Bactericidal effect of plaunotol, a cytoprotective antiulcer agent, against Helicobacter pylori

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In order to investigate the bactericidal effect of plaunotol, an oily antiulcer agent, against Helicobacter pylori, comparative studies were conducted using its derivatives, M-4, M-5, and M-6, whose hydrophobicity decreased in the order of plaunotol > M-6 > M-5 > M-4 by log P determination. Plaunotol rapidly reduced the viability of H. pylori in vitro, and cell death was associated with cell lysis. In addition, plaunotol showed eightfold stronger bactericidal activity against H. pylori than M-6 and M-5, while the compound with the lowest hydrophobicity, M-4, showed no bactericidal activity. The bactericidal activities of plaunotol and its derivatives were related to the hydrophobicity of these compounds. To investigate a possible interaction between these compounds and the cell membrane of H. pylori, their effects on liposomal membranes prepared from phosphatidylethanolamine and cardiolipin, which are known to be present in the membrane of H. pylori, were determined by detection of glucose release from the liposomes. Plaunotol showed eight-fold higher activity than M-6 and M-5, while M-4 showed no activity. The effects of plaunotol and its derivatives on liposomal membrane were therefore related to their bactericidal activities. In addition, it was confirmed that the bactericidal effect of plaunotol against H. pylori was neutralized by the liposomal membrane, and that plaunotol led to an increase in permeability of the membrane, as evidenced by measurement of the leakage of 260 nm absorbing-material from H. pylori. These results suggest that the bactericidal effect of plaunotol against H. pylori is due to the interaction between this compound and the bacterial cell membrane.

Introduction

Plaunotol, an acyclic diterpene alcohol, is often used as a cytoprotective antiulcer agent for gastritis and gastric ulcer in Japan (Kobayashi et al., 1982). This compound acts by enhancing host defences such as gastric mucosal blood flow, gastric bicarbonate secretion and mucus secretion, due to an increased prostaglandin content in the gastric mucosa (Ushiyama et al., 1985). Recently, it has been noted that plaunotol also shows antibacterial activity against Helicobacter pylori, which is associated with gastritis (Morris & Nicholson, 1987), duodenal ulcer (Graham, 1991; Calam, 1994), gastric ulcer (Blaser, 1987), and the epidemic form of gastric carcinoma (Forman et al., 1993). Karita et al. (1994) have reported that triple therapy, consisting of plaunotol, amoxycillin and metronidazole, is clinically effective in eradicating H. pylori with no recurrence of ulcer, although treatment with a single antimicrobial agent has failed to eradicate this
organism (Glupczynski et al., 1987). In our previous study, we demonstrated that plaunotol had the strongest antibacterial activity against H. pylori among the cytoprotective antiulcer agents. In addition, plaunotol induced a rapid reduction of culture turbidity with an extensive loss of viability, causing autolysis and deformation of treated cells (Koga et al., 1996). These results suggest that plaunotol might interact with a cell surface component, but the mechanism of its antibacterial effect is still unclear. In order to investigate this, comparative studies were conducted using its derivatives, M-4, M-5, and M-6, whose hydrophobicity decreased in the order of plaunotol > M-6 > M-5 > M-4 by log P determination.

**Materials and methods**

**Agents**

Plaunotol (mol.wt 306.5) was extracted from a Thai medical plant called plau-noi, by Sankyo Co., Ltd. Its derivatives, which showed different hydrophobicities, M-4 (mol.wt 350.4), M-5 (mol.wt 322.5), and M-6 (mol.wt 320.5), were synthesized by Sankyo, as shown in Figure 1. All of the compounds were dissolved in dimethyl sulfoxide (DMSO) and then diluted in this to give the desired concentrations. L-α-phosphatidylethanolamine (PE) (Sigma Chemical Co., St. Louis, USA) and cardiolipin (CL) (Sigma) were used for the preparation of liposomes. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Tokyo Kasei Kogyo Co., Ltd, Tokyo, Japan) was used as a silylating reagent.

**Bacteria**

*H. pylori* CPY 2052 was kindly supplied by Dr M. Karita (School of Medicine, Yamaguchi University, Yamaguchi, Japan). The MIC of plaunotol against this strain was the same as the MIC₉₀ of plaunotol against a standard strain and 10 clinical isolates (Koga et al., 1996). Stock cultures were stored at −80°C in brucella broth (Cockeysville, MD, USA) supplemented with 2% heat-inactivated fetal bovine serum (FBS). Stock cultures containing about 3 × 10⁶ cfu/mL of *H. pylori* CPY 2052 were inoculated into

![Figure 1. Structures of plaunotol and its derivatives.](image)
200 mL of brucella broth containing 2% FBS in 300 mL sterile glass Erlenmyer flasks, to give about $10^4$ cfu/mL. After microaerobic incubation for 40 h at 37°C on a gyratory shaker at 110 rpm, the logarithmic growth culture was harvested by centrifugation at room temperature for 10 min at 950g, and the cells were washed and suspended in 50 mM phosphate-buffered saline (PBS, pH 7.2), to give a slightly turbid suspension ($10^7$ cfu/mL) (Koga et al., 1996). The bacterial suspension was used for the following experiments.

**Lipophilicity**

Lipophilicity is expressed as the logarithm of the partition coefficient ($\log P$) in n-octanol/phosphate buffer (pH 7.2). The flask-shaking method used was as previously described (Cloux et al., 1988). The buffer phase was extracted by ethyl acetate, and concentrated 100-fold. The octanol and buffer phase were silylated with BSTFA and pyridine. The analytes in both phases were determined by gas-liquid chromatography (GC14A, Shimadzu Corporation, Kyoto, Japan) using a methylsilicate glass column (40 cm x 1.2 mm internal diameter). Oven temperatures were 250°C for plaunotol, M-5, and M-6, and 260°C for M-4. Injection and detector temperatures were 320°C. The carrier gas was helium (20 mL/min for plaunotol, M-5, and M-6, and 45 mL/min for M-4).

**Determination of MICs**

Stock cultures of bacteria were grown on brain heart infusion agar (Difco Laboratories, Detroit, USA) supplemented with 7% horse blood, at 37°C for 3 days, in GasPak jars (Becton Dickinson Microbiology Systems, Cockeysville, USA) with Campy Paks (Becton Dickinson). The MICs were measured on brain heart infusion blood agar by the two-fold dilution method. The inoculum size of bacteria was $10^4$ cfu/spot.

**Bactericidal assay**

The bactericidal effects of plaunotol and its derivatives were determined using an in-vitro killing assay (Knapp & Melly, 1986). Nine millilitres of the bacterial suspension was mixed with 1 mL of a stock solution of these compounds dissolved in 1% DMSO. For the control, 9 mL of the suspension was mixed with 1 mL of 1% DMSO. The mixture was kept at room temperature for 1 h and the viable cells were then determined by the plate count technique. Serial ten-fold dilutions were made in brucella broth supplemented with 2% FBS, and a 0.1 mL portion of each, from neat to $10^5$ dilution, was plated in duplicate onto brain heart infusion agar containing 10% horse serum and 20 mg/L of 2, 3, 5-triphenyltetrazolium chloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (Queiroz, Mendes & Rocha, 1987). Colonies were counted after 4 days of incubation at 37°C under microaerobic conditions and were expressed in cfu/mL. The turbidity was monitored at 550 nm in a Coleman spectrophotometer (Coleman Instruments, Maywood, USA). The changes in turbidity were expressed as the ratio of the initial turbidity.
Preparation of liposomes

Liposomes were prepared by mixing chloroform solutions of PE and CL. The molar ratio of PE:CL was 1:0.2 (Imai, Inoue & Nojima, 1975). The solvent was removed under reduced pressure, and the dried film was dispersed in PBS. When liposomes containing glucose were prepared, the dried film was dispersed in 50 mM Tris-HCl (pH 7.2) with 0.3 mM glucose (Kinsky et al., 1969).

Assay for glucose release from liposomes by plaunotol and its derivatives

Liposomes were used after the untrapped glucose was removed by dialysis against 50 mM Tris-HCl (pH 7.2) with 0.15 M NaCl. The final concentration of the liposome suspension was 500 μM. Four hundred and fifty μL of this liposome suspension and respectively, 50 μL of each of plaunotol and its derivatives dissolved in 10% DMSO (to give a final concentration of 1% DMSO), were added to tubes. After incubation at room temperature for 1 h, the reaction mixtures were centrifuged at 10000g for 30 min (4°C), and the glucose content of the clear supernatant was monitored spectrophotometrically by the change in 578 nm absorbance, indicating oxidation of ammonium salt of 2,2'-azino-di-3-ethyl-benzothiazoline-6-sulphonic acid in the presence of glucose oxidase and peroxidase (Miskiewicz, Arnett & Simon, 1973). The extent of plaunotol-induced release of the glucose marker is expressed as per cent maximum glucose release, and was calculated from the expression: ((glucose release in the presence of plaunotol – blank control)/(total amount of glucose – blank control)) × 100. The total amount of glucose trapped in the liposomes was determined by adding 0.1 mL of liposome preparation to 0.1 mL of 10% Triton X-100, heating in a boiling-water bath for a few seconds, and cooling to room temperature prior to glucose assay.

Investigation of influences of liposomes on plaunotol-induced bactericidal effect

Eight millilitres of bacterial suspension was mixed with 1 mL of liposome suspension at the concentrations 0, 17.5 or 35.1 μM, before adding 1 mL of 196 μM plaunotol solution. The changes in viability and turbidity were determined as described above.

The effect of plaunotol on leakage of 260 nm-absorbing material from H. pylori

This was measured according to the method described by Greenway & Dyke (1979) in the presence and absence of plaunotol. Bacteria grown to mid-exponential phase were harvested by centrifugation for 10 min at 950g, washed in ice-cold 50 mM Tris-HCl (pH 7.5), and resuspended in similar buffer to give an absorbance value of 1.5 at 550 nm. The bacterial suspensions were then incubated at 30°C with plaunotol at 40.6, 81.6, and 163 μM dissolved in ethanol. The final concentration of ethanol was 1% v/v. After 30 min, 2 mL of these suspensions was cooled rapidly to 0°C and centrifuged. One mL of the supernatant was then carefully removed, and the absorbance at 260 nm was measured. The total amount of 260 nm-absorbing material present in the bacteria was measured by boiling bacterial samples for 15 min. The samples were then cooled and centrifuged, and the absorbance at 260 nm of the supernatant was measured. The release of material was expressed as a percentage of the material released by boiling.
Bactericidal effect of plaunotol against \textit{H. pylori}

Results

Lipophilicity

Plaunotol showed the highest hydrophobicity among the compounds. Log $P$ of plaunotol was 4.7. The log $P$ of the derivatives were as follows: 0.03 for M-4, 3.4 for M-5, and 3.6 for M-6. The hydrophobicity of M-6 decreased by replacement of a hydroxyl side chain of plaunotol with a carboxyl side chain; moreover, that of M-5 decreased by decreasing the number of double bonds, and that of M-4 decreased by replacement of a methyl side chain of M-6 with a carboxyl side chain.

Antibacterial activities of plaunotol and its derivatives against \textit{H. pylori}

Plaunotol showed the highest antibacterial activity against \textit{H. pylori} CPY 2052, with an MIC of 20.4 $\mu$M. The MICs of the derivatives were as follows: $>571$ $\mu$M for M-4, 155 $\mu$M for M-5, and 78.0 $\mu$M for M-6.

Bactericidal effects of plaunotol and its derivatives

Figure 2 shows the changes in viability and culture turbidity of \textit{H. pylori} CPY 2052 exposed to plaunotol and its derivatives, M-4, M-5, and M-6, respectively. Plaunotol at 19.6 $\mu$M rapidly reduced bacterial viability and culture turbidity. M-6 at 150 and 300 $\mu$M and M-5 at 149 and 298 $\mu$M, respectively, also rapidly reduced bacterial viability and culture turbidity. M-4, however, showed little bactericidal activity at the concentration of 274 $\mu$M.

Glucose release from liposomes by plaunotol and its derivatives

Figure 3 illustrates the glucose release from liposomes induced by plaunotol, M-4, M-5, and M-6, respectively. It can be seen in Figure 3(a) that 65.3 $\mu$M of plaunotol resulted in a release of about 20% of glucose from liposomes. A two-fold increase in the plaunotol concentration resulted in a release of over 80% of the glucose. Figure 3(b) shows that 1830 $\mu$M of M-4 resulted in no release of glucose from liposomes. Figure 3(c) shows that 496 $\mu$M of M-5 resulted in a release of about 20% of glucose from liposomes. A two-fold increase in M-5 concentration resulted in a release of over 50% of the glucose. Figure 3(d) shows that 499 $\mu$M of M-6 resulted in a release of about 20% of glucose from liposomes. A two-fold increase in M-6 concentration resulted in a release of over 70% of the glucose.

Effects of liposomes on plaunotol-induced bactericidal effect

Reductions in both viability and turbidity of bacteria were observed in the presence of plaunotol at the concentration of 19.6 $\mu$M. Addition of liposomes at 3.51 $\mu$M neutralized the plaunotol-induced bactericidal effect. However addition at 1.75 $\mu$M had little effect (Figure 4).

The effect of plaunotol on leakage of 260 nm-absorbing material from \textit{H. pylori}

Plaunotol at 40.6, 81.6, and 163 $\mu$M showed 0.9%, 19.7% and 54.0% release,
Figure 2. Bactericidal effects of plaunotol and its derivatives. *H. pylori* CPY 2052 was exposed to plaunotol (a), M-4 (b), M-5 (c), and M-6 (d). (a) Plaunotol: 0 (●), 9.79 (■), and 19.6 μM (▲); (b) M-4: 0 (●), 68.5 (○), 137 (△), and 274 μM (□); (c) M-5: 0 (●), 74.4 (○), 149 (△), and 298 μM (□); and (d) M-6: 0 (●), 74.9 (○), 150 (△), and 300 μM (□). After further incubation, the viability and culture turbidity were determined at each point.
Bactericidal effect of plaunotol against *H. pylori*

![Graphs showing glucose release from liposomes by plaunotol and its derivatives](image)

Figure 3. Glucose release from liposomes by plaunotol and its derivatives. The results depicted in (a), (b), (c), and (d) were the effect of plaunotol, M-4, M-5, and M-6, respectively. Liposomes were prepared from mixtures containing PE and CL (1:0.2, molar ratio). Liposomes were used at a concentration of 450 μM. Glucose release was determined spectrophotometrically as described in the text. The reactions were run in 50 mM Tris-HCl at pH 7.2.

respectively. This release of 260 nm-absorbing material was dependent on the plaunotol concentration.

Discussion

Some hydrophobic compounds are known to interact with bacterial membrane lipids (Lien, Hansch & Anderson, 1968; Hansch & Glave, 1970), and it has been reported that their bactericidal effects are linearly related to their hydrophobicity. Therefore, we used derivatives of plaunotol, M-4, M-5, and M-6, which showed different hydrophobicities. Plaunotol, which is the most hydrophobic among the compounds, had the strongest bactericidal activity against *H. pylori*. In addition, the bactericidal effects of plaunotol and its derivatives were linearly related to their hydrophobicity, suggesting that these compounds interact with the cell membrane of *H. pylori*.

Polyunsaturated fatty acids have bactericidal activity, and are known to be inserted into the bacterial phospholipid bilayer (Galbraith et al., 1971; Mabrey & Sturtevant, 1977). Study of fatty acids and their derivatives has revealed that alcohols have higher bactericidal effect than fatty acids of the same carbon length; that stronger bactericidal
Figure 4. Effect of liposomes on the plaunotol-induced bactericidal effect. *H. pylori* CPY 2052 was incubated with plaunotol at concentrations of 0 (■) and 19.6 μM (△), and with plaunotol plus phospholipids at concentrations of 1.75 (●) and 3.51 μM (○). After further incubation, the viability and culture turbidity were determined at each point.

activity is associated with more double bonds; and that dicarboxylic acids have significantly less activity than the corresponding chain length single carboxylic acids (Wyss, Ludwig & Joiner, 1945; Kabara et al., 1972; Kondo & Kanai, 1977). Plaunotol, which is an alcohol, had a greater bactericidal effect than M-6, which is an organic acid. M-6, which has four double bonds, had a greater bactericidal effect than M-5, which has three double bonds. M-4, which is a dicarboxylic acid, showed no bactericidal effect against *H. pylori*. This tendency resembles that of fatty acids and their derivatives, although the structure of plaunotol is different from that of a fatty alcohol. The interaction between chemical structure and hydrophobicity is unclear. One possibility is that plaunotol was incorporated into *H. pylori* phospholipid.

The cell membrane phospholipid of *H. pylori* consists of PE (57.7%), CL (21.5%), phosphatidylglycerol (12.6%), phosphatidylserine (2.0%), and phosphatidylcholine (1.6%) (Hirai et al., 1993). In order to investigate the relationship between plaunotol and its derivatives and the cell membrane of *H. pylori*, we examined the effects of the compounds on the liposome membranes prepared from PE and CL, by measuring glucose release from the liposomes. Plaunotol disrupted the liposome membranes at 131 μM. M-6 and M-5 showed about eightfold less activity than plaunotol. The strengths of effects of the compounds on the liposome membranes were closely related to their bactericidal effect against *H. pylori*. These results suggested that plaunotol interacted with the cell membrane.

The concentration of plaunotol that showed an effect on liposome membranes (131 μM), was higher than the concentration that showed a bactericidal effect against *H. pylori* (19.6 μM). The reason for this is unknown, but it may be related to the molar ratio of the compounds and phospholipid of the cell membrane or liposome membranes. According to HsuChen & Feingold (1973), 1 molecule of polymyxin B per five molecules of phospholipid released over 80% of glucose in liposomes. In the present study, the molar ratio of plaunotol and phospholipid using liposomes is about one molecule per 3.5 molecules of phospholipid, indicating that the effect of plaunotol on liposome membranes was similar to that of polymyxin B. This suggests again that plaunotol interacts with the cell membrane. The concentration of phospholipid, at 450 μM, used
in this study was very high, but this was necessary to detect glucose release from the liposomes.

In order to confirm that plaunotol interacts with the cell membrane, we examined whether liposomes neutralized the plaunotol-induced bactericidal effect. This was demonstrated by the addition of liposomes at 3.51 μM. It is probable that the neutralizing effect of liposomes was due to the formation of extracellular complexes with plaunotol. Kodicek & Worden (1945) reported that a membrane component, lecithin, showed protective activity against the bactericidal activity of fatty acids, by forming complexes with them.

The antibacterial activity, as well as bactericidal effects, of plaunotol and its derivatives against H. pylori were related to the strength of their effects on the liposome membranes, indicating that their antibacterial activity may be due to their effects on bacterial membrane at low concentrations. These compounds may therefore be incorporated into the cell membrane, resulting in alteration of its permeability. Thompson, Cockayne & Spiller (1994) and Khulusi et al. (1995) have reported that polyunsaturated fatty acids showed antibacterial activity against H. pylori by incorporation into the cell membrane. Low concentrations of fatty acids decrease the entry of essential nutrients and show an antibacterial effect. Further incorporation of a larger concentration of fatty acids leads to cell lysis and bactericidal effects.

Further evidence for the effect of plaunotol on the permeability of H. pylori membrane is the dose-dependent loss of 260 nm-absorbing material. Greenway & Dyke (1979) and Raychowdhury, Goswami & Chakrabarti (1985) have shown that chemicals that cause alteration in membrane permeability increase the leakage of 260 nm-absorbing material from bacterial preparations. This material was identified as consisting of nucleotides and amino acids, and represented pool material (Salton, 1951). Greenway & Dyke (1979) considered that before the 260 nm-absorbing material was released from treated bacteria, low-molecular-weight substances such as hydrogen ions were released. Raychowdhury et al. (1985) have indicated that increased membrane permeability may be due to incorporation of polyunsaturated fatty acids into membrane lipids and a resultant change in the fluidity of the membrane. Plaunotol incorporated into membranes alters their permeability, and may also change the fluidity of the membrane.

Based on these experimental data, we suggest that the bactericidal effect of plaunotol against H. pylori might be due to a change in fluidity, with associated increased membrane permeability, as a result of the interaction between this compound and the bacterial cell membrane. Bactericidal effects on other systems such as membrane protein cannot be excluded (Sheu & Freese, 1972), and further investigation may be necessary.

References


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