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Effectiveness of Pirotiodecane, Absorption Enhancer, on Nasal Absorption in Rabbits

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Summary: The absorption enhancing effect of 1-[2-(decylthio) ethyl] azacyclopentan-2-one (Pirotiodecane), on drug permeation across rabbit nasal mucosa was studied. The nasal epithelial mucosa was isolated from rabbit nasal septum and mounted in an Ussing chamber to allow for monitoring of the membrane resistance (Rm), and the permeation of fluorescein isothiocyanate-labeled dextran (FD-4, M.W. 4,400 Da). Treatment with 0.05, 0.1, and 0.2% Pirotiodecane for 60 min decreased Rm, and increased the cumulative amount of FD-4 permeated in a concentration-dependent manner, suggesting that Pirotiodecane possesses passively a disassembly of tight junction to enable the enhanced FD-4 permeation. The remarkable increase in plasma concentration of FD-4 was also observed in intranasal co-administration with 1% Pirotiodecane in rabbits. The Rm was virtually maintained after the removal of Pirotiodecane, although recovery of Rm was not seen. On the other hand, the increase in plasma concentration of FD-4 with intranasal co-administration of 1% Pirotiodecane in rabbits in vivo was not observed in FD-4 administration at 15–60 min after administration of 1% Pirotiodecane alone. It was concluded that Pirotiodecane possesses a relatively short absorption enhancing effect through nasal epithelial.

Key words: FITC-labeled dextran (FD-4) transport; rabbit; nasal; Pirotiodecane; absorption enhancer; Ussing chamber

Introduction

Drugs are selectively transported across epithelial and endothelial cellular sheets, and this occurs either by mainly transcellular transport through the cell or by paracellular flux through tight junctions (TJs). TJs are responsible for preventing the diffusion of solutes through the intercellular space between adjacent cells, but not simply impermeable barriers, hence allowing the transport of some solutes including drugs.1) Nasal administration has the advantage of providing direct entry of a drug into the systemic circulation through the extensive capillary networks without the first pass metabolisms in the liver.2) Furthermore, the nasal route is often selected as a dosing route of a peptide drug for systemic application, because the nasal cavity has the following advantages that proteolytical activity is poor3,4) and the structural barrier is leakier5,6) than that in the gastrointestinal tract. However, in a case of peptide or protein-like compounds whose molecular weights are 1,000 Da or higher, the bioavailability is reduced to 0.5–5%.7,8) As one of the strategies to improve bioavailability, it has been noted that the membrane permeability is improved by absorption enhancers such as bile acids,9–12) fatty acids,12) surfactants13,14) and chelating agents.12) Pirotiodecane is an absorption enhancer developed by focusing on cysteine, which is a keratin-constituting amino acid of the cuticle as well as a pyrrolidone derivative that is a moisturizing component in the skin.

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Previous reports suggested that Pirotiodecane has an enhancing effect on percutaneous absorption of a large number of drugs such as 5-fluorouracil and indomethacin. Abe et al. reported that the combined use of Pirotiodecane and 2-hydroxypropyl-β-cyclodextrin improved the nasal absorption of insulin (36,000 Da as a hexamer) or buserelin without any adverse effect on the nasal mucosa. In their studies, the mechanism of action remains unknown, although it is shown that Pirotiodecane alone possesses the enhancing effect on the nasal absorption of these compounds described above.

In the present study, we examined the relationship between membrane permeation of fluorescein isothiocyanate-labeled dextran (FD-4, M.W. 4,400 Da) and changes in membrane resistance of rabbit nasal mucosa induced by Pirotiodecane based on in vitro electrophysiological analysis. We also examined the enhancing effect of Pirotiodecane on nasal absorption of FD-4 in the rabbit.

**Materials and Methods**

**Materials:** Pirotiodecane was gifted from Hisamitsu Pharmaceutical Co., Inc., Japan. FD-4 and urethane were purchased from Sigma-Aldrich, USA. Polyoxymethylene hydrogenated castor oil 60 (HCO-60) was purchased from Nikko Chemicals Co., Ltd., Japan. Sodium pentobarbital solution (Nembutal) was purchased from Dainippon Pharmaceutical Co., Inc., Japan. FD-4 and urethane were of analytical grade or purchased from Dainippon Pharmaceutical Co., Ltd., Japan. Polyoxymethylene hydrogenated castor oil 60 (HCO-60) was relatively stable and ready to use. The PD of 4.7 ± 1.4 mV, Isc of 107.6 ± 30.1 μA/cm², and Rm of 43.6 ± 1.3 Ω·cm² were observed for stabilized nasal membrane (mean ± S.E.).

After the nasal mucosa was mounted on the chamber and incubated for 90 min, the HCO-60% of the mucosal side compartment was replaced with 0.5% FD-4 with or without Pirotiodecane. 0.5% FD-4 did not affect electrophysiological parameters. Thereafter, every 10 min, 200 μL-sample of buffer was obtained from the serosal side compartment, and every time the same volume of fresh HCO-60%/KHBB was added to make up the portion used. The measurement of FD-4 concentrations was performed with a plate reader fluorescent photometer (PerSeptive Biosystems, Series 400, MA, USA), at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

**Effects of Pirotiodecane in vitro:** The enhancing effects of Pirotiodecane on permeation of FD-4 were expressed as a cumulative amount of FD-4 permeated per membrane area (μg/cm²) for 60 min after the addition of Pirotiodecane. An area under the curve of the cumulative amount of FD-4 was calculated from time-course profile. The cumulative amount of FD-4 at 60 min after the addition of Pirotiodecane was subtracted to a cumulative amount in the absence of Pirotiodecane from that in the presence of Pirotiodecane. Apparent permeation coefficient (Papp) was calculated as follows:

\[
Papp = \frac{dQ}{dt \times A \times C_0}
\]

dQ/dt is the transport rate (steady state from 40 to
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60 min) transport rate, μg/sec) and corresponds to the slope of the regression line, \( C_o \) is the initial concentration in the donor chamber (μg/mL), and \( A \) is the membrane area. The membrane area was calculated from the exposed area of the Ussing chamber (0.67 cm²). The changes in \( R_m \) when treated with Pirotiodecane were expressed as the difference (\( \Delta R_m \)) from the \( R_m \) value just before the addition of Pirotiodecane (at 90 min). Furthermore, an area under the ratios of \( R_m \)-time curve (Rm-AUC) was calculated as follows:

\[
\text{Rm-AUC} = \frac{\text{\( \Delta R_m \) AUC}_{90-150} \text{ control}}{\text{\( \Delta R_m \) AUC}_{90-150} \text{ control}} \times 100
\]

The \( \Delta R_m \) AUC_{90-150} was the calculated area under the \( \Delta R_m \) curve from 90 min to 150 min.

At first, for investigation of in vitro reversibility, the preincubation period with KHBB was 90 min after removing the nasal mucosa from the rabbits. But recovery of the reduction in \( R_m \) with Pirotiodecane was not confirmed after the removal of Pirotiodecane. It was considered to be one of the causes that the long-time aqueous condition in vitro was severe for the nasal mucosa usually exposed to the air. Hence, the preincubation period with KHBB was shortened to 60 min, according to the methods described by Bechgaard et al., in order to keep the mucosal damage to a minimum. At the 60 min when the electrophysiological parameters became stabilized, the KHBB of the mucosal side compartment was replaced with Pirotiodecane solution and the electrophysiological parameters were measured. After the treatment with Pirotiodecane for 60 min, the compartments were washed with KHBB twice gently, followed by addition of KHBB to the mucosal side and the serosal side. Then the electrophysiological parameters were measured continuously for 60 min.

Effects of Pirotiodecane in vivo: The concentrations of Pirotiodecane and FD-4 solutions were used at 1% and 1 mg/kg, respectively. The concentration of Pirotiodecane was reported to promote the nasal absorption of insulin or buserelin, without tissue damage. The methods of administration of Pirotiodecane and FD-4 solutions were according to the method of Vermehren et al. Male Japanese white rabbits (Japan Laboratory Animals, Inc., Tokyo, Japan), weighing 2.5–3.0 kg, were used without anesthesia. All animal experiments complied with the standards set out in the guidelines of Tokyo University of Science. Pirotiodecane and FD-4 were administered at the same time or FD-4 was administered at 15, 30, or 60 min after the administration of Pirotiodecane. The dosing volume was fixed to 75 μL/nostril, which was reported to induce no irritation to the nasal mucosa. About 500 μL of the heparinized blood was collected from the auricle vein at 2, 5, 10, 15, 20, 30, 45 and 60 min after the administration of FD-4. After the blood samples were centrifuged, plasma was diluted 5-fold with KHBB. The plasma concentrations of FD-4 were determined by a fluorescent spectrophotometer (JASCO, FP6500, Tokyo, Japan), at an excitation wavelength of 492 nm and an emission wavelength of 515 nm (Lower limit of quantification: 6.1 ng/mL). After all the procedures had been completed, the animals were sacrificed by a lethal injection of Nembutal.

Statistical method: All results are expressed as mean ± SEM and statistically analyzed by Bartlett’s test for homoscedasticity, followed by Williams’, Shirley’s or Dunnett’s multiple test. \( P<0.05 \) was defined as statistically significant.

Results

Effects of Pirotiodecane on membrane resistance (Rm) and membrane permeability of FD-4

Changes in the Rm of nasal mucosa when treated with 0.01 to 0.2% Pirotiodecane were shown in terms of the \( \Delta R_m \) (Fig. 1). Under the treatment of 0.01 and 0.025% Pirotiodecane conditions, no marked change was observed when compared with control conditions (0.6% HC HCO-60/KHBB), but treatment with 0.05, 0.1, and 0.2% Pirotiodecane remarkably decreased Rm. In particular, in treatment with 0.2% Pirotiodecane, Rm

Fig. 1. Effects of various concentrations of Pirotiodecane on \( \Delta R_m \) of the rabbit nasal mucosa

The nasal mucosa was pre-incubated with KHBB. After 90 min, the KHBB was replaced by a solution containing Pirotiodecane. Controls were treated with 0.6% HCO-60/KHBB. \( \Delta R_m \): difference from the \( R_m \) value just before the addition of Pirotiodecane. ◄: control (without Pirotiodecane), ◄: 0.01%, ◄: 0.05%, ◄: 0.1%, ◄: 0.15%, ◄: 0.2%. Data represent mean±S.E. (n=3–10 for each condition). *\( p<0.05 \); significant difference from the control (Williams’ or Shirley’s test).
### Table 1. The cumulative amount and apparent permeation coefficient (Papp) of FD-4 in rabbit nasal mucosae treated with various concentrations of Pirotiodcane.

<table>
<thead>
<tr>
<th>Pirotiodcane concentration</th>
<th>AUCa (μg · min/cm²)</th>
<th>Pappb (10⁻⁶ cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>427.9 ± 135.5</td>
<td>1.11 ± 0.20</td>
</tr>
<tr>
<td>0.01%</td>
<td>352.8 ± 72.7</td>
<td>1.40 ± 0.37</td>
</tr>
<tr>
<td>0.025%</td>
<td>545.4 ± 56.3</td>
<td>1.97 ± 0.14*</td>
</tr>
<tr>
<td>0.05%</td>
<td>500.8 ± 91.5</td>
<td>1.95 ± 0.38*</td>
</tr>
<tr>
<td>0.15%</td>
<td>524.2 ± 89.8</td>
<td>2.29 ± 0.16*</td>
</tr>
<tr>
<td>0.2%</td>
<td>658.9 ± 159.9</td>
<td>2.70 ± 0.41*</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E. (n = 3–10). *p < 0.05; significant difference from the control (Shirley’s test).

a The cumulative amount of FD-4 was expressed as an AUC calculated from time-course profile.

b Papp was calculated from the following equation \( Papp = \frac{dQ}{dt \times A \times C_0} \) where \( dq/dt \) is the transport rate (μg/sec), and \( C_0 \) is the initial concentration in the donor chamber (μg/mL), and A is the area of the membrane (cm²).

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...decreased immediately after the addition of Pirotiodcane.

The AUC of the cumulative amount of FD-4 permeated per area against time and Papp is listed in Table 1. Permeability of the FD-4 in the control condition was increased slightly (about 25%) by treatment with 0.025, 0.05 and 0.1% Pirotiodcane, and increased about 50% by treatment with 0.2% Pirotiodcane, but these increases were not significant. The Papp values of FD-4 treated with Pirotiodcane increased in a concentration-dependent manner.

The Rm-AUCs were calculated from the ΔRm curve of 0.01 to 0.2% Pirotiodcane treatments. The ratios of the Rm-AUC changed by the Pirotiodcane treatment to the Rm-AUC in the control and the cumulative amount of FD-4 permeated per membrane area at 60 min were in a concentration-dependent manner (Fig. 2A). Moreover, when the cumulative amount was compared with the Rm-AUCs for 60 min (Fig. 2A and Fig. 2B), a good correlation was obtained (\( R^2 = 0.80, p < 0.05 \)).

**In vitro reversibility:** Figure 3 shows the reversibility of TJ that had been widened by Pirotiodcane. When the membranes were treated with 0.05, 0.1, and 0.2% Pirotiodcane for 60 min, Rm decreased in a concentration-dependent manner. Recovery of Rm was not seen in spite of removal of Pirotiodcane, but Rm was virtually maintained after Pirotiodcane was removed.

**Effects of Pirotiodcane in vivo:** The effects of Pirotiodcane in vivo are shown in Fig. 4. Nasal administration of 1 mg/kg FD-4 immediately after nasal administration of 1% Pirotiodcane resulted in a significant increase in the plasma concentration of FD-4 compared to administration of FD-4 alone (control conditions). When Pirotiodcane and FD-4 were simultaneously administered, the area under the plasma FD-4 concentration curve (AUC) of FD-4 was 88.0 ± 3.46 μg · min/mL. The AUC was about 6-fold as high as those in the control condition (14.4 ± 1.58 μg · min/mL). When FD-4 was administered at 15, 30 and 60 min after administration of Pirotiodcane, the AUC of FD-4 in such groups (21.5 ± 4.04, 19.8 ± 6.52 and 23.3 ± 2.48 μg · min/mL, respectively) was similar to that of the control conditions.

**Discussion**

In the present study, using the nasal mucosa of rabbits in vitro, we examined the relationships between...
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Furthermore, Pirotiodecane enhanced the cumulative amount of FD-4 permeated in a concentration-dependent manner (Fig. 2A). Moreover, the cumulative amount was correlated well with ratios of the Rm-AUCs by the Pirotiodecane treatment to the Rm-AUC in control condition (Fig. 2B, $R^2 = 0.80, p < 0.05$). Tomita et al. demonstrates that a specific transporter for hydrophilic probes such as FD-4 exists in the colon and has probably played a role in excretion.21 There is a possibility that a specific transporter similar to the specific transporter exists in the nasal mucosa of rabbits. Hosoya et al. indicated that fluxes of FD-4 at the concentration of 0.5% from the mucosal side and the serosal side were equal in the nasal mucosa by the Ussing Chamber system.6 Hence, it was considered that if a special transportation system exists in a nasal membrane, it might be saturated in the concentration of FD-4. When the FD-4 concentration was 0.5% or more, the penetration pathway(s) other than the paracellular route is also suggested to be negligible. Nakamura et al. has reported that a permeation of FD-4 correlates to AUC of trans-epithelial electrical resistance (TEER) in nasal membrane treated with sodium caprate22 and β-sitosterol, which promotes absorption of FD-4 by paracellular flux through tight junctions and β-sitosterol β-D-glucoside, which promotes by mainly transcellular transport through the cell, and does not affect nasal membrane TEER.23 Therefore, in the present study, it was suggested that the cumulative amount of FD-4 permeated could be regarded as the cumulative amount of the paracellular route.

1% Pirotiodecane significantly enhanced the absorption of FD-4 administered immediately after the administration of Pirotiodecane, but the result for FD-4 administered 15 to 60 min after Pirotiodecane administration was not different from control (Fig. 4). Since a marked absorption enhancing effect was observed in a relatively short period of several minutes in vivo, we can expect its effectiveness as an absorption enhancer.

Bagger et al. reported that when 1% sodium glycocholate was administered simultaneously with peptide T to rabbits via the nasal route, AUC of peptide T increased to about 7-fold that of the control conditions.20 On the other hand, in our study, AUC of FD-4 was increased to about 6-fold that of the control conditions when Pirotiodecane and FD-4 were simultaneously administered (88.0 µg·min·mL vs. 14.4 µg·min·mL in the control condition). Considering that the molecular weight of FD-4 (MW: 4,400) is higher than that of peptide T (MW: 857), Pirotiodecane has an absorption enhancing effect on a higher molecular compound than sodium glycocholate.20 Pirotiodecane seems to possess a potential in the enhancement of nasal absorption, hence expecting intranasal application to high molecular weight peptides or proteins in delivery.
Complete recovery of the reduction in $R_m$ of the rabbit nasal mucosae mounted on an Ussing Chamber with Pirotiodecane was not confirmed after the removal of Pirotiodecane, but $R_m$ at the time when Pirotiodecane was removed was mostly maintained (Fig. 3). As the reason why that complete recovery was not observed in vitro, the following factor can be considered. Pirotiodecane is poorly soluble in water and extremely difficult to dissolve in water. Since both mucosal and serosal sites were in contact with aqueous solvents, Pirotiodecane was thought to tend to remain in the tissue even after it was washed out. Pirotiodecane may sufficiently exert its effect in the tissue even at a low concentration, and it takes much time to restore the membrane to control condition. Perhaps there is a possibility that the nasal mucosae exposed to Pirotiodecane for a long time may have extracted membrane constituent.\textsuperscript{20} In order to clarify these points, further studies are required in the future.

In conclusion, Pirotiodecane remarkably enhanced the nasal absorption of FD-4 in vitro and in vivo, although complete recovery of membrane resistance after removal of Pirotiodecane was difficult to observe. Pirotiodecane seems to possess a potential for absorption enhancement of nasally administered peptides and proteins, even though further studies remain to clarify its safety and mechanism.

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