SECRETORY MECHANISMS OF GREPAFLOXACIN AND LEVOFLOXACIN IN THE HUMAN INTESTINAL CELL LINE CACO-2

HIROAKI YAMAGUCHI, IKUKO YANO, YUKIYA HASHIMOTO, and KEN-ICHI INUI

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan

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ABSTRACT

Grepafloxacin and levofloxacin transport by Caco-2 cell monolayers was examined to characterize the intestinal behavior of these quinolones. The levels of transcellular transport of \[^{14}C\]\textregistered grepafloxacin and \[^{14}C\]\textregistered levofloxacin from the basolateral to the apical side were greater than those in the opposite direction. The unidirectional transport was inhibited by the presence of excess unlabeled quinolones, accompanied by increased accumulation. The inhibitory effects of cyclosporin A plus grepafloxacin on basolateral-to-apical transcellular transport and cellular accumulation of \[^{14}C\]\textregistered grepafloxacin were comparable to those of cyclosporin A alone, indicating that the transport of grepafloxacin across the apical membrane was mainly mediated by P-glycoprotein. On the other hand, basolateral-to-apical transcellular transport of \[^{14}C\]\textregistered levofloxacin in the presence of cyclosporin A was decreased by unlabeled levofloxacin, grepafloxacin, and enoxacin, accompanied by significantly increased cellular accumulation. The organic cation cimetidine, organic anion \(p\)-aminohippurate, and the multidrug resistance-related protein (MRP) modulator probenecid did not affect the transcellular transport of \[^{14}C\]\textregistered grepafloxacin or \[^{14}C\]\textregistered levofloxacin in the presence of cyclosporin A. The basolateral-to-apical transcellular transport of levofloxacin in the presence of cyclosporin A showed concentration-dependent saturation with an apparent Michaelis constant of 5.6 mM. In conclusion, these results suggested that basolateral-to-apical flux of quinolones was mediated by P-glycoprotein and a specific transport system distinct from organic cation and anion transporters and MRP.

Quinolone antibacterial drugs have been clinically used to treat various bacterial infections. Most quinolone antibacterial drugs are rapidly absorbed from the intestine, with a bioavailability of close to 90%, and then penetrate well into most body tissues and fluids (Sörgel et al., 1989a; Wollson and Hooper, 1989). However, the bioavailabilities of ciprofloxacin and norfloxacin are 50 to 80% and 30 to 40%, respectively (Sörgel et al., 1989a; Soppe et al., 1992). Ofloxacin was shown to be absorbed by a carrier-mediated mechanism using intestinal perfusion studies (Prieto et al., 1988). Absorption of enoxacin was suggested to be dependent on the membrane potential by rat intestinal brush-border membrane vesicles (Iseki et al., 1993; Hirano et al., 1994). On the other hand, it has been suggested that the intestine plays an important role not only as an absorption tissue but also as an elimination tissue or absorption barrier. At least 10.6% of i.v. administered ciprofloxacin (200 mg) is eliminated by intestinal secretion (Sörgel et al., 1989b, 1991), and temafloxacin shows significant gastrointestinal secretion into feces in humans (Granneman et al., 1991). The pharmacokinetics of ciprofloxacin was suggested to involve one or more active transport mechanisms in the intestine, as well as at the biliary levels, in rats (Dautrey et al., 1999). Thus, the precise mechanisms underlying the intestinal absorption or secretion of quinolones remain to be clarified.

The human colon adenocarcinoma cell line Caco-2 has been used as a model to study the epithelial function of the intestine. This cell line forms confluent monolayers of well-differentiated enterocyte-like cells with functional properties of transporting epithelia (Hidalgo et al., 1989). This cell line has been used in a number of studies to characterize the intestinal transport of quinolones. Cornet et al. (1997) demonstrated that the uptake of sparflaxin was a passive diffusion mechanism by the brush-border membrane of Caco-2 cells. Griffiths et al. (1994) showed that transepithelial secretion of quinolones involved common mechanisms of accumulative transport at the basolateral membrane followed by facilitated exit across the apical membrane in Caco-2 cells. Using the same model, Cormet-Boyaka et al. (1998) demonstrated a net secretory flux of sparflaxin and showed that sparflaxin transport from the apical to the basolateral membrane increased in the presence of verapamil, a P-glycoprotein modulator. Reported transport mechanisms for quinolones in the Caco-2 cell line were different between investigators and the quinolones used.
We previously reported that ofloxacin interacts with the organic cation transport system in rat renal brush-border membrane (Okano et al., 1990). We also reported that levofloxacin and grepafloxacin are specifically transported from the basolateral to the apical side in the kidney epithelial cell line LLC-PK₁ and have higher affinity for the transport system on the apical membrane, a system distinct from the H⁺/organic cation antiport system (Matsuo et al., 1998). Grepafloxacin and levofloxacin are absorbed with good bioavailability, 72% for grepafloxacin (Effthymiopoulos et al., 1997) and approximately 100% for levofloxacin (Fish and Chow, 1997). Intestinal and kidney epithelia frequently have the same transporters, for example, peptide transporter PEPT1 (Liang et al., 1995; Saito et al., 1995) and glucose transporter SGLT1 (Hediger and Rhoads, 1994).

In this study, the transport characteristics of grepafloxacin and levofloxacin by Caco-2 cells were examined in comparison with transport by LLC-PK₁. We investigated whether carrier-mediated processes are involved in the transport of quinolone antibacterial drugs and what kinds of transporters contribute to transport in Caco-2 cells.

Experimental Procedures

Materials. D-[3H]Mannitol (828.8 GBq/mmol) was purchased from NEN Life Science Products, Inc. (Boston, MA). [14C]Grepafloxacin (1.17 GBq/mmol) and unlabeled grepafloxacin were kindly supplied by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), [14C]Levo-

Grepafloxacin and levofloxacin are absorbed with good bioavailability, 72% for grepafloxacin (Effthymiopoulos et al., 1997) and approximately 100% for levofloxacin. In the present study, Caco-2 cells were used between passages 33 and 49.

Measurements of Transcellular Transport, Cellular Accumulation, and Efflux. Transcellular transport and accumulation of [14C]grepafloxacin and [14C]levofloxacin were measured using monolayer cultures grown in Transwell chambers. The composition of incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 CaCl₂, 0.5 MgCl₂, 5 d-glucose, 5 HEPES (pH 7.4). The pH of the medium was adjusted with a solution of HCl or NaOH. After removal of the culture medium from both sides of the monolayers, the cell monolayers were preincubated with incubation medium (2 ml each side) at 37°C for 15 min. Then, 2 ml of incubation medium containing the radioactive substrate was added to either the basolateral or apical side, with 2 ml of nonradioactive incubation medium to the opposite side, and the monolayers were incubated for specified periods at 37°C. D-[3H]Mannitol (5 μM, 22.8 kBq/ml), a compound that is not transported by the cells, was used to calculate the paracellular flux and the extracellular trapping of [14C]grepafloxacin (5 μM, 5.8 kBq/ml) and [14C]levofloxacin (5 μM, 5.4 kBq/ml). For transport measurements, aliquots of the incubation medium on the other side were taken at specified times, and the radioactivity was counted.

For accumulation studies, the medium was removed by aspiration at the end of the incubation period, and the monolayers were rapidly washed twice with 2 ml of ice-cold incubation medium on each side. The filters with monolayers were detached from chambers, the cells on the filters were solubilized with 0.5 ml of 1 N NaOH, and the radioactivity in aliquots was counted. The radioactivity of the collected medium and the solubilized cell monolayers was determined in ACS II (Amersham International, Buckinghamshire, UK) by liquid scintillation counting.

For efflux studies, the monolayers were preincubated at 37°C for 15 min with 2 ml of the incubation medium. After removal of the medium, cells were incubated with 2 ml of incubation medium containing [14C]grepafloxacin (5 μM) or [14C]levofloxacin (25 μM) and D-[3H]mannitol (5 μM) for 30 min. After incubation, the medium was aspirated and the monolayers were rapidly rinsed twice with 2 ml of ice-cold incubation medium. The cells were then incubated with the medium for a specified period at 37°C or 4°C. Aliquots of the incubation medium on both apical and basolateral sides were taken, and the radioactivity was counted. To measure the amount of residual quinolones remaining in the cells, the medium was aspirated and the monolayers were rapidly rinsed twice with 2 ml of ice-cold incubation medium. The cells were solubilized in 0.5 ml of 1 N NaOH, and the radioactivity was determined as described above.

Measurement of Intracellular Volume. The equilibrium accumulation of sulfanilamide was determined according to the general procedures described for the accumulation experiment, and equilibrium was reached within 5 min. The intracellular volume was calculated from the equilibrium accumulation (15 min) and the external concentration of sulfanilamide (Saito et al., 1986).

Calculation of Clearance. Initial uptake clearance and efflux clearance were calculated by dividing the amount of drug transport at 1 min by the extracellular and intracellular drug concentrations at 0 min, respectively. Intracellular drug concentration was calculated...
by dividing the intracellular amount of the drug by the intracellular volume of Caco-2 cells.

**Protein Assay.** The protein contents of the cell monolayers solubilized in 1 N NaOH were determined by the method of Bradford (1976) using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) using bovine \( \gamma \)-globulin as the standard.

**Statistical Analysis.** Statistical significance of differences between mean values was calculated using the nonpaired \( t \) test. Multiple comparisons were performed with Scheffe’s test after ANOVA. \( P < .05 \) was considered significant.

## Results

**Transcellular Transport and Cellular Accumulation of Grepafloxacin and Levofloxacin.** We first evaluated the transcellular transport and cellular accumulation of \(^{14}C\)grepafloxacin and \(^{14}C\)levofloxacin by Caco-2 cell monolayers. The basolateral-to-apical transport of each quinolone was much greater than the apical-to-basolateral transport at each sampling point (Fig. 2, A and C). Transcellular transport of \(^{14}C\)grepafloxacin and \(^{14}C\)levofloxacin from the basolateral to the apical side was significantly decreased and that in the opposite direction was significantly increased except at 15 min for grepafloxacin and at 15 and 60 min for levofloxacin in the presence of an excess of each unlabeled quinolone. The levels of cellular accumulation of \(^{14}C\)grepafloxacin and \(^{14}C\)levofloxacin from both apical and basolateral sides were significantly increased in the presence of an excess of each unlabeled quinolone (Fig. 2, B and D, \( P < .05 \)).

The basolateral-to-apical transcellular transport of \(^{14}C\)grepafloxacin was 2.5-fold greater than that of \(^{14}C\)levofloxacin, and levels of cellular accumulation of \(^{14}C\)grepafloxacin from both sides were 5- to 10-fold greater than those of \(^{14}C\)levofloxacin.

**Uptake from Apical and Basolateral Membranes.** The basolateral-to-apical transcellular transport consists of entry into the cell across the basolateral membrane and expulsion across the apical membrane. To elucidate which transmembrane transport contributes to the unidirectional transport of quinolones, we measured the time courses of \(^{14}C\)grepafloxacin and \(^{14}C\)levofloxacin uptake from apical and basolateral membranes. Uptake of \(^{14}C\)grepafloxacin from the apical membrane was greater than that from the basolateral membrane at each sampling point (Fig. 3A).

\(^{14}C\)Levofloxacin uptake from the basolateral membrane was greater than that from the apical membrane before 5 min, and was almost the same at 15 min due to efflux through the apical membrane (Fig. 3B). Grepafloxacin uptake from the apical side was 10-fold greater than that of levofloxacin at 1 min.

**Efflux of Grepafloxacin and Levofloxacin.** Next, we examined the efflux characteristics of \(^{14}C\)grepafloxacin and \(^{14}C\)levofloxacin from Caco-2 cell monolayers. Efflux of each quinolone from the apical membrane was much greater than that from the basolateral membrane, and this was not ap-

![Fig. 2. Transcellular transport and cellular accumulation of grepafloxacin (A, B) and levofloxacin (C, D) by Caco-2 cell monolayers. The monolayers were incubated at 37°C with 5 \( \mu \)M \(^{14}C\)grepafloxacin or \(^{14}C\)levofloxacin added to either the basolateral ([]) or the apical (\( \triangle \)) side in the absence (open symbols) or presence (solid symbols) of 1 mM grepafloxacin or 5 mM levofloxacin. After incubation, the radioactivity on the opposite side was measured. After a 60-min transport measurement, accumulation was determined in the absence (open columns) or presence (dotted columns) of 1 mM grepafloxacin or 5 mM levofloxacin. Each point or column represents the mean \( \pm \) S.E. of three monolayers. \( * \) \( P < .05 \), significantly different from corresponding open symbol.](image)
parent at 4°C (Fig. 4, A and C). The intracellular levels of \([^{14}C]\)grepafloxacin and \([^{14}C]\)levofloxacin decreased rapidly with time at 37°C and were significantly less than those at corresponding time points at 4°C (Fig. 4, B and D).

**Clearance of Grepafloxacin and Levofloxacin Transport.** Using the results from the uptake and efflux studies (Figs. 3 and 4), we calculated the initial transport clearance of grepafloxacin and levofloxacin across each membrane by Caco-2 cell monolayers. The intracellular volume of Caco-2 cells obtained from the sulfanilamide accumulation experiment was \(4.05 \pm 0.08 \mu l/mg\) of protein (mean ± S.E. of three monolayers). As shown in Table 1, the clearance of grepafloxacin from the apical medium to cell (CLA-C) was significantly greater than that from the basolateral medium (CLB-C) or the clearance from cell to the apical medium (CLC-A), whereas no significant difference was observed between CLA-C and CLB-C or CLC-A of levofloxacin. Furthermore, the levels of clearance from cells to the apical medium (CLC-A) of grepafloxacin and levofloxacin were significantly greater than those to the basolateral medium (CLC-B). The levels of clearance from the basolateral medium to cell (CLB-C) of both quinolones were much greater than those from cell to the basolateral medium (CLC-B).

**Effects of Various Compounds on Grepafloxacin Transcellular Transport and Cellular Accumulation.** We examined the effects of quinolones, the organic cation cimetidine (Okano et al., 1990), the organic anion \(p\)-aminohippurate (Hori et al., 1993), the multidrug resistance-
related protein (MRP) modulator probenecid (Hollo et al., 1996; Barrand et al., 1997), and the P-glycoprotein inhibitor cyclosporin A (10 μM) on the unidirectional transport of grepafloxacin. Cyclosporin A (10 μM) was enough to inhibit the P-glycoprotein-mediated transport (Ito et al., 1997). Unlabeled grepafloxacin, levofloxacin, and cyclosporin A significantly decreased [14C]grepafloxacin transport, and unlabeled grepafloxacin significantly increased the accumulation (Table 2). The effects of cyclosporin A plus grepafloxacin on transepithelial transport and accumulation were almost the same as those of cyclosporin A alone. However, probenecid, cimetidine, and p-aminohippurate affected neither the transcellular transport nor accumulation of [14C]grepafloxacin.

**Effects of Various Compounds on Transcellular Transport and Cellular Accumulation of Levofloxacin in the Presence of Cyclosporin A.** In contrast to grepafloxacin, the basolateral-to-apical transport of levofloxacin was not affected by cyclosporin A (10 μM) (control, 86.8 ± 2.7; cyclosporin A, 78.0 ± 2.6 pmol/cm²/60 min; mean ± S.E. of three monolayers). Therefore, another transport system distinct from P-glycoprotein may contribute to the unidirectional transport of levofloxacin. The effects of various compounds on the transepithelial transport and cellular accumulation of levofloxacin were examined in the presence of cyclosporin A (10 μM) to avoid the contribution of P-glycoprotein-mediated transport. The transepithelial transport of [14C]levofloxacin was decreased by all quinolones examined, accompanied by significant increases in cellular accumulation (Table 3). However, probenecid, cimetidine, and p-aminohippurate did not affect transcellular transport or cellular accumulation of [14C]grepafloxacin.

**Concentration Dependence of Grepafloxacin Transcellular Transport.** To characterize the basolateral-to-apical transport of quinolones, the concentration dependence of grepafloxacin transcellular transport by Caco-2 cell monolayers was examined. Figure 5A shows the basolateral-to-apical transcellular transport of grepafloxacin at 60 min as a function of substrate concentration. The relationship between concentration and the basolateral-to-apical flux rate appeared to approach saturation. The kinetic parameters were calculated using the following equation: \( V = \frac{V_{\max} \cdot S}{K_m + S} \), where \( V \) is the transport rate (nmol/cm² per 60 min), \( S \) is the substrate concentration in the medium (mM), \( K_m \) is the Michaelis-Menten constant (mM), and \( V_{\max} \) is the maximum velocity.

**Discussion**

The gastrointestinal secretion of quinolone antibacterial drugs is an important route, although these drugs are well absorbed from the intestine (Sörge et al., 1991). The detailed mechanisms involved in the intestinal transport of quinolones are still unknown. In our study, to investigate the mechanisms of intestinal absorption and secretion of quinolones, the transport characteristics of grepafloxacin and levofloxacin were examined by Caco-2 cell monolayers. Both grepafloxacin and levofloxacin showed much higher levels of transport from the basolateral to the apical side than in the opposite direction, which corresponds to intestinal secretion (Fig. 2, A and C). This unidirectional transport was decreased in the presence of the corresponding unlabeled quinolone, accompanied by increased accumulation (Fig. 2). The \( \text{CL}_{C,A} \) of grepafloxacin and levofloxacin was much larger than \( \text{CL}_{C,B} \), although \( \text{CL}_{B,C} \) was comparable to or less than \( \text{CL}_{A,C} \) (Table 1). These results indicated that the unidirectional transport of grepafloxacin and levofloxacin was due to expulsion across the apical membrane and that the specific transport from cells to the apical side existed for both quinolones in Caco-2 cells. In addition, because the \( \text{CL}_{B,C} \) values of both quinolones were significantly larger than \( \text{CL}_{C,B} \) (Table 1), an accumulative mechanism at the basolateral membrane may exist in Caco-2 cells. However, the possibility that the intracellular binding of quinolones caused smaller \( \text{CL}_{C,B} \)

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**Table 1**

Effect of various compounds on the transcellular transport and cellular accumulation of levofloxacin by Caco-2 cell monolayers in the presence of cyclosporin A.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transcellular Transport</th>
<th>Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/cm²/60 min</td>
<td>pmol/mg of protein/60 min</td>
</tr>
<tr>
<td>Control</td>
<td>94.3 ± 2.8</td>
<td>16.8 ± 0.6</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>77.8 ± 2.2*</td>
<td>26.3 ± 0.9*</td>
</tr>
<tr>
<td>Grepafloxacin</td>
<td>69.5 ± 3.3*</td>
<td>26.4 ± 1.9*</td>
</tr>
<tr>
<td>Enoxacin</td>
<td>78.5 ± 0.5</td>
<td>36.3 ± 0.7*</td>
</tr>
<tr>
<td>Probenecid</td>
<td>83.6 ± 2.6</td>
<td>21.6 ± 1.0</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>85.6 ± 1.9</td>
<td>19.6 ± 0.6</td>
</tr>
<tr>
<td>p-Aminohippurate</td>
<td>90.2 ± 0.3</td>
<td>22.9 ± 0.4</td>
</tr>
</tbody>
</table>

* *P < .05, significantly different from control.

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**Table 2**

Effect of various compounds on the transepithelial transport and cellular accumulation of grepafloxacin by Caco-2 cell monolayers.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transcellular Transport</th>
<th>Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/cm²/60 min</td>
<td>pmol/mg of protein/60 min</td>
</tr>
<tr>
<td>Control</td>
<td>265.4 ± 10.8</td>
<td>43.0 ± 3.2</td>
</tr>
<tr>
<td>Grepafloxacin</td>
<td>155.7 ± 8.0*</td>
<td>60.5 ± 1.5*</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>182.7 ± 4.9*</td>
<td>44.5 ± 1.2</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>189.4 ± 8.8*</td>
<td>51.9 ± 1.7</td>
</tr>
<tr>
<td>Cyclosporin A +</td>
<td>165.5 ± 20.7*</td>
<td>60.5 ± 1.1*</td>
</tr>
<tr>
<td>Grepafloxacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probenecid</td>
<td>198.4 ± 0.9</td>
<td>41.4 ± 2.2</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>221.5 ± 10.5</td>
<td>46.2 ± 3.5</td>
</tr>
<tr>
<td>p-Aminohippurate</td>
<td>233.6 ± 4.9</td>
<td>48.6 ± 1.1</td>
</tr>
</tbody>
</table>

* *P < .05, significantly different from control.
cannot be excluded. Griffiths et al. (1994) reported that the active transepithelial secretion of quinolones involved common accumulative transport at the basolateral membrane followed by facilitated exit across the apical membrane, consistent with our results.

Caco-2 cell monolayers retain P-glycoprotein on the apical membrane, which functions as an ATP-dependent drug efflux pump (Hunter et al., 1993). Cormet-Boyaka et al. (1998) demonstrated a net secretory flux of sparfloxacin in Caco-2 cell monolayers modulated by verapamil, a P-glycoprotein inhibitor. We previously reported that quinolones are transported by P-glycoprotein, using LLC-GA5-COL150 cell monolayers that overexpress human P-glycoprotein on the apical membrane (Ito et al., 1997). The transcellular transport of grepafloxacin was inhibited by cyclosporin A, a P-glycoprotein inhibitor, to the same extent as cyclosporin A plus grepafloxacin (Table 2). On the other hand, unlabeld levofloxacin and grepafloxacin further inhibited [14C]levofloxacin transport in the presence of cyclosporin A (Table 3). Moreover, this transport showed concentration-dependent saturation (Fig. 5B). These results indicated that most of the grepafloxacin transport in the secretory direction was mediated by P-glycoprotein and that levofloxacin was transported by another P-glycoprotein and a specific transport system, which is distinct from organic cation and organic anion transporters and MRP.

We reported that quinolones are specifically transported from the basolateral to the apical side in the kidney epithelial cell line LLC-PK1 by a specific mechanism and have higher affinity for the transport system on the apical membrane, a system distinct from the H+/organic cation antiport system and P-glycoprotein (Matsuo et al., 1998). In the present study, levofloxacin had higher affinity for the apical membrane in Caco-2 cells, because 1 mM levofloxacin inhibited the basolateral-to-apical transport, accompanied by increased accumulation (Table 3). The apparent $K_m$ value for saturable basolateral-to-apical transport of levofloxacin in the presence of cyclosporin A was 5.6 mM (Fig. 5B), which was larger than the apparent $K_m$ of the saturable transcellular transport of levofloxacin reported using LLC-PK1 cells (0.6 mM) (Matsuo et al., 1998). Therefore, it is likely that levofloxacin has lower affinity to the transport system in Caco-2 cells than in LLC-PK1 cells. It remains to be clarified whether the transport systems in these two cell lines are the same.

Although the absorptive mechanisms were not fully evaluated in the present study, uptake of grepafloxacin from the apical membrane was greater than that from the basolateral membrane (Fig. 3). In contrast, uptake of levofloxacin from the basolateral membrane was greater than that from the apical membrane at time points before 5 min (Fig. 3). These results suggested that a specific transport system may contribute to the uptake of grepafloxacin from the apical membrane in Caco-2 cells. The precise mechanisms involved in uptake of quinolones remain to be clarified.

In conclusion, our results suggest that gastrointestinal secretion of quinolone antibacterial drugs is mediated by P-glycoprotein and a specific transport system, which is distinct from organic cation and organic anion transporters and MRP.
References


Send reprint requests to: Professor Ken-ichi Inui, Ph.D., Department of Pharmacy, Kyoto University Hospital, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: inui@kuhp.kyoto-u.ac.jp