1 INTRODUCTION

The bacterium *Helicobacter pylori* (Hp) is linked to several gastric diseases and in particular, to the progression to gastric cancer\(^1\). In 1994, the World Health Organization/International Agency for Research on Cancer classified *Hp* as a “definite carcinogen” based on epidemiological findings\(^2\). While the pathogenic roles of *Hp* are not yet fully understood, epidemiologic studies do overwhelmingly agree on the protective effects of fruits and vegetables in reducing the risk of gastric cancer\(^3\). In support of this trend, we have reported on the suppressive effects of Japanese plum juice on *Hp*-induced chronic gastritis\(^4\).

Auraptene (AUR), a coumarin derivative of citrus fruit, is a promising chemopreventive agent against skin, tongue, esophagus, and colon carcinogenesis in experimental animals\(^5-8\). Previously, we investigated the role of AUR in attenuating the biochemical responsiveness of inflammatory leukocytes using a 12-O-tetradecanoylphorbol-13-acetate-treated mouse skin model\(^9\). Moreover, we demonstrated that AUR directly activated macrophage activities and exerted part of its chemopreventive activities through enhancement of immune function\(^10\). Recently, we showed that the dietary feeding of AUR could inhibit colitis-related mouse colon carcinogenesis\(^11\).

Mongolian gerbils (MGs) can be readily infected with *Hp*, making them good experimental models for clarifying the role of *Hp* in chronic active gastritis, peptic ulcers, intestinal metaplasia, and gastric cancer\(^12,13\). We have established a gastric carcinogenesis model using these animals, and demonstrated that *Hp* infection enhanced development of gastric cancer induced by chemical carcinogen treatment\(^14\). On the other hand, *Hp* motility has an important role in colonization of the gastric mucosa\(^15-17\). As such, we hypothesize that inhibition of *Hp* motility can be expected to diminish its colonization of the gastric mucosa\(^4,18,19\), and found AUR in a screen for mobility.

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**Citrus Auraptene Reduces *Helicobacter pylori* Colonization of Glandular Stomach Lesions in Mongolian Gerbils**

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**Abstract:** We examined the efficacy of auraptene (AUR), an antioxidant agent of a citrus coumarin derivative, for suppressing gastric inflammation introduced by *Helicobacter pylori* (*Hp*) infection in Mongolian gerbils (MGs). *Hp*-infected MGs were placed on diets containing 100 or 500 ppm AUR for 7 weeks. Real-time PCR was used to estimate the *Hp* population in glandular stomach lesions, and a histological assessment of inflammation was performed. At a dose of 500 ppm, AUR reduced the *Hp* population to 21.9±12.0% of the control group (*p*<0.05). However, no apparent differences were seen in hematoxylin and eosin sections between AUR-administered and control groups. We conclude that dietary supplementation with 500 ppm of AUR suppresses *Hp* colonization, but does not reduce gastric inflammation.

**Key words:** auraptene, *Helicobacter pylori*, gastritis, Mongolian gerbil
inhibiting agents (data not shown).

In the present study, we investigated the protective effects of AUR on \textit{Hp} colonization of the gastric mucosa, as well as its therapeutic effects on \textit{Hp}-induced glandular stomach lesions in MGs. The suppressive effects of AUR on \textit{Hp} proliferation are also discussed.

2 EXPERIMENTAL
2.1 Animals and Diet

A total of 27 specific pathogen-free 9-week-old male MGs (\textit{Meriones unguiculatus}; MGS/Sea, Kyudo Co., Ltd., Fukuoka, Japan) were housed in plastic cages with wood chips for bedding. The cages were placed in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. The animals were fed a pelleted basal diet (NF, Oriental Yeast Co., Ltd., Fukuoka, Japan) and water \textit{ad libitum}. AUR was isolated from natsumikans (\textit{Citrus natsudaidai} Hayata) in Wakayama Prefecture (Japan) as previously described\cite{10} and AUR-containing diets were prepared by adding AUR at concentrations of 100 or 500 ppm to the basal food, NF, by Oriental Yeast Co., Ltd.

2.2 Bacteria

\textit{Hp} strain ATCC43504 (American Type Culture Collection, Manassas, VA, USA) was cultured in Brucella Broth (Becton Dickinson Co., Franklin Lakes, NJ, USA) containing 7% heat-inactivated fetal bovine serum for 2 days at 37°C under microaerophilic conditions sustained by Anaero Pack Campylo (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). The bacterial morphology and motility of the \textit{Hp} broth cultures were examined by phase contrast microscopy. Inoculum cultures containing approximately $2 \times 10^7$ cfu were delivered intra-gastrically using an oral catheter to MGs fasted 24-h prior. Brucella Broth (vehicle) was administered to uninoculated MGs as a control.

2.3 Experimental Protocol

The experimental design for \textit{Hp} infection and administration of AUR to MGs is shown in Fig.1. A total of twenty-seven MGs were divided into four groups A, B, C and D. In the first and second experimental weeks, \textit{Hp} was inoculated into three groups, B, C and D, while the control group A received Brucella Broth alone as the vehicle. From the third experimental week to the end of the experiment at 10 weeks, group A (vehicle alone) and group C (\textit{Hp} inoculated) were fed a 500 ppm AUR-containing diet, and group B (\textit{Hp} inoculated) was given a 100 ppm AUR-containing diet. The other \textit{Hp} inoculated group, group D, was given basal food during the entire experimental period. All MGs were euthanized by deep ether anesthesia at the end of the study. The animals were laparatomized and exsanguinated from the inferior vena cava, followed by excision of their stomachs. A 3 mm square tissue fragment was excised from the antrum of each glandular stomach and immediately frozen in liquid nitrogen for genomic DNA analysis. The residual tissues were fixed in 10% neutral buffered-formalin, embedded in paraffin blocks, and routinely processed for histopathological examination. The experimental protocols, animal care, and treatment were approved by the Committee for Animal Studies at Wakayama Medical University.

2.4 Histopathological examination

Tissue sections were stained with hematoxylin and eosin. Immunohistochemical staining was also done for histological assessment of \textit{Hp}-infection using an indirect immunoperoxidase method with 1/100 diluted anti-\textit{Hp} serum (DAKO, Glostrup, Denmark) and 1/100 diluted anti-rabbit Ig (DAKO). The degree of chronic active gastritis was graded according to a modified Updated Sydney System\cite{21} by scoring the following parameters: mononuclear cell infiltration (0-3; 0, normal; 1, mild infiltration into lamina propria; 2, moderate infiltration into lamina propria; 3, marked infiltration into lamina propria and multiple lymphoid follicle formation); \textit{Hp} density (0-3; 0, none; 1, mild \textit{Hp} density; 2, moderate; 3, marked).

2.5 Serum levels of anti-\textit{Hp} antibodies

The serum IgG antibody titer against \textit{Hp} is believed to be an early and reliable indicator of \textit{Hp} infection\cite{24}. The anti-\textit{Hp} IgG levels in the serum of each animal were measured by enzyme-linked immunosorbent assay using an I-DQ75 ELISA kit (Eiken Chemical Co., Ltd., Tokyo, Japan). HRP-conjugated anti-mouse immunoglobulin (DAKO) was used as the secondary antibody. Optical density at 492 nm (OD$_{492}$) was measured spectrophotometrically. A value greater than 1.0 OD was defined as positive for \textit{Hp} infec-
2.6 Quantitative real-time polymerase chain reaction

Genomic DNA was extracted from the glandular stomach tissue of MGs using the DNeasy tissue kit (QIAGEN, Hilden, Germany). For quantification of relative *Hp* levels, real-time PCR of *Hp* specific urease A and gerbil specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with the QuantiTect SYBR Green PCR kit (QIAGEN) and iCycler iQ system (BIO-RAD, Hercules, CA, USA). The primer sequences for urease A and GADPH are given in Table 1. Specificity of the PCR reaction was confirmed using the melting program provided with the iCycler iQ system. Relative quantitative analysis of *Hp* was performed as previously described.

2.7 Statistical analysis

The Kruskal-Wallis test was applied to determine any significant differences in urease A gene quantity, and anti-*Hp* IgG antibody titers. Differences with a *P* value less than 0.05 were considered significant.

### 3 RESULTS

#### 3.1 Body weight and intake of AUR

Administration of AUR did not affect food intake or body weight (Fig. 2). The averages of AUR intake in *Hp*-free 500 ppm AUR-administered group A, *Hp*-infected 100 and 500 ppm AUR-administered groups B and C were 2.6, 0.51 and 2.5 mg/day/head, respectively.

#### 3.2 Pathological findings

All the MGs in the *Hp*-inoculated groups B, C and D were shown by microscopic examination to have severe gastritis with erosion and marked infiltration of inflammatory cells and lymphoid follicle formation (Fig. 3b, 3c and 3d). Fundic glands were destroyed and diminished in their volume, while foveolar epithelia exhibited marked hyperplasia, resulting in an apparent increase in gastric mucosa thickness compared to the control group A (Fig. 3a). Aggregates of neutrophils were sometimes found in the dilated gastric pit (Fig. 3f). A small number of heterotopic submucosal proliferative glands were also identified in a few members of the *Hp*-inoculated groups (Fig. 3g). Intestinal metaplasia was hardly observed. From the immunohistochemistry, *Hp* colonization was still evident after AUR administration (Fig. 3h). No significant differences were seen in the inflammation and *Hp* colonization scores between the groups given AUR and the *Hp*-inoculated control groups (Table 2). No evidence of gastritis, fundic gland atrophy, or mucosal hyperplasia was found in any of the *Hp*-free animals of control group A (Fig. 3a, 3e).

#### 3.3 Antibody Titer

The anti-*Hp* antibody titers in all *Hp*-inoculated groups B, C and D were greater than the cut off values (Table 2). There were no significant differences in antibody titers among the three groups.

#### 3.4 Quantification of *Hp*

Real-time PCR was performed to quantify the urease A gene in the *Hp* genome. The average of relative urease A gene levels of glandular stomach in 100 ppm and 500 ppm AUR-treated groups B and C, and *Hp*-inoculated control

<table>
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<tr>
<th>Primer</th>
<th>Gene</th>
<th>Sequence</th>
<th>Product Length</th>
<th>Accession No.</th>
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<tbody>
<tr>
<td>Ure-F</td>
<td>Urease A</td>
<td>TGTTGGCGACAGACGGTTCAAATC (sense) GCTGTCCGCTGCAATGTCTAAGC (antisense)</td>
<td>120 bp</td>
<td>M60398</td>
</tr>
<tr>
<td>Ure-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gap-F</td>
<td>GAPDH</td>
<td>AACGGCACAGTAGCTGAGCTGAC (sense) CAACATACTCGGACCACCCGATCG (antisense)</td>
<td>118 bp</td>
<td>AB040445</td>
</tr>
<tr>
<td>Gap-R</td>
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**Fig. 2** Growth Curve of Mongolian Gerbils Administered AUR. Dietary administration of AUR did not affect body weight.
group D were 116±51.3%, 21.9±12.0% and 100±42.4% (average±SD), respectively. The reduction of urease A gene levels by 500 ppm AUR was significant (P<0.05) ([JH]). No amplification of urease A gene was detected in the Hp-free animals of control group A.

4 DISCUSSION

It has been reported that AUR has chemopreventive effects on cancer development in the oral cavity, colon, and liver using the rat or mouse carcinogenesis models [6-8,11]). No studies have been reported to date on its effect on gastric carcinogenesis. However, there are several reports showing AUR exerting anti-inflammatory or stimulating effects on the immune system [9,23,24]. Through the activation of miscellaneous immunocompetent cells represented by...
Effects of Auraptene on Hp-induced Gastritis

Macrophages\textsuperscript{25}, AUR is believed to suppress the inflammation triggered by carcinogenic substances. AUR has also been suggested to stimulate IL-2 secretion by T cells or the whole immune network by various cytokines. Recently, gastric carcinogenesis was associated with persistent chronic inflammation and the subsequent mucosal atrophy caused by Hp infection\textsuperscript{26}. Based upon a large number of experimental and epidemiological studies, it is widely believed that the eradication of Hp should prevent gastric cancer\textsuperscript{27-28}. The effect of many anti-inflammatory drugs on Hp-infected gastritis have been verified using animal models, with some of them exhibiting molecular and biologic advantages with possible chemopreventive effect against Hp-associated gastric carcinogenesis\textsuperscript{29-31}. We also determined that a selective cyclooxygenase-2 inhibitor may act as a chemopreventive agent for stomach cancer by controlling COX-2 expression or activity\textsuperscript{30}.

In the present study, we examined the suppressive effects of AUR on acute and chronic inflammation of the glandular stomach using Hp-infected MGs as a model. We have a plan to verify the anticancer effects of AUR using Hp-related gastric carcinogenesis models in MGs, for which the present study serves as a short-term pilot trial. Prior to the present work, we had already demonstrated the direct effect of 500 ppm AUR in inhibiting Hp motility \textit{in vitro} (unpublished data), which guided us to use doses of AUR 100 and 500 ppm in the current study. The histological findings in the H&E sections did not differ between the AUR-treated groups and the control (Fig. 3a-3g), while all animals exhibited marked chronic active inflammation, erosion, and lymphoid follicle formation with the same inflammation scores (Table 2). The effect of AUR on Hp colonization was not mirrored in the immunohistochemical evaluation (Fig. 3h). Irrespective of these results, the Hp colonization rates as determined by real-time PCR of Hp urease A gene were clearly diminished in the 500 ppm AUR administration group (p<0.05). Rokbi et al. had previously demonstrated the utility of real-time PCR for the detection and quantification of Hp gene expression in the gastric mucosa\textsuperscript{30}. Clayton et al. also found PCR amplification of the Hp urease A gene to be a highly sensitive and specific method for the diagnosis of Hp infection\textsuperscript{31}. As such, our data from real-time PCR reliably demonstrate the reduction of Hp colonization. There are several possible reasons why the reduction in Hp colonization did not reduce gastric inflammation. First, the dose of AUR may have been insufficient for complete eradication of Hp. Secondly, the duration of AUR treatment may have been too short to improve the gastric lesions even though it reduced Hp colonization. Imiuro et al. had reported that administration of garlic extract in a basal diet for 6 weeks was effective in reducing gastritis in a dose-dependent manner in Hp-inoculated MGs\textsuperscript{30}. Tanaka et al. showed that addition of 100 or 500 ppm AUR to the drinking water for 8 weeks showed clear anticancer activity against 4NQO-treated rat tongue carcinogenesis\textsuperscript{32}. Kohno et al. studied the same doses of AUR in dietary feeding for 20 weeks for their chemopreventive effects on colitic cancer and both significantly inhibited the occurrence of colonic adenocarcinoma and reduced colitis\textsuperscript{33}. Judging from these findings, we believe that the 7-week treatment period and the doses of AUR used in this study may have been insufficient to suppress Hp-induced inflammation. Thirdly, the method of AUR administration might not have been optimal. We previously demonstrated the efficacy of a Japanese apricot (CJA) concentrate using the same animal model, in which CJA was given in the drinking water\textsuperscript{34}. It may be better for the intra-gastric administration of test substances to dissolve them in the drinking water than mixing them into the diet, as the drinking water has easier access to, and penetration into the gastric mucosa. In this study, AUR administration was done with dietary feeding because AUR was not readily soluble in water. Several mechanisms may explain how AUR exerts its suppressive effect on Hp proliferation in Hp-infected MGs. AUR was reported to have an inhibitory effect on the cell proliferation activity of mouse tongue mucosa induced by 4NQO exposure\textsuperscript{35}. We had previously found that 500 ppm AUR suppressed the motility of Hp with an apparent deformity of bacterial shape (unpublished data), suggesting a possible inhibitory effect on Hp proliferation. AUR may also inhibit Hp proliferation by simula-

![Fig. 4 Relative Levels of the Urease A Gene in Glandular Stomach of Mongolian Gerbils. Values were set at 100% for the Hp inoculated control group and expressed as mean ± SD. Note the decrease in relative urease A gene levels in the 500 ppm AUR-administered group. *p<0.05, by Kruskal-Wallis test.](image-url)
tion of the host immune system".

5 CONCLUSION
Dietary administration of AUR reduced the Hp colonization rates by mechanisms that have yet to be determined. Although the results in the present study did not demonstrate the modifying effect of AUR on Hp-related gastric lesions, we are optimistic. Taken together with previous reports, our findings show that AUR may suppress Hp-related gastritis and improve gastric lesions, which in turn may inhibit gastric cancer development.

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