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SHORT COMMUNICATION

Comparison of the Effects of Hydrophobicity, Amphiphilicity, and α-Helicity on the Activities of Antimicrobial Peptides

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ABSTRACT **Multiple linear regression was used to quantify the dependence of the antimicrobial activity of 13 peptides upon three calculated or experimentally determined parameters: mean hydrophobicity, mean hydrophobic moment, and α-helix content. Mean hydrophobic moment is a measure of the amphiphilicity of peptides in an α-helical conformation. Antimicrobial activity was quantified as the reciprocal of the measured minimal inhibitory concentration (*MIC*) against *Escherichia coli*. One of the peptides was magainin 2, and the remainder were novel peptides designed for this study. The multiple linear regression results revealed that the amphiphilicity of the peptides was the most important factor governing antimicrobial activity compared to mean hydrophobicity or α-helix content. A better regression of the data was obtained using ln(1/*MIC* + *constant*) as the dependent variable than with either 1/*MIC* or ln(1/*MIC*). These results should be useful in designing peptides with higher antimicrobial activity.**

Key words: hydrophobic moment, peptide–cell membrane interactions

INTRODUCTION

Many antimicrobial peptides have an amphiphilic character when their secondary structure is α-helical. For example magainin,[1] cecropin,[2] sarcotoxin,[3] andropin,[4] and bombinin-like peptides[5] are amphiphilic when arranged in an α-helix. It is generally believed that the amphiphilic α-helical structure of these peptides leads to the formation of ion channels across the cell membrane. For instance, magainin 2 has been demonstrated to form ion channels in artificial lipid bilayer membranes.[6]

Previous studies of amphiphilic α-helical antimicrobial peptides have demonstrated the importance of the degree of amphiphilicity,[7–9] α-helicity,[10] and hydrophobicity,[9,11] to antimicrobial activity. These studies did not, however, attempt to quantitatively compare the effect of these three parameters on antimicrobial activity. In the present study, such a comparison is made based on experimental and calculated data for 13 antimicrobial peptides which include magainin 2. We demonstrate that the mean hydrophobic moment, a measure of amphiphilicity, has far more influence on antimicrobial activity than α-helicity or mean hydrophobicity.

MATERIALS AND METHODS

Design of Peptides

The sequence of the complete set of peptides in this study is shown in Figure 1. Schiffer-Edmundson helical wheel diagrams[12] are shown in Figure 2 for two of the peptides (A2 and M1, selected because these had the lowest and highest antimicrobial activity, respectively; see Results and Discussion). The antisense peptide was derived from the cDNA sequence of magainin 2 by reading the noncoding strand in the same reading frame as the magainin 2 gene.[1] The antisense peptide tends to have hydrophobic amino acids at positions corresponding to polar amino acids in magainin 2, and vice versa. Stop codons derived from serine sense codons are encoded at positions 1 and 16 of the antisense peptide. We chose to replace the stop codons with one of the three alternative serine antisense codons so that we could make an antisense peptide that was the same length as magainin 2. The A, B, and C analogs were made by replacing cysteine at position 15 by alanine or lysine, the glutamine at position 18 by glycine, and the tyrosine at position 4 by lysine. Cysteine was replaced by alanine to overcome a reduction in activity of the antisense peptide due to the oxidation of this residue (see the antimicrobial activity results). The replacement of glutamine with glycine was

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Magainin 2	G	I	G	K	F	L	H	S	A	K	K	F	G	K	A	F	V	G	E	I	M	N	S
Antisense	G	I	H	Y	L	S	H	K	S	F	S	K	F	F	C	G	V	Q	K	F	T	N	S
A1	G	I	H	Y	L	S	H	K	S	F	S	K	F	F	A	G	V	Q	K	F	T	N	S
A2	G	I	H	Y	L	S	H	K	S	F	S	K	F	F	A	G	V	G	K	F	T	N	S
B1	G	I	H	Y	L	S	H	K	S	F	S	K	F	F	K	G	V	Q	K	F	T	N	S
B2	G	I	H	Y	L	S	H	K	S	F	S	K	F	F	K	G	V	G	K	F	T	N	S
C1	G	I	H	K	L	S	H	K	S	F	S	K	F	F	K	G	V	Q	K	F	T	N	S
C2	G	I	H	K	L	S	H	K	S	F	S	K	F	F	K	G	V	G	K	F	T	N	S
P1	A	I	H	N	F	A	H	K	S	F	A	K	F	F	R	A	V	K	K	F	A	N	A
P2	A	I	H	N	L	A	H	K	S	L	A	K	L	L	R	A	V	K	K	L	A	N	A
P3	G	I	H	N	F	A	H	K	S	F	A	K	F	F	R	A	V	K	K	F	A	N	S
M1	A	I	H	N	L	A	H	K	L	L	K	K	L	L	R	A	V	K	K	L	A	N	A
M2	K	I	H	K	L	A	H	K	L	L	K	K	L	L	K	A	V	K	K	L	A	K	A

Fig. 1. Amino acid sequences of the peptides studied. All of the peptides are oriented so that residue 1 represents the amino terminus.

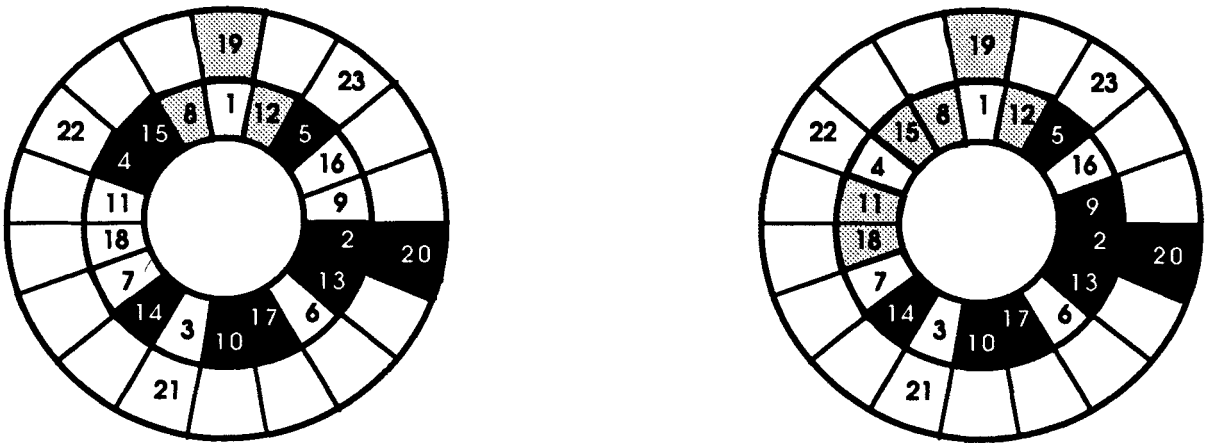

Fig. 2. Schiffer–Edmundson helical wheel diagrams for two peptides A2 and M1. The distribution of positively charged lysines (light gray), weakly hydrophilic or weakly hydrophobic amino acids (white), and other hydrophobic amino acids (black) are as they would appear if the peptides were completely α-helical. Amino acids are numbered starting from the amino terminus (see Fig. 1).

made to study the effect of α-helicity on antimicrobial activity (glutamine has more of a tendency to be in an α-helix than glycine[13]). The substitution of lysine for tyrosine-4 was done to further expand the putative hydrophilic face and to increase the mean hydrophobic moment (see Fig. 2).

Peptides P1, P2, and P3 were designed by generating additional antisense peptides to magainin 2 based on the degeneracy of the genetic code for the magainin 2 cDNA. The selection of possible codons was based on increasing the α-helical content (according to the Chou–Fasman parameters[13]) and the mean hydrophobic moment (see equation under Calculations) of the peptides. All the phenylalanines in P1 were replaced by leucines to obtain P2. This was done to lower the hydrophobicity. The end groups in P1 were changed to those in magainin 2 to obtain P3. Analogs M1 and M2 were designed on the basis of analog P2 to further increase the α-helix content and mean hydrophobic moment.

TABLE I. Peptide Data Used in the Multiple Linear Regression Analyses*

Peptide	*H*	*M*	α	*MIC* (μg/ml)	1/*MIC* (ml/μg)
Magainin 2	0.173	0.455	0.22	20	0.0500
Antisense peptide	0.090	0.367	0.27	60†	0.0167†
Analog A1	0.105	0.352	0.42	100	0.0100
Analog A2	0.160	0.325	0.21	400	0.0025
Analog B1	0.013	0.444	0.44	40	0.0250
Analog B2	0.070	0.417	0.30	40	0.0250
Analog C1	−0.060	0.518	0.35	20	0.0500
Analog C2	−0.006	0.487	0.38	20	0.0500
Analog P1	0.046	0.560	0.56	10	0.1000
Analog P2	0.018	0.546	0.57	5	0.2000
Analog P3	0.005	0.569	0.49	5	0.2000
Analog M1	−0.020	0.660	0.61	2.5	0.4000
Analog M2	−0.130	0.736	0.84	3	0.3333

*H is mean hydrophobicity, M is mean hydrophobic moment, α is α-helix fraction, and *MIC* is minimal inhibitory concentration.
†Dithiothreitol added at 1 mM. The antisense peptide lost activity when dithiothreitol was not added.

TABLE II. Multiple Linear Regression for Three Forms of the Dependent Variable with *H*, *M*, and α as Independent Variables*

Form of dependent variable	r^2
1/*MIC*	0.839
ln(1/*MIC*)	0.884
ln(1/*MIC* + 0.015)	0.931

*The square of the multiple-correlation coefficient, r^2, is the fraction of the total variance of the dependent variable which is contributed by its regression upon the independent variables.[17]

TABLE III. Multiple Linear Regression of ln (1/*MIC* + 0.015) With Different Combinations of Independent Variables

Independent variables	r^2	Standard error of estimate of ln(1/*MIC* + 0.015)
H	0.460	0.786
M	0.909	0.322
α	0.665	0.618
H, *M*	0.931	0.296
M, α	0.910	0.336
H, α	0.671	0.643
H, *M*, α	0.931	0.311

Calculations

The mean hydrophobicity of each peptide was calculated by averaging the hydrophobicity of each amino acid using the hydrophobicity consensus scale of Eisenberg et al.[14] The mean hydrophobic moment of each peptide containing N residues was calculated using the following formula of Eisenberg et al.[15]:

$$M = \frac{\left[\left(\sum_{n=1}^{N} H_n \sin(\delta n)\right)^2 + \left(\sum_{n=1}^{N} H_n \cos(\delta n)\right)^2\right]^{1/2}}{N}$$

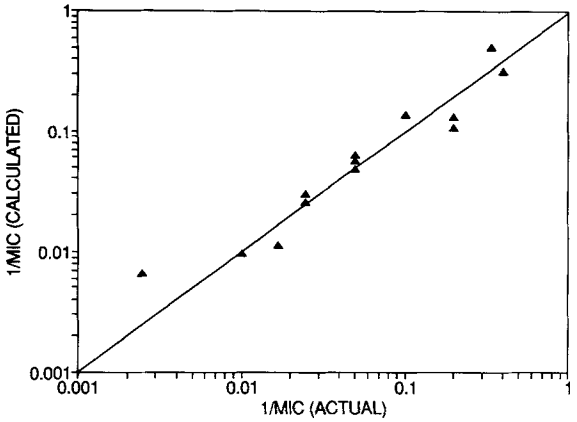

Fig. 3. Log–log plot of calculated versus actual results of 1/*MIC* for the 13 peptides using three independent variables in the multiple linear regression with ln(1/*MIC* + 0.015) as the dependent variable.

in which H_n is the hydrophobicity of the nth residue, and δ is 100°. In making this calculation it is assumed that entire peptide is in an α-helix. Multiple linear regression was performed using Quatro Pro software.

Synthesis of Peptides

The peptides were synthesized, purified, and analyzed for purity by the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center. Solid phase synthesis was done using Fmoc chemistry. Purification was carried out by HPLC on a C_{18} column. The amino acid composition of each peptide was verified by complete hydrolysis followed by cation exchange chromatography.

Determination of Antimicrobial Activity

Solutions of peptides were prepared from the pure powder in deionized water, filter sterilized (0.45 μm pore size), and stored at −20°C. Solutions of the an-

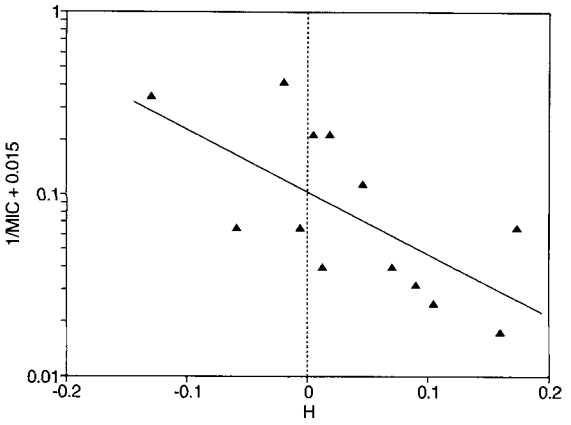

Fig. 4. Influence of *H*, mean hydrophobicity, on ln(1/*MIC* + 0.015). The linear regression line is shown.

tisense peptide of magainin 2 and dithiothreitol were prepared just before use. To prepare the microbial culture, 6 ml of TSB (tryptic soy broth, Difco Laboratories) was inoculated with *Escherichia coli* D31. When the culture reached an OD_{600nm} close to 0.6, 1 ml of sterile glycerol was added in the tube. The stock culture was stored at −20°C in aliquots of 1 ml. The colony-forming units (CFU)/ml of each stock solution was determined by plating on LB (Luria broth) agar (10 g Tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar per liter). The in vitro antimicrobial activities of the peptides were tested by the microdilution technique[16] with TSB used as growth medium. In each well of the plate (96-well tissue culture plate), 100 μl of 2-fold concentrated peptide was added to 100 μl of medium containing 10^5 CFU/ml (prepared with the stock solution of microorganism). The plate was incubated 18 h at 37°C. The minimal inhibitory concentration (*MIC*) was the minimal concentration of peptide for which no turbidity could be seen after 18 h.

Determination of Secondary Structure

Secondary structures of the peptides were determined using CD spectroscopy. The CD spectra were recorded on an AVIV circular dichroism spectrophotometer (Model 62 DS) using a quartz cell of 10 mm path length over the wave length range 190–250 nm. Solutions were prepared by mixing 25 mM potassium phosphate buffer at pH 7.0 and trifluoroethanol in a 60:40 ratio. The concentration of the peptides in their final solutions was 30 μg/ml. The spectra were analyzed by PROSEC software to estimate the secondary structure.

RESULTS AND DISCUSSION

Data for mean hydrophobicity (*H*), mean hydrophobic moment (*M*), α-helix content (α), and minimal inhibitory concentration (*MIC*) against *E. coli* are given in Table I for 13 peptides. Mean hydrophobic moment measures the amphiphilicity of the peptide in an α-helix.[15] The CD measurements were made in 40% trifluoroethanol in water to induce α-helix formation; thus, the results for α-helix content do not represent the secondary structure of the peptide in the membrane but instead indicate the *relative* tendency to form an α-helix. Antimicrobial activity is quantified as 1/*MIC* so that higher activity correlates with higher 1/*MIC*.

Multiple linear regression was performed with several functions of 1/*MIC* as the dependent variable and with one, two, or three independent variables—mean hydrophobicity, mean hydrophobic moment, and α-helix content. The dependent variable forms that were tested were the following: 1/*MIC*, ln(1/*MIC*), and ln(1/*MIC* + *constant*). Values of the square of the multiple-correlation coefficient (r^2) for the three dependent variable forms are shown in Table II. The constant 0.015 was found by trial and error to be the constant that gave the highest value of r^2. From the results in Table II, it is clear that the form ln (1/MIC + 0.015) gives the best fit of the data. The regression equation with this form is as follows:

$$\ln(1/MIC + 0.015) = 3.023H + 9.551M + 0.201\alpha - 7.462$$

A plot of calculated versus actual 1/*MIC* values for this regression is given in Figure 3 (shown as a log–log plot to spread out the data at lower values of 1/*MIC*). When each independent variable is normalized by dividing by the maximum value observed for that variable (asterisks denote normalized values), the following regression equation results:

$$\ln(1/MIC + 0.015) = 0.523H^* + 7.030M^* + 0.169\alpha^* - 7.462$$

This normalized equation indicates that the mean hydrophobic moment has much more influence on antimicrobial activity than either mean hydrophobicity or α-helix content. Since the equation is normalized, the effect of a given ΔM^* can be compared to the effect of the same ΔH^* or $\Delta\alpha^*$. Thus, for $\Delta M^* = \Delta H^* = \Delta\alpha^*$, 1/*MIC* is much more affected by ΔM^* than by ΔH^* or $\Delta\alpha^*$.

Selected statistics calculated for the regressions with one, two, or three independent variables are shown in Table III. The highest value of the square of the multiple-correlation coefficient (r^2) was obtained with all three independent variables or with H and M as independent variables. Thus, based on the value of r^2 for the regression with three variables, 93% of the variance of ln(1/*MIC* + 0.015) is contributed from these three variables. Except for the regression with *H* as the only independent variable, we can reject the null hypothesis that $r = 0$ at the 1% level of significance.[17]

The standard error of the estimate of ln(1/*MIC* + 0.015) is the lowest or very close to the lowest for all the regression cases when M is present as an inde-

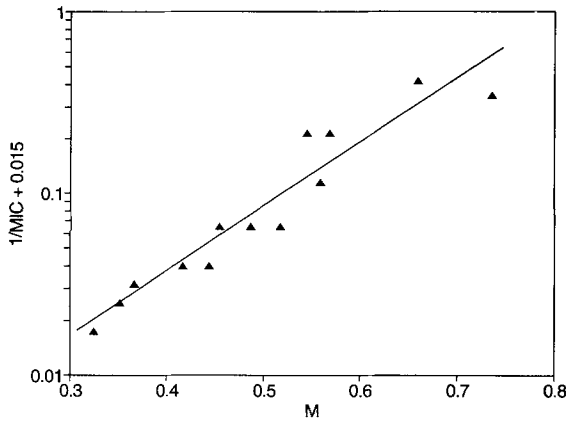

Fig. 5. Influence of *M*, mean hydrophobic moment, on ln(1/*MIC* + 0.015). The linear regression line is shown.

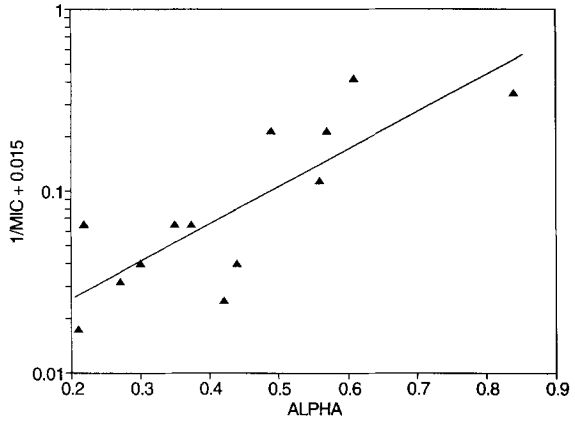

Fig. 6. Influence of alpha (α), fraction of α-helix, on ln(1/*MIC* + 0.015). The linear regression line is shown.

pendent variable (Table III). This again indicates the predominance of the effect the mean hydrophobic moment.

The importance of the mean hydrophobic moment is illustrated graphically in plots of ln(1/*MIC* + 0.015) versus *H*, *M*, and α (Figs. 4, 5, and 6). Experimentally determined 1/*MIC*s were used in these plots. The deviation of ln (1/*MIC* + 0.015) from the linear regression line is generally much less when plotted against *M* compared to when plotted against *H* or α.

These results imply that the amphiphilicity of the peptide in an idealized α-helix is the most important factor governing antimicrobial activity. This further implies that the membrane can induce the formation of an α-helix even when α-helix formation is not as favorable for the amino acids contained in the peptide. These results should be helpful in designing peptides with even higher antimicrobial activity.

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REFERENCES

1. Zasloff, M. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequences of a precursor. Proc. Natl. Acad. Sci. USA 84:5449–5453, 1987.
2. Boman, H.G., Hultmark, D. Cell-free immunity in insects. Annu. Rev. Microbiol. 41:103–126, 1987.
3. Okada, M., Natori, S. Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae. J. Biol. Chem. 260: 7174–7177, 1985.
4. Samakovlis, C., Kylsten, P., Kimbrell, D.A., Engstrom, A., Hultmark, D. The andropin gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaster*. EMBO J. 10:163–169, 1991.
5. Gibson, B.W., Tang, D., Mandrell, R., Kelly, M., Spindel, E.R. Bombinin-like peptides with antimicrobial activity from skin secretions of the Asian toad, *Bombinia orientalis*. J. Biol. Chem. 266:23103–23111, 1991.
6. Cruciani, R.A., Barker, J.L., Durell, S.R., Raghunathan, G., Guy, H.R., Zasloff, M., Stanley, E.F. Magainin 2, a natural antibiotic from frog skin, forms ion channels in lipid bilayer membranes. Eur. J. Pharmacol. 226:287–296, 1992.
7. Lee, S., Mihara, H., Aoyagi, H., Kato, T., Izumiya, N., Yamasaki, N. Relationship between antimicrobial activity and amphiphilic property of basic model peptides. Biochem. Biophys. Acta 862:211–219, 1986.
8. Blondelle, A.E., Houghten, R.A. Design of model amphipathic peptides having potent antimicrobial activities. Biochemistry 31:12688–12694, 1992.
9. Bessalle, R., Gorea, A., Shalit, I., Metzger, J.W., Dass, C., Desiderio D.M., Fridkin, M. Structure-function studies of amphiphilic antibacterial peptides. J. Med. Chem. 36: 1203–1209, 1992.
10. Chen, H.C., Brown, J.H., Morell, J.L., Huang, C.M. Synthetic magainin analogues with improved antimicrobial activity. FEBS Lett. 236:462–466, 1988.
11. Ando, N., Ochiai, J., Hemmi, J., Numao, N. Synthesis and antibacterial activity of magainin 2 analogs. Peptide Chem. 27:209–214, 1990.
12. Schiffer, M., Edmundson, A.B. Use of helical wheel to represent the structures of proteins and to identify segments with helical potential. Biophys. J. 7:121–135, 1967.
13. Chou, Y.P., Fasman, G.D. Empirical predictions of protein conformation. Annu. Rev. Biochem. 47:251–276, 1978.
14. Eisenberg, D., Weiss, R.M., Terwilliger, T.C., Wilcox, W. Hydrophobic moments and protein structure. Faraday Symp. Chem. Soc. 17:109–120, 1982.
15. Eisenberg, D., Schwarz, E., Komaromy, M., Wall, R. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. 179:125–142, 1984.
16. Jones, R.N., Barry, A.L., Gavan, T.L., Washington, J.A. In: "Manual of Clinical Microbiology." Lennette, E.H., Balows, A., Hausler, W.J., Shadomy, H.J., (eds.), 4th ed. Washington, D.C.: American Society for Microbiology, 1985: 972–977.
17. Neville, A.M., Kennedy, J.B. "Basic Statistical Methods for Engineers and Scientists." Scranton, PA: International Textbook Co., 1964: 215,314.