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Study of Structure-activity Relationship in Aurein 1.2 Analogs

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Abstract: Two new analogs of aurein 1.2 antimicrobial peptide were synthesized and the antimicrobial activities were investigated. The results showed that the activity of G1R/F3W analog was higher than the native peptide and the F3W analog. Circular dichroism studies also showed that the secondary structure of the F3W was concentration-dependent, whereas, there was no such relationship seen in the case of G1R/F3W analog. It has been proposed that G1R/F3W activity was based on a single mechanism (snorkeling), while Aurein 1.2 and F3W utilized the snorkeling mechanism at low concentrations (0-0.01 mM) and the carpet mechanism at higher concentrations (0.01-0.1 mM). This study suggests that one pay attention to the concentration of biomolecules in peptide-based drug design.

Key words: Antimicrobial peptide, Aurein 1.2, carpet model, snorkeling model, two-stage mechanism

INTRODUCTION

Antimicrobial peptides are a class of molecules characterized by their antibacterial effects. It is believed that the mechanism of their action is related to the destruction of the cell membrane (Zasloff, 2002; Devine and Hancock, 2002). This group of peptides has great importance because of several practical considerations such as rapid killing of bacteria and the wide-spectrum of their activities (Alexandra and Marr, 2006; Ganz and Lehrer, 1999).

Aurein 1.2 is a 13-residue antimicrobial peptide with a high propensity of the α-helix formation in 2,2,2-trifluoroethanol (TFE) (Rozek *et al*., 2000a, b). The mechanism of its action is not yet fully understood. However, it is known that it interacts with phospholipids (Bowie *et al*., 2003). Different models have been proposed to describe the action mechanism of aurein 1.2, such as carpet model (Ernesto *et al*., 2005). detergent-like model (Separovic, 2004) and snorkelling model (Sarah *et al*., 2007).

It is clearly known that the peptide hydrophobicity (White, 1998) and the presence of positively charged residues are the main factors in its antimicrobial activity (Dathe *et al*., 2001).

Several studies indicate that Trp may be an important determinant of antimicrobial activity (Hans *et al*., 2002; Jordan *et al*., 2005). Trp and Phe residues are generally listed in textbooks as hydrophobic amino acids. But, in contrast with the side chain of Phe, Trp has a sizeable dipole and quadrupole moment as well as hydrogen-bonding potential. In antimicrobial peptides, substitution of Trp by Phe (W replaced with F) led to a reduction in their antimicrobial activity (Subalakshmi *et al*., 2000). It is believed that Phe 3 and Phe 13 are important for the interaction of aurein 1.2 with the hydrophobic regions of the membrane lipids (Wang *et al*., 2005). Furthermore, these aromatic residues have been known to act as membrane anchors (Rozek *et al*., 2000b).

Another factor that affects antibacterial activity has been the presence of positively charged residues (Dathe *et al*., 2001). Recently, arginine substitution in the amphipathic peptides and several of their analogs, were found to have no significant effect upon the peptide conformation but led to increased antimicrobial activity (Zasloff, 2002). Moreover, these studies suggested that differences between the positions of positive charge on arginine side-chains may be linked to the membrane lipid selectivity and thereby to differences in the antibacterial action of amphipathic peptides (Zasloff, 2002).

It was therefore decided to synthesize certain analogs of aurein 1.2 to clarify its structure-activity relationship. Thus in the present study first, Phe 3 of aurein 1.2 was replaced by Trp (designated as F3W) and second analog designated as G1R/F3W wherein Gly residue at position 1 was substituted by an Arg residue. In this study, Aurein 1.2 and its analogs possessed free C-termini.

MATERIALS AND METHODS

Protected amino acids, resins and all other synthesis reagents were obtained from Bachem (Germany). All other

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chemicals were of analytical or reagent grade (Merck, GmbH, Darmstadt, Germany). Peptides were synthesized by solid phase peptide synthesis according to Fmoc (9-fluorenylmethoxycarbonyl) methodology (Fields *et al*., 1992; Furka *et al*., 1991). The peptides were assembled manually, using a fitted glass reaction vessel containing 2-chlorotrityl chloride resin. Amino acid couplings were accomplished using O-Benzotriazol-1-yl-N, N, N', N'-tetramethyluronium Tetrafluoroborate (TBTU), N-ethyl diisopropylamine and Fmoc deprotection was performed using 20% piperidine in dimethylformamide. Completion of coupling was monitored carefully by the Kaiser test. The final peptides were cleaved from the resin with trifluoroacetic acid (98% TFA).

The peptides were purified via high-performance liquid chromatography (HPLC) (Pharmacia, Sweden) on a Vydac C18 reverse-phase column using a water-acetonitrile gradient containing ~0.1% TFA, Then lyophilized in freeze dryer. These peptides were pure 95% as verified by HPLC on analytical Vydac C 18 reverse-phase (RP) column. Purified samples were assayed by several methods: amino acid analysis (Amino Tech, Germany) (Smith, 1997). HPLC (Carr, 2002) and electrospray mass spectrometry (Kaltashov and Eyles, 2005) (done at Mass Spectrometry unit, Glasgow University, UK).

The antimicrobial activity of the peptides assessed in terms of Minimum Inhibitory Concentration (MIC) using the standard microdilution susceptibility test. The small culture of *Listeria monocytogenes* (PTCC1163), *Leuconostoc mesenteroides* (PTCC1059) and *S. epidermidis* (PTCC1114) grew overnight. A fresh culture medium was inoculated with a small aliquot of the overnight culture and incubated at 37°C until the culture reached to its logarithmic stage (A600~ 0.5, 9×10^8 cells mL^{-1}). The culture diluted to A600 ~ 0.001, 10^6 cells mL^{-1} and dispensed into a 96-well plate, introducing ~10^5 cells per well (90 μL each). Then 10 μL aliquots of the peptide at different concentrations (three assays for each) added to the cultures, allowing the minimum inhibition concentration (MIC) to be measured. The plate then further incubated overnight at 37°C. A_{620} values have read using an Ultra Micro plate Reader (Hancock, 1997).

CD spectra recorded on a JASCO J-715 spectropolarimeter; model J-715(Japan) equipped with a temperature controller using quartz cells (1 mM, 10 mM). The spectra recorded at 25°C in the ranges 190-250 nm at a scan rate of 60 nm^{-1}. Peptide solutions prepared by dissolving lyophilized peptide in deionized water. The concentrations of the peptide in aqueous solutions were in the range 0.006 to 0.1 mM.

Three scans for each sample performed and the sizeable noise in the data smoothed using the JASCOJ-715 software, including the fast Fourier-transform noise reduction routine, which allows the decrement of most noisy spectra without distorting their peak shapes. Ellipticity values obtained in millidegrees directly from the instrument and converted to the molecular ellipticity, $[\theta]_{MRW}$, expressed in $deg.cm^2.dmol^{-1}$ (Woody, 1992). Peptide concentrations measured by Waddell's approach (Waddell, 1956).

RESULTS AND DISCUSSION

The Table 1 shows the MIC values of 10, 20 and 22 μg mL^{-1} for G1R/F3W, F3W analogs and aurein 1.2, respectively against Leuconostoc mesenteroides.

The reverse-phase HPLC retention time of the G1R/F3W analog (58 min) is shorter than that of F3W analog (70 min) (Table 1). Houghten and DeGraw (1987) have proposed that C18 column in HPLC could be considered as a model for the lipid membrane behavior. Therefore, the shorter retention time of G1R/F3W analog indicates that this peptide is more hydrophilic than F3W analog due to its Arg residue. Antimicrobial assays results of the F3W analog are shown in Fig. 1. As is illustrated in the first step of the antimicrobial assay, the increase in the concentration of F3W analog and Aurein 1.2 leads to a dramatic decrease in the observed O.D. values (Fig. 1a). However, this trend significantly differs in the second step and the peptide antimicrobial activity decreases. Thereafter, in the third step, the antibacterial effect of the peptide is resumed. It is already known that there is a good correlation between the structural changes and the functional properties of peptides. Antibacterial activity of G1R/F3W analog is routine (Fig. 1b). It shows the increase in the concentration of G1R/F3W analog leads to a dramatic decrease in the observed O.D. values and graph is exponential.

Table 1: Mic, Helix percentage and retention time values shows against leuconistoc mesenterides

Sequence	Name	[a] $MIC_{(1059)}$	$MIC_{(1114)}$	$MIC_{(1163)}$	[b] % Helix	[c] RT(17°C)	[d] RT(25°C)	[e] RT(28°C)
GLWDIIKKIAESF	F3W	20	25	22	80	90	60	70
RLWDIIKKIAESF	G1R/F3W	10	12	10	57	60	59	58
GLFDIIKKIAESF	Aurein 1.2	22	25	25	80	78	55	60

[a]MIC is the lowest concentration of peptide that reduces growth by more than 50% (μg mL^{-1}), [b]Helix percentage calculated from the CD spectra, [C]Retention time on a reverse phase HPLC column of HPLC as a measure hydrophobicity at 17°C (38, 39), [d]Retention time on a reverse phase HPLC column of HPLC at 25°C, [e]Retention time on a reverse phase HPLC column of HPLC at 28°C

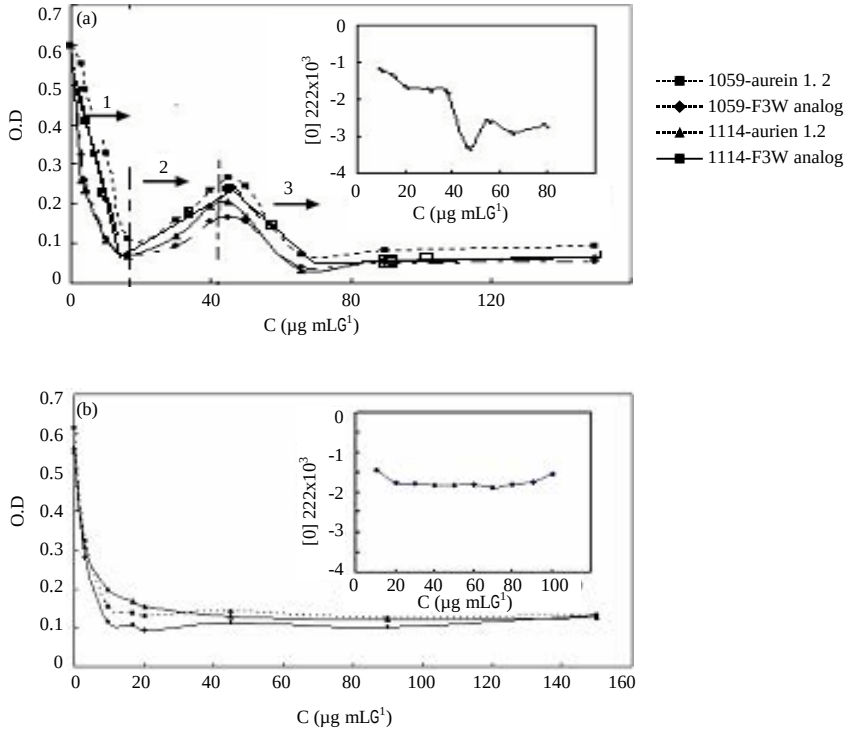

Fig. 1 (a-b): antimicrobial 1163, 1045, 1114 assay: 1a) an increase in the aurein 1.2 and F3W peptide concentration led to a dramatic decrease of O.D. values in the first phase. However, this pattern significantly differs in the second phase wherein the inhibitory effect of the peptide is partially removed. The dominant trend in the third phase follows a decrease in order, reaching a plateau after 60 µg mL^{-1}, 1b) an increase in the G1R/F3W peptide concentration led to a decrease of O.D values.

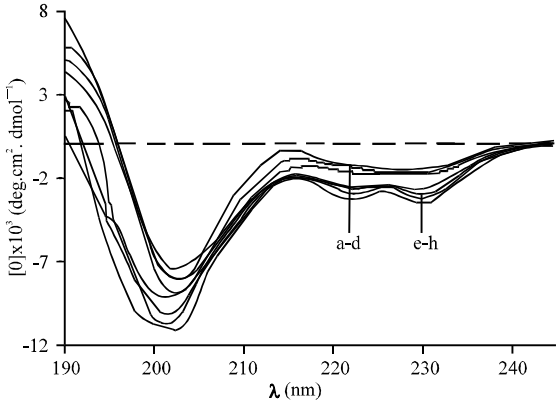

Fig. 2: CD spectra of F3W analog in deionized water at various concentrations. F3W analog concentration: (a) 0.066 mM (b) 0.098 mM (c) 0.13 mM (d) 0.19 mM (e) 0.24 mM (f) 0.4 mM (g) 0.37 mM (h) 0.5 mM (observed as same as aurein 1.2)

Subsequently, the CD spectra are used to assess the relationship between the antimicrobial effect and the secondary structure of F3W analog. Figure 2 shows the CD spectra of the F3W in water. A strong negative band at ~ 200 nm, indicating the presence of different unordered states, characterizes the spectra of the peptide at different concentrations. The CD spectra of F3W analog changes slightly with increasing the concentration from 0.06 to 0.5 mM. In addition, a shoulder appears at ~ 222 nm. This suggests a conformational change that probably is due to peptide association.

Concentration range studied in circular dichroism is of the same order as in antimicrobial assay. Our results also reveal that at lower concentrations (5 to 20 µg mL^{-1}) the peptide is an indication of an unordered state. An increase in the concentration of the peptide above 20 µg mL^{-1} leads to a decrease in the OD intensity and at the same time a red shift in the maximum wavelength at ~ 200 nm. In addition, there is a remarkable increase in the

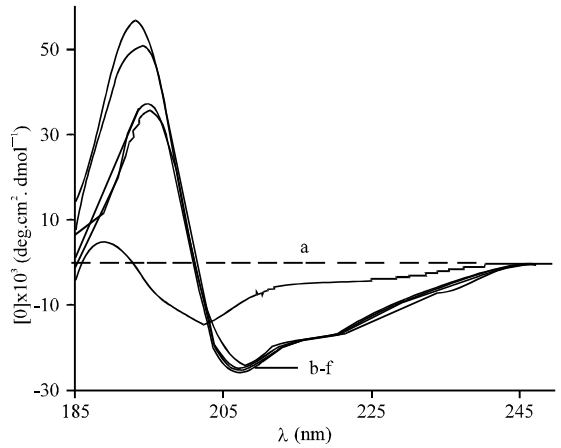

Fig. 3: CD spectra of aurein 1.2 in the absence or in the presence of various amounts of SDS. none (a) 1 mM (b) 3 mM (c) 5 mM (d) 8 mM (e)10 mM (f) and 20 mM (g) (for F3W analogs is similar)

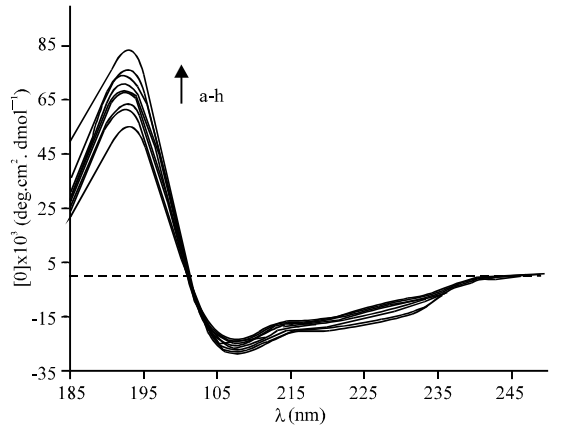

Fig. 4: CD spectra of different concentrations of peptides in 10 Mm SDS: 0.2 mM (a) 0.18 mM (b) 0.15 mM (c) 0.12 mM (d) 0.11 mM (e) 0.07 mM (f) 0.05 mM (g) 0.04 mM (m) 0.03 mM (n) and 0.01 mM (h)

intensity of the Far UV -CD spectra in the region between ~215 and ~240 nm. It is known that in this region the ordered structures have a distinguishable negative peak. It is justifying the contention that at higher concentrations, the peptide's secondary structure is leading to association of its molecules thereby to lower antibacterial activity. These observations show the structure-activity relation of the peptide.

In order to examine the behavior of aurein 1.2 in a membrane mimicking media, the CD spectra obtained at presence of different concentrations of Sodium Dodecyl Sulfate (SDS) at concentrations ranging from 1 to 10 mM. (Fig. 3).

The helix propensity increases with the increase in the SDS concentration. Combined appearance of a positive band at ~195 nm and a double minimum at ~208 and 222 nm suggests the presence of a helical structure in both peptide analogs. It is known that the intensity of the 222 nm band is proportional to the degree of helicity (Chen *et al*., 1974). The degree of helicity of both analogs is estimated by Johnson (1986) method and our results show 80 and 57% helicity for F3W and G1R/F3W analogs, respectively (Table 1).

The induction of α-helical structure for F3W occurs below the CMC (8 mM) of the SDS, implying that the formation of α-helix is unrelated to the SDS micelles formation.

Furthermore, the relationship between the secondary structure and concentration of the peptide (from 0.006 to 0.2 mM) at presence of 10 mM concentration of SDS in 25°C is shown in Fig. 4. Based on these results, we propose that there is an isodichroic point at 202 nm, indicating the possibility of equilibrium between the two structurally different populations of F3W (Holtzer and Holtzer, 1992). This result observes for aurein 1.2, too.

It seems that G1R/F3W analog has a stronger interaction with the membrane mainly due to the positive charge of Arg residue. It has been suggested before that the higher positive charge of the antimicrobial peptides may lead to the snorkeling mechanism for their function (Holtzer and Holtzer, 1992; Segrest *et al*., 1991; Strandberg and Killian, 2003). This also suggests that the positively charged residues (Arg and Lys); electrostatically interact with the negatively charged head groups of the membrane lipids that cause the hydrophobic part of the peptide penetrates better into the lipid membrane.

There is no shoulder in Fig. 1. This indicates G1R/F3W analog do not uses carpet mechanism. It seems this analog has no potential for association, however, it has antibacterial activity. Data analysis indicates that the function of F3W analog and Aurein 1.2 depends on their concentration. Consequently, we propose that the activity of F3W analog and Aurein 1.2 follows the snorkeling mechanism in the first phase (0-12 μM). It also agrees with the observations of phoenix group (Denmison *et al*., 2006). Then, in the second phase where the bactericidal efficiency of F3W is partially reduced, it is proposed that the hydrophobic character of this amphipathic peptide make it susceptible to self-association (Yang *et al*., 2006). Previously, Lee and Hodges (2003) have shown that the amphipathic peptides have a potential for self-association, as are indicated by a decrease in HPLC retention times. At low temperatures, they reported a

nonlinear relationship between peptide retention time and temperature. Present data show (Table 1) the retention time of Aurein 1.2 and F3W increases in higher temperature that this indicates peptide self-association. This is confirmed by the CD data which show point of the formation of more ordered structures as the increase of concentration (Fig. 2).

Furthermore, the third phase of the antimicrobial assay where the antimicrobial activity recovered from 30-60 μM, suggests that there is a threshold concentration of the peptide for its optimal function. This pattern is characteristic of the amphipathic peptides in accordance with carpet model (Shai, 1999).

According to the carpet model, first the peptides bind onto the surface of the target microbial cell membrane. Subsequently, the membrane is covered by a 'carpet-like' cluster of peptides. Initial interaction with the negatively charged target membrane is electrically driven. In the second step, after the threshold concentration has been reached, the peptides permeate the membrane. This model further suggests that the membrane breaks into pieces and leads to lysis of the microbial cell when a threshold concentration of the peptide is reached. High local concentration on the surface of the membrane depends upon the type of the target membrane and can occur either after all the surface of the membrane is covered with peptide monomers, in other words, antimicrobial peptides that associate on the surface of the membrane, can form a local carpet (Shai, 1999).

Altogether, these observations indicate that in this phase F3W analog operates by the carpet model. Furthermore, the standing disputes regarding the activity mechanism and discrepancies in reported data by others can be resolved in terms of a two-state mechanism. Aurein 1.2 and F3W analog at the low concentrations (12 μM, <22 ug mL^{-1}) operate the snorkeling mechanism, while at the higher concentrations (30 μM, >47 ug mL^{-1}) the carpet model is the determining mechanism.

The proposed two-stage mechanism leads also to new insights into the antimicrobial activity of the peptides. For example in a previous study by other researchers (Wang *et al*., 2005), it has been shown that substitution of Phe by Trp at position 13 of aurein 1.2 causes a significant decrease in antimicrobial activity, without any structural change. Based on this proposed two-stage mechanism, one may conclude that the low antimicrobial activity of the F3W analog can be related to its reduced potential for self-association due to the presence of TRP residue in the C-terminal region of aurein 1.2. This idea is supported further by the imposed steric hindrance of the TRP residue introduced in the peptide. Thus, an optimum hydrophobicity is required for dimerization control and maintenance of antimicrobial activity of the peptide.

CONCLUSION

There is no single mechanism of action that appears to fit all observed AMP behaviors (Li *et al*., 2006) and the antimicrobial peptide appears to act by fundamentally different mechanisms depending on their lengths (Bolintineanu and Kaznessis, 2011; Chen and Mark, 2011). However overall our data show a two-stage concentration-dependent mechanism between the activity and the structure of F3W analog where at low concentrations; snorkeling mechanism seems to be operative, while the carpet mechanism is dominant at higher concentrations. The suggested two-step mechanism is further supported by results from the CD spectra, which shows a concentration-dependent secondary structural change and observation of a dichroic point in the presence of SDS. These results also indicate that the antimicrobial activity of G1R/F3W is higher than the F3W analog, probably due to the introduced positively charge of the Arg residue. These observations not only resolve the discrepancies in data reported by others, but also suggests that concentration of macro molecules must be consider in drug peptide design.

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