On the Antibacterial Activity of Normal and Reversed Magainin 2 Analogs against Helicobacter pylori

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Magainin 2 is an antimicrobial peptide isolated from the skin of Xenopus laevis. We have tested the antibacterial activities of normal and reversed magainin 2 analogs against two strains of Helicobacter pylori (ATCC 43526, ATCC 43579), compared with those against Escherichia coli (ATCC 25922) and Staphylococcus aureus (ATCC 25923). Among these analogs, MSI-78A showed the strongest activity against H. pylori. The MIC (minimum inhibitory concentration) values were almost the same as those against E. coli and S. aureus. No or lesser activity was observed in all the reversed peptides compared to the corresponding normal magainin 2 analogs. Based on the CD (circular dichroism) measurement, the more active peptide tends to show a higher α-content. The positivelycharged five amino acids (KILKK) positioned at the C terminus on the amphipathic α-helical structure play important roles in exerting the strong activity against H. pylori. This indicates that the net charge of the cell surface in H. pylori may be more negative than that of E. coli, though both strains belong to the same genus.

Key words magainin 2 analogs; antibacterial activity; H. pylori; hemolysis

We have an interest in developing simple methods for determining peptide leads with a desired function from naturally occurring amino acid sequences, followed by the design of a new molecule. In the previous study, we reported the antibacterial activity of normal and reversed magainin 2 analogs against E. coli (ATCC 25922) and S. aureus (ATCC 25923), respectively. In that, the reversed peptides, that is, with an opposite amino to carboxy orientation (vice versa) of the naturally occurring amino acid sequence (i.e., normal peptide), showed similar activity to that of the normal peptide. Impressively, both MSI-78, which enhanced the amphipathic nature of magainin 2 (vide infra), and the reversed peptide 87-ISM showed the same activity against E. coli, though their stereochemistry is completely different. This result motivated us to further examine their activity against other gram negative bacteria.

Among various bacteria, we decided to test their antibacterial activity against H. pylori, because it belongs to the same genus as E. coli and is strongly associated with both peptic ulcers and chronic gastritis. This strain is a microaerophilic, urease-positive, spiral or curved bacterium, which was first isolated from the gastric mucosa of patients with gastritis. In the treatment, double or triple therapy with antibiotics and proton pump inhibitors have in general been investigated, while the occurrence of a resistant strain to antibiotics has been already reported. At present, novel non-crossing agents with antibiotics well characterized have been intensively screened.

Magainin 2 is a peptide composed of 23 amino acid residues, isolated from the skin of the Xenopus laevis. It interacts directly with the biological membrane to lyse various bacteria from gram negative and positive microorganisms, except H. pylori, rather than via specific membrane receptors. Since this discovery, there have been reported a number of synthetic magainin 2 analogs, including a magainin 2 amide composed of all d-amino acids, all of which have been studied to understand the structure–activity relationship and to develop a clinically useful agent using either the replacement, deletion or addition of amino acids in magainin 2. As a result, the amphipathic α-helical structure of magainin 2 has been clarified as an important factor in the antibacterial activity, although the precise antibacterial mechanism on the microorganisms has not yet been defined. Inclusively, active analogs have been obtained by enhancing either the hydrophobicity, the α-helix tendency or the amphipathic nature of the α-helical structure. Based on the knowledge of the magainin 2 mechanism of antibacterial action, therefore, in the analogs may exist a novel non-crossing agent with antibiotics.

In this study, we report the antibacterial activities of magainin 2 analogs against two strains of H. pylori (ATCC 43526, ATCC 43579), compared with those against E. coli (ATCC 25922) and S. aureus (ATCC 25923). We also performed their CD (circular dichroism) measurement to determine the activity–structure relationship.

MATERIALS AND METHODS

Peptides Peptides used in this study were synthesized by solid-phase methods using a peptide synthesizer (model 430A or 431A, Applied Biosystems, Inc., CA, U.S.A.), and the procedure was described previously. The synthetic peptides are shown in Table 1.

Bacteria and Cells E. coli (ATCC 25922) and S. aureus (ATCC 25923) were obtained from American Type Culture Collection (ATCC, MD, U.S.A.). Two strains of H. Pylori (ATCC 43526, ATCC 43579) are kind gifts from Dr. T. Nagate of Taisho Pharmaceutical Co., Ltd. Human red blood cells (RBCs) were collected from human blood of normal donors.

Antibacterial Activity To measure the antibacterial activity...
activity of the peptides against microorganisms, we used the micro-dilution broth method to determine the minimum inhibitory concentration (MIC). The procedures in *E. coli* and *S. aureus* were reported previously. In the case of *H. pylori*, Mueller–Hinton broth containing 5% fetal bovine serum (FBS) was used as the medium, and was cultured in a jar conditioned with Campylo Pak (Dia Iatron) for 48 h. Briefly, *H. pylori* strains were inoculated on a Brucella agar plate containing 10% horse serum, and cultured at 37 °C for 48 h. The bacterial colonies collected were diluted to 10⁷ colony forming unit (CFU)/ml with 0.9% saline. The peptides were dissolved in distilled water, then diluted with Mueller–Hinton broth.

To the solution of the peptides, the suspension solution of each bacterium was added to make 10⁶ CFU/100 ml/well. The mixture was incubated at 37 °C for 48 h. The MIC value of the peptides was determined by observation.

**CD Measurement** All measurements were carried out on a Jasco model J-500A spectropolarimeter, in conjunction with a temperature controller (HAAKE, Berlin, Germany). Peptides were dissolved in 40% (v/v) trifluoroethanol (Kanto Chemicals, Tokyo, Japan)–50 mM potassium phosphate buffer, pH 7.0, at a concentration of 0.1 mg/ml, and were measured in a 1 mm path length cell. The wavelength scanned was 250—190 nm (1 nm interval), and was analyzed from 240 to 205 nm. The temperature was controlled at 25 °C. The α-helical contents were estimated by the method of Provencher & Glockner for the secondary structure using the computer program CONTIN. The results were summarized in Table 2. At first, the Mag 2 amide (1) was slightly active (1024 µg/ml), with no detectable activity at the concentration of 2048 µg/ml in the case of 2 Gam amide (2), that is, the reversed peptide of the Mag 2 amide (1). Next, we tested the activity of D1 (3), which enhanced the hydrophobicity of magainin 2, and that of the reversed peptide (4). The latter peptide (4) showed no activity against *H. pylori* (ATCC 43526), but moderate activity against *H. pylori* (ATCC 43579). This difference may be due to bacterial properties, because the growth rate of the latter bacterium under our culture conditions was faster than the former. Thus, we evaluated the effect of D35 (5), in which a Gly residue is replaced with Ala to enhance the α-helical tendency of Mag 2 amide (1), and also the effect of the reversed peptide (6). A faint activity (512 or 1024 µg/ml) was observed in D35 (5), but no detectable activity in 53D (6). The difference in the activity between the three normal and reversed magainin 2 analogs was similar to that in the case of *S. aureus* described previously. In addition, the MIC values observed in this study are higher in comparison with those against *S. aureus* (Table 2). Therefore, we assumed that magainin 2 analogs, either with enhanced hydrophobicity

### Table 1. The Amino Acid Sequences of Magainin 2 Analogs

<table>
<thead>
<tr>
<th>No.</th>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mag 2 amide</td>
<td>GIGKFLHSAKKFGKAFGVIGEGMS-CNH₂</td>
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<tr>
<td>2</td>
<td>2 Gam amide</td>
<td>SNMIEGYFAGKFKKHFKGSGG-CNH₂</td>
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<tr>
<td>4</td>
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<td>D35</td>
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<tr>
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<td>53D</td>
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<tr>
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<td>1K87-ISM</td>
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<td>15</td>
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### Table 2. Antibacterial Activity of Magainin 2 Analogs

<table>
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<tr>
<th>No.</th>
<th>Peptide</th>
<th>E. coli (ATCC25922)</th>
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<th>H. pylori (ATCC43526)</th>
<th>H. pylori (ATCC43579)</th>
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<tr>
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<td>2048</td>
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<tr>
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<td>MSI-78K2</td>
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<td>64</td>
<td>2048</td>
<td>2048</td>
</tr>
<tr>
<td>10</td>
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<td>1024</td>
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or an α-helical tendency, express insufficient antibacterial activity against H. pylori, similarly to S. aureus, as reported previously.\textsuperscript{2,3}

Thus, we had great interest in the effect of the amphipathically x-helical magainin 2 analogs on H. pylori. We evaluated the activity of MSI-78 (7) and its reversed peptide 87-ISM (8). The MSI-78 (7) had potent activity against S. aureus strains, including methicillin-resistant S. aureus (MRSA), and is presently in clinical trials as a topical antibacterial for the treatment of infected diabetic foot ulcers.\textsuperscript{13,14} Furthermore, this peptide has been reported not to cause resistance against S. aureus in an in vitro assay. As shown in Table 2, MSI-78 (7) was found to show relatively stronger in vitro antibacterial activity against two strains of H. pylori, while no detectable activity was observed in 87-ISM (8). The MIC values (64 μg/ml) against H. pylori are 4-fold higher than that (16 μg/ml) against E. coli. In order to clarify their differences, they examined their antibacterial activity against E. coli under the same conditions as H. pylori cultured in the presence of 5% fetal bovine serum, because the influence of the serum was already reported in another study.\textsuperscript{18,19} However, the MIC value of MSI-78 (7) against E. coli was unaffected in our in vitro assay (data not shown), indicating the possibility that the killing mechanism of MSI-78 (7) against E. coli and H. pylori is different. In addition, similar results were also obtained in the case of 87-ISM (8).

Lastly, we examined the activity of MSI-78A (13) and its reversed peptide, A87-ISM (14), against the bacteria. The MSI-78A (13) exhibited 16~32 times stronger activity than 87-ISM (14) against H. pylori (Table 2). In addition, MSI-78A (13) was 2~4 times more active against H. pylori than E. coli, whereas A87-ISM (14) showed the opposite results.

A relatively good relation between the x-content and activity of magainins has long been known.\textsuperscript{1,3} We originally designed the peptides, MSI-78A (13) and A87-ISM (14), respectively, in order to enhance the α-helical tendency of MSI-78 (7) or 87-ISM (8). Of these, MSI-78A (13) showed the strongest activity against H. pylori. To clarify this, we measured their α-helical contents in CD spectrum (40% trifluoroethanol solution), including that of D35 (5) and S3D (6). As shown in Table 3, the ratios of the α-helical content of MSI-78 (7), 87-ISM (8), MSI-78A (13), A87-ISM (14), D35 (5) and S3D (6) are 61, 35, 78, 59, 56 and 29%, respectively. The order is MSI-78A (13) > MSI-78 (7) > A87-ISM (14) > D35 (5) > 87-ISM (8) > S3D (6). Based on these values, the more strongly active peptides tend to show the higher ratio. However, their relationship is not always parallel, because A87-ISM (14) and D35 (5) are less active than MSI-78 (7), in spite of having the same α-helical content as MSI-78 (7). This suggests that the antibacterial activity of magainin 2 analogs against H. pylori is strongly dependent on the individual amino acid sequence, differing from the case of E. coli. It is interesting that similar relationships were observed in the case of S. aureus.

To further examine the structure-activity relationship on the MSI-78 (7) sequence, we investigated the in vitro activity of other deleted analogs. In a previous report,\textsuperscript{2} we described that the hydrophobic profiles between the 1-17 amino acid sequence (GIGKFLKKAKKKFQGAFV) in the MSI-87 and 6-22 amino acid sequence (VFAK-GFKKAKKLFKGIG) in 87-ISM have been shown to be almost pided upon each other. In addition, their peptides (i.e., 9 and 10) showed similar, but not the same, antibacterial activity against E. coli (Table 2). Keeping this in view, we examined the effect of MSI-78K2 (9), 2K87-ISM (10), MSI-78K1 (11) and 1K87-ISM (12) against H. pylori. As a result, all the peptides did not show detectable activity (Table 2). In addition to that, we examined the activity of MSI-78A2 (15), which deleted the five amino acids at the C terminus of MSI-78A (13). The activity against H. pylori was remarkably reduced (Table 2). Taking these into consideration, at least five amino acids (KILKK) positioned at the C terminus of MSI-78 (7) and MSI-78A (13) are necessary for expressing the strong antibacterial activity. Based on this, the net charge of the cell surface in H. pylori should be more negative than that of E. coli, though both strains are gram negative.

We have demonstrated the hemolytic activity of the peptides to RBCs. Among the analogs, MSI-78A (13) showed the strongest hemolytic activity to RBCs, whereas all the reversed peptides or shortened peptides showed no or slight activity (Table 4).

In conclusion, the susceptibility of normal and reversed magainin 2 analogs to two strains of H. pylori was fairly different; that is, the normal analogs were, in general, more strongly active than the corresponding reversed analogs, respectively, unlike the case of E. coli. We are the process
of generating an improved MSI-78A (13) analog, without such side-effect as hemolytic activity.

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REFERENCES