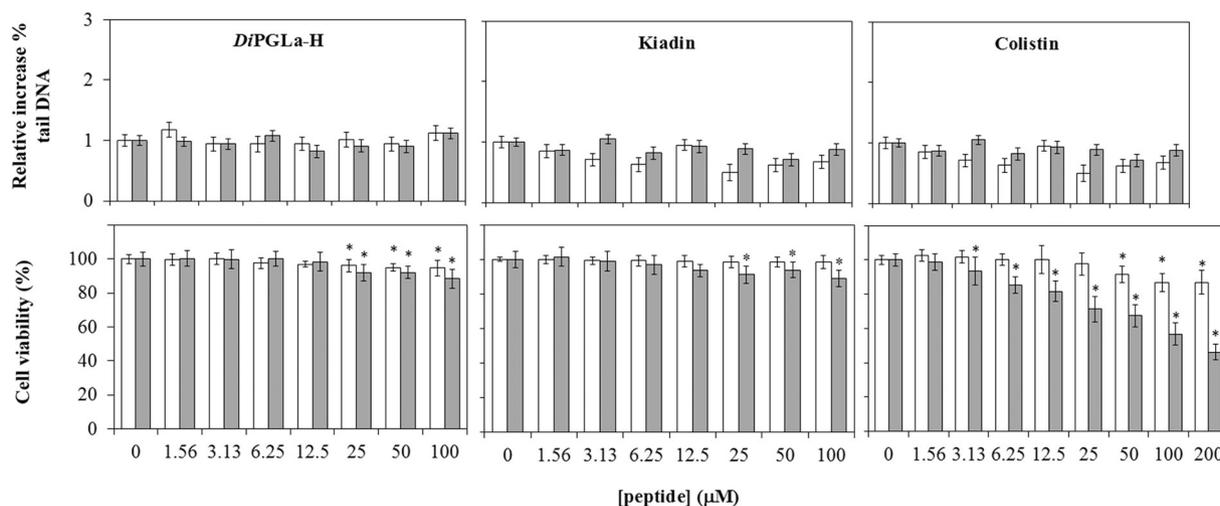


Fig. 3. The effect of *diPGLa-H*, *kiadin* and *colistin* on cell viability and induced DNA damage after exposure for 4 and 24 h. Viability of human peripheral blood leukocytes (HPBLs, bottom panels) was determined by differential staining with AO/EtBr after exposure to *diPGLa-H*, *kiadin* up to 100 μM , and *colistin* up to 200 μM , for 4 (white columns) and 24 h (grey columns), and is expressed as mean values \pm SD. DNA damage was assessed using the alkaline comet assay after exposure to peptides *diPGLa-H*, *kiadin* and *colistin* up to 100 μM (upper panels) for 4 and 24 h and is expressed as mean values of relative increase of % DNA in comet tail \pm SE. *Statistically significant compared to corresponding control ($P < 0.05$).

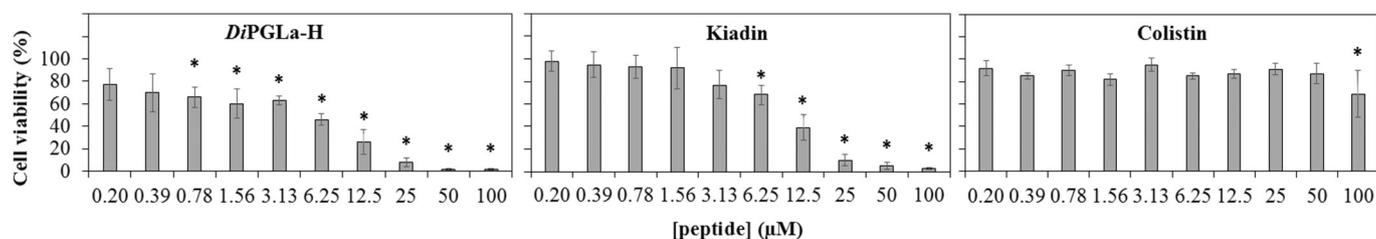


Fig. 4. The effect of *diPGLa-H*, kiadin and colistin on cell vitality as assessed by the MTT assay. Cells were plated in 96-well tissue culture plates and the viability of fibroblasts was determined by colorimetric MTT assay after exposure to *diPGLa-H*, kiadin and colistin up to 100 μM for 24 h. Data are expressed as mean values \pm SD. *Statistically significant compared to corresponding control ($P < 0.05$).

turn structure could contribute to the observed alteration of the CD spectrum in the presence of LUVs (increased intensity of the band at ~ 225 nm) [57], an effect that appears to be especially strong for kiadin.

4. Discussion and conclusions

We have made use of a database of anuran AMP sequences, as well as computational methods [15,21] to design AMPs with broad spectrum antibacterial activity, and high selectivity with respect to circulating blood cells. We have shown that by doubling the size of a small but poorly active peptide we could significantly increase its potency without affecting this selectivity, while our previous experience suggested that introducing a glycine residue at a key sequence positions could further improve it [13,22]. PGLa-H, the shortest PGLa-like natural peptide reported to have antimicrobial activity [19], in our hands was inactive, likely due to the 10-fold higher bacterial load used in our assay with respect to the original report. We speculated that a sequential tandem repeat, *diPGLa-H* would improve helix formation and bacterial membrane interaction, leading to membrane lysis and bacterial killing. These features are however often correlated to an increased cytotoxicity for host cells, and our design method was intended to minimize this.

Both *diPGLa-H* and the Gly substituted kiadin showed a potent antibacterial activity against one Gram-positive and four Gram-negative species. MIC values were between 1 and 12 μM and most often ≤ 3 μM , and the MBC values were close to the MIC, indicating a bactericidal activity. Furthermore, activity was in several cases comparable for standard laboratory strains and multidrug resistant clinical isolates. Exceptions were *E. coli* and *K. pneumoniae*, and it would be interesting to identify the underlying features of the bacterial membrane or mechanisms that result in this reduced susceptibility.

Our Mutator algorithm for peptide selectivity optimization [14] suggested only a small selectivity advantage could be achieved, with respect to erythrocytes, by substituting a valine with glycine in kiadin, but the SI value of *diPGLa-H*, at 93, was already very close to the maximum output of 95 for this algorithm. In any case, we did observe a decreased haemolysis for kiadin with respect to *diPGLa-H* (Table 2). Furthermore, *in vitro* cytotoxicity and genotoxicity assays carried out on human peripheral blood cells confirmed that both peptides were not toxic at concentrations significantly higher than the MIC. This was in contrast to the last resort antibiotic colistin and the bee venom peptide melittin, both likely acting by a membranolytic mechanism.

The higher toxicity shown by *diPGLa-H* and kiadin to human fibroblasts, as assessed using the MTT assay, suggests a possible differential effect with respect to non-circulating cells, but it may in part be methodological, due to the higher membrane surface density of these peptides acting on smaller number of large, adhered cells. In any case, it underlines the advisability of carrying out several different types of toxicity assessment in evaluating HDPs.

CD studies indicate that both our peptides undergo a transition from a mostly random coil structure in aqueous solution ($\sim 10\%$ helix content) to a substantially helical structure in anisotropic environments such as $>30\%$ TFE or SDS micelles. It was interesting to observe that while spectra had the characteristic shape of a lone α -helix in these environments [46], in the presence of a model anionic membrane (PG/dPG LUVs) the altered spectral shape suggested helix association may occur. Some peptides, like temporins A and B, and also human LL-37, are known to aggregate in bulk solution or in the external bacterial layers, and this can inhibit antibacterial activity as it restricts the peptides' capacity to reach cytoplasmic membrane [58–60]. Furthermore, peptide aggregation favours the formation of toroidal pores for human LL-37 [61,62]. Another possibility is that the conformation changes from that of a

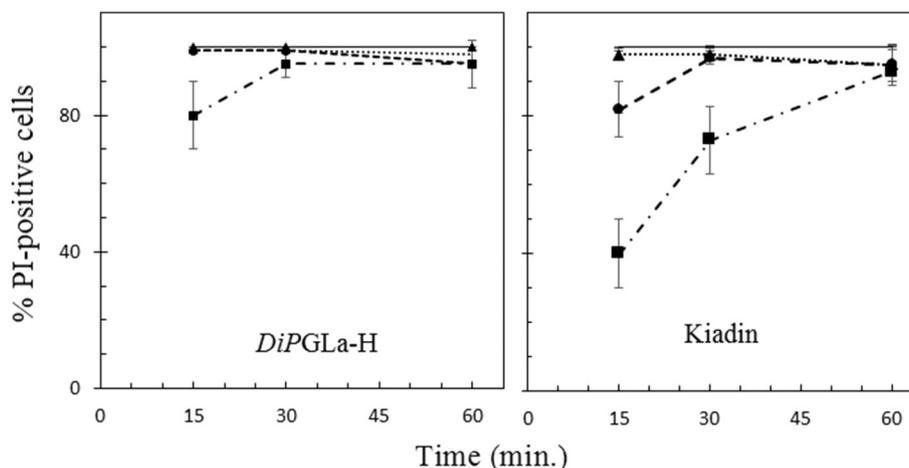


Fig. 5. Bacterial membrane integrity after treatment with *diPGLa-H* and kiadin. Percentage of fluorescent cells (PI positive) measured by flow cytometry after incubation of *E. coli* ATCC 25922 cells with 0.5 μM (---), 1 μM (-.-.-), and 2 μM (.....) *diPGLa-H* and kiadin is shown. Concentration of melittin was 5 μM (—). The background level of permeabilized cells, obtained using untreated samples, was always lower than 3% and was subtracted to the corresponding peptide-treated sample. Data are expressed as the average of % PI-positive cells \pm SD for three independent experiments.

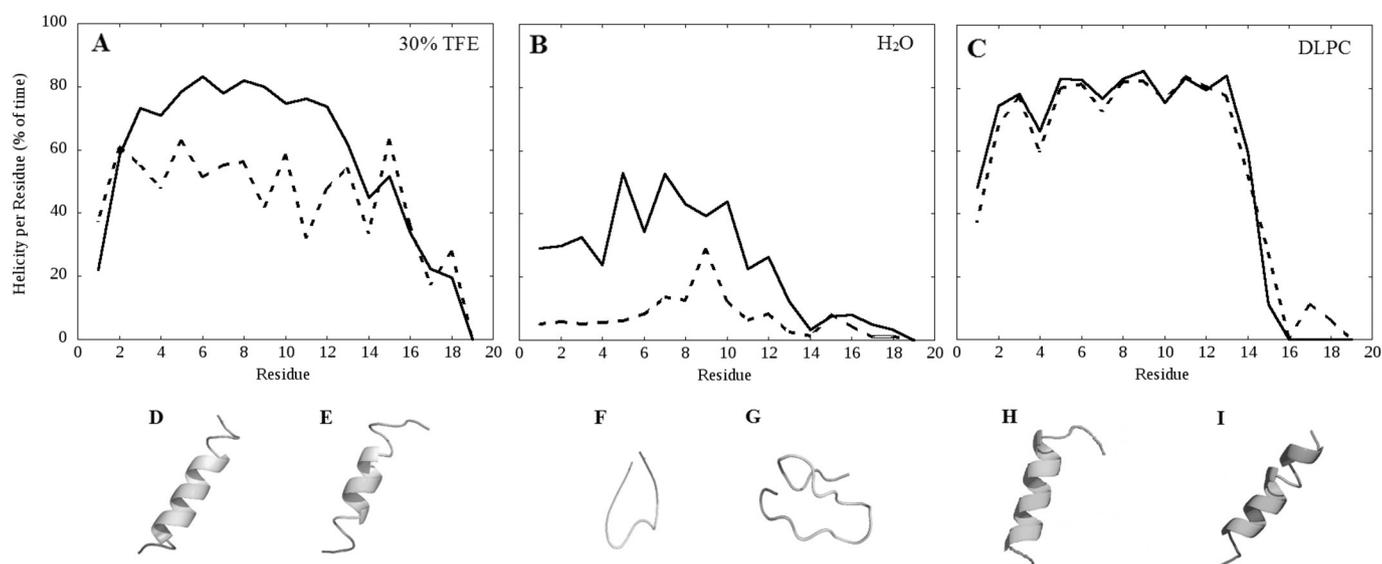


Fig. 6. Percent of MD simulation time for *diPGLa-H* (—) and *kiadin* (---) found in a helical conformation. A) 30% TFE in water; B) water only, C) DLPC. The structures at the bottom correspond to conformations of the peptides after 120 ns: D) *diPGLa-H* in 30% TFE; E) *kiadin* in 30% TFE; F) *diPGLa-H* in H₂O; G) *kiadin* in H₂O; H) *diPGLa-H* in lipid bilayer; I) *kiadin* in lipid bilayer.

canonical α -helix on membrane interaction. MD simulations carried out on both peptides agreed substantially with CD studies, with respect to the helix content in the presence of 30% TFE, but also suggested that *kiadin* could show a significant content in π -helix. This conformation has been indicated as having an increased negative ellipticity at around 225 nm [57], which might in part explain the shape of its CD spectrum in the presence of LUVs. NMR data suggest that both peptides appear to be α -helical but that *kiadin* appears less so and possibly more kinked (unpublished results).

MD simulations in different environments indicate that *kiadin* has a greater flexibility than *diPGLa-H*, and can access more types of conformation (π -helix and turns), suggesting it is more flexible. Conformational flexibility in α -helical, amphipathic and positively charged peptides has been reported to improve peptide activity and selectivity towards bacterial cells [63,64], which was also observed for *kiadin* vs *diPGLa-H*, but more extensive mode-of-action and simulation studies need to be carried out, to assess its significance in this case.

While there are many peptide antibiotics that are effective *in vitro* against both Gram-positive and Gram-negative bacteria, including multi-drug resistant isolates [7,65–67], *diPGLa-H* and *kiadin* are interesting as possible lead compounds due to their reduced cytotoxicity for circulating blood cells *in vitro*, especially in comparison to the last resort antibiotic colistin. Therefore, further analysis to determine their suitability and *in vivo* studies on animal models are warranted. Furthermore, it was important to confirm the effectiveness of design methods and algorithms that can significantly reduce the synthetic effort required to obtain such useful lead compounds.

Author contributions

Conceptualization, DJ; methodology, TR, GG, NI, JS, MB; investigations, TR, GG, IG-B, MT; simulations, LZ, MM; writing original draft and editing DJ, AT, GG, TR, IG-B, MT, LZ; analysis, DJ, AT; supervision and funding, DJ, LZ, AT.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbmem.2016.11.011>.

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