

## Comparative structure-activity relationships of indolicidin and its analogues

Sung-min Kim, Bishnu Prasad Joshi, Chuda Raj Lohani, and Keun-hyeung Lee.\*

Bioorganic Chemistry Lab, Department of Chemistry, Inha University, 253-Yonghyun-Dong, Nam-Gu, Incheon, 402-751, South Korea

E-mail:leekh@inha.ac.kr

### Introduction

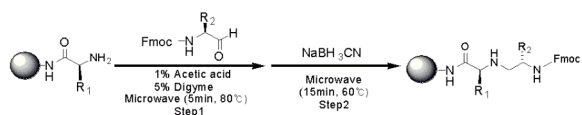
Indolicidin consisting of 13 amino acids (ILPWKWPWWPWR-NH<sub>2</sub>) has been isolated from the cytoplasmic granules of bovine neutrophils. This peptide exhibits a broad spectrum of antibacterial activity and has great activity against fungi, protozoa, and HIV-1 virus.

Indolicidin has received attention due to its unique primary structure containing several Trp and Pro amino acids. Several researchers have suggested that indolicidin might adopt a turn structure. To investigate the effect of a turn structure on the activity, we designed and synthesized indolicidin analogues containing reduced amide bond(s).

We investigated the structure and antibacterial and hemolytic activity of the peptide analogues to provide insight into the structure activity relationships.

### Results and Discussion

Indolicidin and its analogues were synthesized using solid phase Fmoc Chemistry with microwave irradiation method (Scheme 1). As shown in Table 1, the amide bond(s) in the position of 1 and 9 was replaced by reduced amide bond. Synthesized crude peptides were purified by semi-prep column and then the product was characterized by HPLC and mass spectrometer (Table 1, Fig. 1).

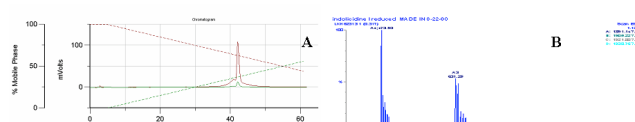


**Scheme 1.** Synthesis scheme for reductive alkylation reaction with microwave irradiation method in solid phase synthesis.

The secondary structures were measured using CD spectroscopy. As shown in figure 2, the CD spectrum of indolicidin revealed that indolicidin adopted a turn structure in the presence of SDS micelles on the basis of the negative ellipticity at 225 nm. However, ID-W and ID-I, W, which have a reduced amide bond at 9<sup>th</sup> amide position did not have turn structure in the presence of SDS micelles.

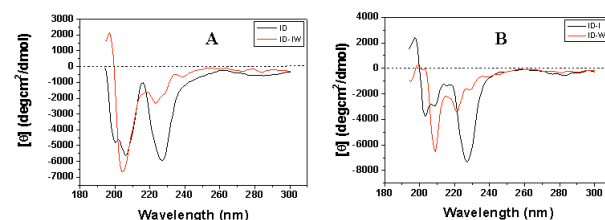
**Table 1.** The characteristics of indolicidin and its analogues containing reduced amide bonds.

Name	Sequence	Net charge (pH 7.4)	Retention time (min)	Calculated mass (measured mass)
ID	ILPWKWPWWPWR-NH <sub>2</sub>	+4	47.6	1905.03 (1905.14)
ID-I	I <sub>v</sub> [CH <sub>2</sub> NH]ILPWKWPWWPWR-NH <sub>2</sub>	+5	45.0	1891.03 (1891.14)
ID-W	ILPWKWPWW <sub>v</sub> [CH <sub>2</sub> NH]PWR-NH <sub>2</sub>	+5	38.6	1891.03 (1891.22)
ID-IW	I <sub>v</sub> [CH <sub>2</sub> NH]ILPWKWPWW <sub>v</sub> [CH <sub>2</sub> NH]PWR-NH <sub>2</sub>	+6	36.6	1877.03 (1876.97)



**Fig. 1.** Characterization of ID-I by (A) HPLC and (B) ESI mass (Calculated mass ( $M+H^+$ ) = 1891.03) and observed ( $M+H^+$ ) = 1891.14).

Interestingly, ID-I, where the first amide bond is replaced by a reduced amide bond, might adopt turn structure on the basis of the negative ellipticity band at 225 nm. Overall the results indicated that the incorporation of reduced amide bond at 9<sup>th</sup> amide bond destroyed the turn structure stabilized by the sequence (WPW).



**Fig. 2.** CD spectrum of Indolicidin and its analogues in SDS micelles. (A) indolicidin and ID-IW (B) ID-I and ID-W.

The minimum inhibitory concentrations (MICs) of indolicidin and its analogues were measured (Table 2). The MICs of the analogues demonstrated that the incorporation of the reduced amide bond into indolicidin resulted in the retention of antibacterial and antifungal activity.

Further, leakage activities of indolicidin and its analogues were studied with different LUVs based on the bacterial cell membrane composition. Considering the composition of bacterial lipid membrane, we prepared PG/CL (3:1mol/mol) large unilamellar vesicles (LUVs) and investigated the leakage activities based on the emission intensity of calcein dye at 520 nm. As shown in figure 3, all test samples showed similar leakage activities,

**Table 2.** MIC of indolicidin analogues.

Peptides	Minimal Inhibition Concentration (MIC, $\mu\text{g/ml}$ ) <sup>a</sup>				
	<i>B. Subtilis</i> (ATCC 6633)	<i>S. aureus</i> (ATCC 6538)	<i>M. luteus</i> (ATCC 9341)	<i>E. coli</i> (ATCC 2592)	<i>C. albicans</i> (ATCC 36232)
ID	3.00/1.5	12.5/12.5	12.5/25	12.5/25	50 <sup>b</sup> /50 <sup>b</sup>
ID-I	6.25/6.25	12.5/12.5	12.5/25	12.5/25	50/50
ID-W	3.12/6.25	12.5/12.5	12.5/25	12.5/25	100/100
ID-IW	3.12/6.25	12.5/12.5	12.5/25	12.5/25	50/50

The experiment was twice repeated in the same condition. A) after 24hr monitoring B) after 48hr monitoring

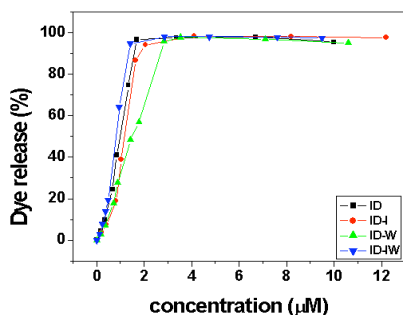


Fig. 3. Leakage activity of indolicidin and analogues for PG/CL (3:1 mol/mol) LUVs.

which is consistent well with the MIC result of test samples. Even though the analogs have a different secondary structure upon lipid membranes, they have a similar mode of antibacterial action of the parent peptide.

We measured hemolytic activity of indolicidin and its analogues as shown in figure 4. Interestingly, ID-IW and ID-W showed a little hemolytic activity and ID-I have 50% hemolytic activity compared to that of indolicidin. Further, considering the composition of lipid membranes of erythrocytes, we prepared PC and PC/Cholesterol (10:1 mol/mol) LUVs, respectively and investigated the leakage activities for these LUVs.

Interestingly, indolicidin and its analogues exhibited similar activity for PC LUVs to that of PG/CL (3:1 mol/mol) and 2 µM of each test sample induced similar maximum leakage (90%). However, the leakage activities for PC/Chol (10:1 mol/mol) LUVs were different. ID-IW had lowest leakage activity among the analogues and ID-W showed a much lower leakage activity than those of ID-I and indolicidin. (Fig. 5.)

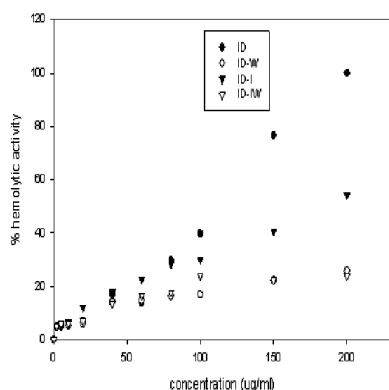


Fig. 4. Hemolytic activity of indolicidin and its analogues.

In conclusion, we successfully synthesized indolicidin analogues with different secondary structures by incorporating reduced amide bonds. Overall studies revealed the effect of turn structure induced WPW and net charge on various activities. The turn structure was not critical for antibacterial activity but critical for hemolytic activity and the leakage activity for PC/Chol LUVs. Also, net positive charge had a decreasing effect on the hemolytic activity.

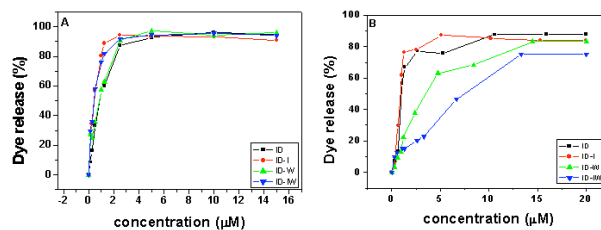


Fig. 5. Leakage assay of indolicidin analogues. (A) PC LUVs dye release percent and (B) PC/Cholesterol (10 : 1 mol/mol) LUVs dye release percent.

### Acknowledgments

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