The 7α-methoxy substituent in cephem or oxacephem antibiotics enhances in vivo anti-Helicobacter felis activity in mice after oral administration

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The in vivo antibacterial activities of 7α-methoxy-cephem or 7α-methoxy-1-oxacephem and their demethoxy congeners after oral administration were compared in a mouse model of gastric infection with Helicobacter felis. The MICs of all four pairs of compounds with and without the 7α-methoxy substituent were within one two-fold dilution for H. felis and Helicobacter pylori, but the 7α-methoxy compounds were at least four-fold more active at bacterial eradication than their demethoxy congeners. Since these compounds are not absorbed after oral administration, they are likely to have gained direct access to the bacteria from the gastric cavity. Thus the 7α-methoxy substituent may enhance in vivo activity by promoting the gastric mucus permeability of the compounds or by allowing the compounds to remain longer in the gastric cavity.

**Introduction**

*Helicobacter pylori* is a human pathogen associated with type B gastritis, peptic ulcer disease and gastric cancer.¹ Its eradication has been shown to prevent gastric and duodenal ulcer recurrence.²,³ Thus, the US National Institutes of Health consensus conference on *H. pylori* recommended all ulcer patients with *H. pylori* infection be treated with antimicrobial agents in addition to gastric antisecretory drugs.⁴ To date, eradication of *H. pylori* from the gastric mucosa has been achieved by combinations of antimicrobial agents such as amoxycillin, clarithromycin and metronidazole together with proton pump inhibitors.⁵⁻⁸ However, during the course of such therapy, bacterial resistance to clarithromycin and metronidazole sometimes occurs, often leading to failure of the treatment.⁹,¹⁰ Although β-lactam-resistant isolates of *H. pylori* have been reported recently,¹¹,¹² they are still rare and β-lactams remain important components of combination therapy for *H. pylori* infection.

We recently developed a mouse helicobacter infection model, in which mice are infected with *Helicobacter felis*. The efficacies of amoxycillin, clarithromycin and metronidazole in *H. felis* eradication in the model were very similar to those reported clinically for *H. pylori* eradication.¹³ With this model, we noted that several 7α-methoxy-cephem or 7α-methoxy-1-oxacephem showed higher rates of eradication in vivo than amoxycillin or clarithromycin even though they were less active in vitro. This suggested the importance of the 7α-methoxy substituent for in vivo activity. In the present study, we selected 7α-methoxy-cephem or 7α-methoxy-1-oxacephem and their demethoxy congeners with similar MICs for *H. pylori* and *H. felis* and compared their in vivo antibacterial activities in the mouse model.

**Materials and methods**

**Drugs and compounds**

Flomoxef, cefmetazole and amoxycillin were obtained from Shionogi & Co. Ltd (Osaka, Japan), Sankyo Co. Ltd (Tokyo, Japan) and Fujisawa Pharmaceutical Co. Ltd (Osaka, Japan), respectively. The demethoxy-flomoxef,
1-thia-flomoxef, demethoxy-1-thia-flomoxef, demethoxy-cefmetazole, compound M-1 and compound H-1 were synthesized at Shionogi Research Laboratories (Osaka, Japan). The chemical structures of these compounds are shown in Figure 1.

**Bacteria**

*H. felis* ATCC 49179 was used for *in vitro* and *in vivo* studies. Two clinical isolates and two standard strains of *H. pylori* were used for *in vitro* antimicrobial testing. Unless otherwise noted in the text, *H. felis* and *H. pylori* were grown in tissue culture flasks (Falcon 3109, Nippon Becton Dickinson Co., Ltd, Tokyo, Japan) containing Columbia broth (Difco Laboratories, Detroit, MI, USA) supplemented with 5% fetal bovine serum (FBS) under microaerophilic conditions at 37°C with constant rotation (45 rpm) for 48 h.

**MIC determination**

Because of poor growth of *H. felis* on the agar, the microbroth dilution method was adopted for MIC determination. No differences in amoxycillin or flomoxef MICs for *H. pylori* strains were seen between agar and broth dilution methods (data not shown). Bacterial cultures of *H. felis* and *H. pylori* were centrifuged and bacteria were suspended in Columbia broth supplemented with 5% FBS at an optical density of 0.9 at 660 nm. The bacterial suspensions were then diluted 50-fold, and 75 μL portions were inoculated into equal volumes of the broth containing serial two-fold dilutions of antimicrobial compounds. The final bacterial density in the assay was c. 10⁶ cfu/mL. Assay plates were incubated for 3 days at 37°C under microaerophilic conditions before MICs were measured.

**Time–kill assay**

For time–kill studies, tissue culture flasks containing 10 mL of the prewarmed broth were inoculated with *H. pylori* at a final bacterial density of c. 10⁶ cfu/mL and incubated at 37°C for 15 min. The antibiotics were added and incubation was continued at 37°C with constant rotation (45 rpm) under microaerophilic conditions. Viable bacterial counting was performed at 0, 3, 6, 9 and 24 h after antibiotic addition by plating serial 10-fold dilutions of 50 μL aliquots of each culture on to Columbia blood agar base supplemented with 5% horse blood. The plates were incubated for 5 days at 37°C under microaerophilic conditions before the colony numbers were counted. The lowest detectable number of organisms in this test was 20 cfu/mL.

**Determination of in vivo antimicrobial activity**

Seven-week-old female specific-pathogen-free BALB/c mice (Clea Japan, Inc., Tokyo, Japan) given free access to

<table>
<thead>
<tr>
<th>Compounds</th>
<th>X</th>
<th>Y</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flomoxef</td>
<td>O</td>
<td>OCH₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demethoxy-flomoxef</td>
<td>O</td>
<td>H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-thia-flomoxef</td>
<td>S</td>
<td>OCH₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demethoxy-1-thia-flomoxef</td>
<td>S</td>
<td>H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefmetazole</td>
<td>S</td>
<td>OCH₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Demethoxy-cefmetazole</td>
<td>S</td>
<td>H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-1</td>
<td>O</td>
<td>OCH₃</td>
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</tr>
<tr>
<td>H-1</td>
<td>O</td>
<td>H</td>
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</tr>
</tbody>
</table>

**Figure.** Chemical structures of compounds used in this study.
autoclaved laboratory chow and water were used for the determination of in vivo antimicrobial activity. H. felis ATCC 49179 was harvested from the broth culture by centrifugation at 3500g for 20 min, resuspended in Brucella broth containing 20% glycerol at an optical density of 0.9 at 660 nm and stored at −80°C until use for inoculation.

Mice (five mice in each group) were inoculated orally with 0.5 mL of the thawed bacterial suspension three times for 3 days (days 0–2). Clearance and eradication tests were performed 2 weeks after H. felis inoculation. For the clearance test, the mice were given oral antimicrobial agents dissolved in 0.3 mL of 0.5% methylcellulose twice on day 16 and were killed the next day (day 17) by cervical dislocation under ether anaesthesia. For the eradication test, the mice were given antimicrobial agents orally twice a day for 5 days (days 16–20). Two weeks after completion of the therapy (day 34), mice were killed as above.

Presence of H. felis in the mouse stomach was detected by the urease test as described previously. The stomach was excised and cut along the greater curvature. The interior was rinsed with sterile saline to remove the remaining debris. The stomach was then cut along the lesser curvature into halves. One half of the gastric samples was homogenized in 1 mL of 10 mM sodium phosphate buffer (pH 6.5). The homogenate was serially diluted two-fold on a 96-well microtitre plate with the same buffer. The same volume of urease test solution (20 mg/mL urea, 0.05% phenol red and 0.2 mg/mL sodium azide in 10 mM sodium phosphate buffer, pH 6.5) was then added to each well. The plate was sealed and incubated for 16 h at 23°C. The gastric homogenate was judged to be urease negative when no significant colour change developed at any dilution.

The 50% eradication or clearance doses of antimicrobial agents were estimated by the logit method.

Results

To evaluate the contribution of the 7α-methoxy substituent of cephem or 1-oxacephem to in vivo antimicrobial activity, we selected four pairs of compounds, with and without 7α-methoxy substituents (Figure 1) that had MICs within one two-fold dilution of each other for H. felis ATCC 49179. Table I shows the MICs of the compounds selected. While compounds M-1 and H-1 had lower MICs for H. felis than for H. pylori, most other compounds exhibited very similar MICs for both H. felis (0.25–0.5 mg/L) and four strains of H. pylori (0.5–1.0 mg/L). All of the compounds in Table I had higher MICs than amoxycillin.

The time–kill curves of flomoxef and its demethoxy congener were compared. Both compounds killed 99.9% of H. pylori cfu after 24 h at their MIC and had very similar time–kill curves.

The in vivo antibacterial activities of the compounds were evaluated in the mouse infection model (Table II). When the compounds were administered twice on the same
day and *H. felis* was quantified in the stomach the next day (clearance test), all of the 7α-methoxy compounds in Figure 1 showed four-fold or more greater eradication activity than their demethoxy congeners. Compounds other than M-1 and H-1 were administered twice daily for 5 days and the mice were killed 14 days after administration (eradication tests). In these tests, the 7α-methoxy compounds also showed much higher activities.

Intravenous administration of flomoxef resulted in extremely low eradication activity compared with oral administration (Table II). These results, together with the fact that flomoxef is not absorbed orally,

\[1, 5, 11, 15, 16\] indicated that the compound gained direct access to the bacteria in the stomach after oral administration.

### Discussion

Comparison of the clearance or eradication activities of compounds with and without the 7α-methoxy substituent, suggested that this substituent of the β-lactam ring enhanced the *in vivo* anti-*H. felis* activity in the mouse model used. The *in vivo* eradication activities of 7α-methoxy compounds tested in this study were much stronger than those of amoxycillin and clarithromycin\[13\] even though their MICs are higher than those of amoxycillin and clarithromycin. Since the methoxy group can be introduced into the 7α position of most cephems, it may have the potential to improve *in vivo* activities of other cephem antibiotics.

Flomoxef and cefmetazole are parenteral antibiotics that are poorly absorbed after oral administration.\[15, 16\] Thus, the compounds used in this study are likely to have gained access to the bacteria on the gastric mucosa directly from the gastric cavity, not via the bloodstream. Indeed, flomoxef administered intravenously showed much lower *in vivo* activity (Table II). For many antimicrobial agents, there are no practical correlations between MICs and the clinical clearance of *H. pylori*\[5\] and these disadvantages can probably be ascribed to difficulties for antimicrobial agents in accessing *H. pylori* in the stomach. The 7α-methoxy substituent may combat this difficulty by promoting permeability of the compounds through gastric mucus or by helping the compounds to remain in the stomach longer.

### References


### Table II. *In vivo* antibacterial activity of cephems or 1-oxacephems and their 7α-methoxy congeners

<table>
<thead>
<tr>
<th>Compounds</th>
<th>50% Clearance dose (mg/kg/dose)a</th>
<th>50% Eradication dose (mg/kg/dose)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flomoxef</td>
<td>1.00</td>
<td>3.67</td>
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<tr>
<td>Demethoxy-flomoxef</td>
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<td>17.4</td>
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<tr>
<td>1-thia-flomoxef</td>
<td>0.97</td>
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<td>&gt;60.0</td>
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<tr>
<td>Cefmetazole</td>
<td>1.00</td>
<td>3.67</td>
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<tr>
<td>Demethoxy-cefmetazole</td>
<td>5.79</td>
<td>58.8</td>
</tr>
<tr>
<td>M-1</td>
<td>0.47</td>
<td>NT</td>
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<tr>
<td>H-1</td>
<td>3.59</td>
<td>NT</td>
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<tr>
<td>Amoxycillin</td>
<td>1.92</td>
<td>9.12c</td>
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<tr>
<td>Flomoxef (iv)c</td>
<td>&gt;15.0</td>
<td>58.8</td>
</tr>
</tbody>
</table>

a Compounds were administered orally twice a day for 1 day and mice were killed on the following day.

b Compounds were administered orally twice a day for 5 days and mice were killed 14 days after administration.

c Eradication dose of amoxycillin has been reported previously.\[13\]

NT, not tested.
7α-Methoxy substituent enhances *H. felis* eradication


