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In-vitro corneal permeation of cannabinoids and their water-soluble phosphate ester prodrugs

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Abstract

Topically administered cannabinoids have been shown to reduce intraocular pressure by interacting with the ocular cannabinoid receptor. Most cannabinoids have very poor aqueous solubility, which limits their pharmaceutical development and usefulness. In this study, permeation of three cannabinoids (arachidonylethanolamide, *R*-methanandamide and noladin ether) and their water-soluble phosphate ester prodrugs across isolated rabbit cornea was investigated in vitro. Hydroxypropyl-β-cyclodextrin (HP-β-CD) was used to solubilize the parent cannabinoids in permeation studies to achieve the required concentration in donor and receiving cells. Highest fluxes were obtained with lipophilic parent compounds administered with HP-β-CD, and the fluxes of phosphate esters were 45–70% that of their corresponding parent compounds. Phosphate esters hydrolysed on the surface of the cornea or during the permeation to release the lipophilic parent compound, which further permeated the cornea. No phosphate esters were detected on the endothelial side of the cornea. Although the phosphate esters had lower fluxes than their corresponding parent compounds in these HP-β-CD formulations, the results are promising and the fluxes of phosphate esters are significantly higher than the fluxes of parent compounds administered as a suspension (due to their low aqueous solubility) without HP-β-CD.

Introduction

Hepler & Frank (1971) reported that smoked marijuana reduces intraocular pressure (IOP). This finding was later confirmed (Cooler & Gregg 1977) by intravenous administration of Δ-9-tetrahydrocannabinol (THC), which is the main active ingredient in marijuana. Discovery of a cannabinoid receptor in 1988 (Devane et al 1988), followed by the characterization of the first endogenous cannabinoid receptor ligand, arachidonylethanolamide (Devane et al 1992), opened up new opportunities in the search for novel compounds as possible anti-glaucoma agents (Järvinen et al 2002). In addition to their IOP lowering effects, cannabinoids also have neuroprotective actions, and both of these actions would be beneficial in the treatment of glaucoma. Pate et al (1995) also observed that endocannabinoids, such as arachidonylethanolamide (AEA), reduce IOP. This IOP decrease was shown to be a local effect, rather than mediated via the central nervous system, and discovery of the ocular CB_1 receptor (Porcella et al 1998) supports this finding. Topical administration of cannabinoids to the eye would therefore be the preferred route of administration, as systemic administration of cannabioids has a higher risk of both psychoactive and cardiovascular effects.

Both classic and endogenous cannabinoids are very lipophilic, and thus poorly water-soluble, molecules. Because of their very low aqueous solubility, topical administration of cannabinoids to the eye requires an oily vehicle or the use of excipients such as polyvinyl alcohol or cyclodextrins in aqueous formulations (Pate et al 1995; Kearse & Green 2000). The aqueous solubility of a drug is very important in topical ophthalmic drug delivery because the instilled volumes should be kept to a minimum in order to minimize the loss of drug due to lacrimal drainage, which is directly proportional to the increase in lacrimal fluid volume. Other important drug factors that affect corneal permeability include partition coefficient, molecular size and degree of ionization (Schoenwald 1990). We recently reported the development of phosphate ester prodrugs

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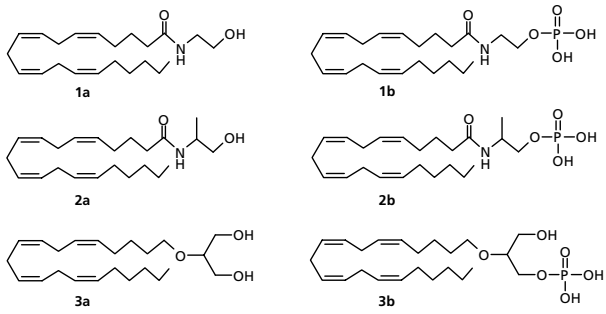

Figure 1 Structures of AEA (**1a**), *R*-methanandamide (**2a**), noladin ether (**3a**) and their phosphate esters (**1b**, **2b** and **3b**).

of AEA, *R*-methanandamide and noladin ether (Juntunen et al 2003a, b). These prodrugs have high aqueous solubilities, and they can be formulated for topical administration at sufficient concentrations in buffered solutions without any solubilizing excipients.

Phosphate ester prodrugs have most commonly been used to increase the aqueous solubility of poorly water-soluble compounds intended for i.v. or i.m. administration. Applications involving their oral administration also exist. However, the topical ophthalmic administration route seems to be a practically unexplored possibility for such prodrugs. One of the few examples of the ophthalmic application of phosphate ester prodrugs is prednisolone phosphate (Musson et al 1991).

Phosphate ester prodrugs are in an ionized form near pH 7.4, which may hinder their permeation across the cornea. However, the IOP-reducing effects of phosphate esters of both *R*-methanandamide and noladin ether in rabbits were comparable to the effects of their parent compounds solubilized by hydroxypropyl-β-cyclodextrin (HP-β-CD) (Juntunen et al 2003a, b). This promising finding led us to study the corneal permeation of these phosphate ester prodrugs in more detail.

In this study, we investigated the permeation of cannabinoids and their water-soluble prodrugs (Figure 1) through rabbit cornea in vitro using side-by-side diffusion cells to clarify whether or not these phosphate ester prodrugs can penetrate the cornea as such, or if they release the more lipophilic parent compound prior to permeating the cornea.

Materials and Methods

Materials

HP-β-CD (Cavasol W7 HP Pharma) was obtained from Wacker-Chemie GmbH, Burghausen, Germany. AEA and *R*-methanandamide were synthesized from arachidonic acid and the corresponding amino alcohol using the method of Abadji et al (1994). Noladin ether was synthesized from arachidonic alcohol as described by Hanuš et al (2001). Phosphate esters of AEA, *R*-methanandamide and noladin ether were prepared as previously reported (Juntunen et al 2003a, b). The structures of compounds investigated in the present study are shown in Figure 1.

Methods

In-vitro corneal permeation study

The permeabilities of these cannabinoids and their phosphate esters were studied using glass diffusion cells at 37°C. Corneas were obtained from albino rabbits (2–3 kg) of both sexes. Animals were killed for other reasons than for this experiment. The study was performed according to ethical approval from the University of Kuopio. The eye was proptosed and the cornea with scleral ring was carefully excised with scissors and placed into 37°C Balanced Salt Solution Plus solution (Alcon Laboratories, Fort Worth, USA). The lenses, together with various tissues of the eye, were removed to leave only the cornea and scleral ring.

The isolated corneas were washed with Tris buffer and mounted on a corneal holder, which was placed between the glass diffusion chambers. When phosphate buffer was used as a vehicle (one experiment), the isolated corneas were washed with phosphate buffer. Preheated (37°C) 20 mM Tris (or phosphate) buffer (3.4 mL) containing 5% HP-β-CD was first added to the receiver cell (endothelial side). HP-β-CD was used in the receiver cell to control for the solubility of penetrated cannabinoids. Then 3.2 mL of the buffered solution containing the test compound (1.4 mM) was added to the donor cell (epithelial side). AEA, *R*-methandamide and noladin ether were each dissolved separately in a 20 mM Tris buffer (pH 7.65 at 37°C) containing 5% HP-β-CD. Phosphate esters were dissolved in 20 mM Tris or phosphate buffer. The osmolality of the solutions was adjusted to 290 mOsm using NaCl. A carbogen bubbling (95% O_2: 5% CO_2) was used to provide mixing of the solutions on both sides. At specified time intervals, 200 μL aliquots were withdrawn from the receiver side and replaced with fresh buffer. Determination of cannabinoid concentrations was made by HPLC.

Enzymatic hydrolysis in cornea homogenate

Fresh bovine eyes were obtained from a local slaughterhouse. The eyes were washed with 0.9% NaCl solution and the corneas were dissected, rinsed with a 0.9% NaCl solution and stored at −80°C until used. The corneas were cut into small pieces with scissors and placed into preweighed centrifuge tubes. Tris buffer (50 mM, pH 7.4 at 37°C) was added to give 16.7% (w/v) solutions. The corneas were then homogenized at 4°C with an Ystral X-1020 homogenizer (Ystral GmbH, Germany). The homogenate was centrifuged for 90 min at 9000 *g* and 4°C, and the supernatant was stored at −80°C until used. Phosphate esters were dissolved in 3 mL of Tris buffer (50 mM, pH 7.4 at 37°C). Each hydrolysis experiment was initiated by adding 1 mL of preincubated (37°C) corneal supernatant. The solution was kept at 37°C and 200 μL aliquots were withdrawn and then added to 400 μL of cold acetonitrile. After mixing and centrifugation, the supernatants were analysed for remaining phosphate ester and for released parent cannabinoid by HPLC.

Hydrolysis in alkaline phosphatase-containing solution
The rate of hydrolysis of **3b** in alkaline phosphatase solution, in both Tris and phosphate buffer, was determined at 37°C. Alkaline phosphatase (Type VII-S: from bovine intestinal mucosa, 3150 units mg^{-1} protein) was purchased from Sigma (St Louis, MO, USA). A total of 0.9 μmol of **3b** in 4 mL of 50 mM Tris or phosphate buffer (pH 7.4) was placed in a water bath at 37 °C, and 2 μL (62 units) of alkaline phosphatase was added. At predetermined time intervals, 200 μL aliquots were removed and 200 μL of acetonitrile was added to stop the enzymatic hydrolysis. After centrifugation (14 000 rpm, 10 min), samples were analysed for remaining phosphate ester **3b** and for released noladin ether **3a** by HPLC.

HPLC analysis
HPLC determinations were performed with a Merck LaChrom HPLC system consisting of an L-7250 autosampler, an L-7100 pump, a D-7000 interface, an L-7455 diode array detector and D-7000 HPLC system manager software (Hitachi, Tokyo, Japan). A Purospher RP-8e (125 × 4.0 mm, 5 μm) (Merck, Darmstadt, Germany) reversed-phase column was used for all analytical HPLC determinations. A mobile phase of acetonitrile and 20 mM phosphate buffer (pH 7.0) at a flow rate of 1.2 mL min^{-1} was used, with a gradient elution that began at 40% acetonitrile and increased to 72% acetonitrile.

Statistical analysis
Differences in steady-state fluxes (n = 3–6) between the compounds were statistically compared using a Kruskall–Wallis test followed by a post-hoc comparison of the means between each compound using a Dunn's test. Prodrugs were compared to their corresponding parent compounds and comparison was also made between parent compounds and between prodrugs. The different formulations of **3a** and **3b** were also compared. A similar statistical analysis was performed on the measured log D-values (n = 3). A significance level of $P < 0.05$ denoted significance in all cases.

Results and Discussion

Our previous in-vivo studies in rabbits showed that phosphate ester prodrugs of both *R*-methanandamide and noladin ether were able to elicit an IOP decrease comparable to the effects of their corresponding parent compounds when dissolved in an aqueous 5% HP-β-CD solution. The question remained whether or not these prodrugs were able to permeate the cornea as such. It is worth noting that although they are ionized at tear fluid pH, they still have apparent octanol/water partition coefficients near optimal values for membrane permeation (Table 1).

In this study, the highest fluxes across isolated rabbit cornea were achieved with cannabinoids solubilized with HP-β-CD, and permeation of the corresponding water-soluble phosphate esters was 45–70% that of the parent compounds (Table 2, Figure 2 A, B and C). However, the differences in steady-state flux values between the prodrugs (**1b** and **3b**) and the corresponding parent compounds (**1a** and **3a**) were not statistically significant. *R*-methanandamide (**2a**) had the highest flux of 54.9 ± 16.1 nmol $(cm^2 \cdot h)^{-1}$; however, in comparison with the permeation characteristics for the parent compounds, one must keep in mind that the complexation with CDs also increases the fluxes (Table 2). To highlight the importance of both aqueous solubility and dissolution rate, **3a** was also administered as a suspension without HP-β-CD. The flux of 3a in this case was reduced to 0.9 ± 0.6 nmol $(cm^2 \cdot h)^{-1}$, which is only 3% of the flux of the same compound with HP-β-CD. The large difference between **3a** suspension and CD formulation is due to slow dissolution of free compound from suspension compared to the fast equilibrium between the complexed and free form in CD formulation.

Phosphate esters **1b** and **2b** had similar fluxes (Table 2) (26.7 ± 7.1 and 24.0 ± 6.7 nmol $(cm^2 \cdot h)^{-1}$, respectively) but **3b** had a lower flux, which corresponds with the lower flux of the parent compound **3a**. The HP-β-CD

Table 1 Physicochemical properties of cannabinoids and their phosphate ester prodrugs

Compound	Solubility at pH 7.4 ($\mu g\,mL^{-1}$)	Log $D_{7.4}$	pK_{a1}	pK_{a2}
1a	0.4[a]	5.97[b]	–	–
1b	> 5000	1.15 ± 0.09[c]	2.68[c]	6.88[c]
2a	–	6.31[b]	–	–
2b	> 5000	1.53 ± 0.02[c]	2.65[c]	6.73[c]
3a	< 0.1	7.05[b]	–	–
3b	> 5000	1.98 ± 0.06[d,e]	–[f]	–[f]

[a]From Jarho et al (1996b). [b]Calculated with ACD labs log D suite. [c]From Juntunen et al (2003a). [d]From Juntunen et al (2003b). [e]$P < 0.05$ compared to **1b** (Kruskall–Wallis test, Dunn's test). [f]Not determined.

Table 2 Steady-state fluxes (J_{ss}) (mean ± s.d.; n = 3–6) for cannabinoids and their phosphate esters through isolated rabbit cornea in vitro (37°C, pH 7.65)

Compound	Vehicle	J_{ss} (nmol $(cm^2 \cdot h)^{-1}$)
1a	5% CD, Tris buffer	39.30 ± 1.94
1b	Tris buffer	26.66 ± 7.06[a]
2a	5% CD, Tris buffer	54.89 ± 16.05[b]
2b	Tris buffer	23.95 ± 6.70[c]
3a	5% CD, Tris buffer	27.61 ± 5.81[d]
3a suspension	Tris buffer	0.89 ± 0.58
3b	Tris buffer	14.07 ± 4.06[d]
3b	Phosphate buffer	4.32 ± 0.69

[a]$P < 0.05$ compared to **3b** (Tris buffer) (Kruskall–Wallis test, Dunn's test). [b]$P < 0.05$ compared to **3a** (CD, Tris buffer) (Kruskall–Wallis test, Dunn's test). [c]$P < 0.05$ compared to **2a** (CD, Tris buffer) (Kruskall–Wallis test, Dunn's test). [d]$P < 0.05$ compared to **3a** (suspension) (Kruskall–Wallis test, Dunn's test).

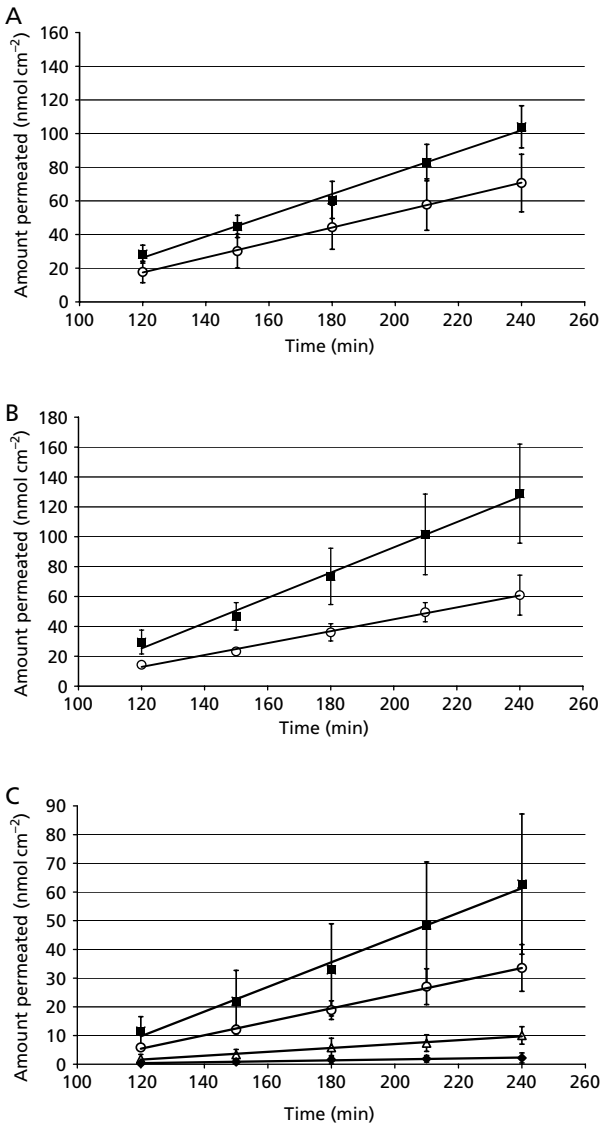

Figure 2 Linear portion of permeation profiles (mean ± s.d.; n = 3–4) for (A) AEA **1a** (■) and prodrug **1b** (○), (B) *R*-methanandamide **2a** (■) and prodrug **2b** (○) and (C) HU-310 **3a** (■), prodrug **3b** (○), **3b** in phosphate buffer (△) and **3a** suspension (◆) through isolated rabbit cornea (pH 7.65).

concentration used in this study had previously been determined to be optimal for corneal permeation of **1a** (Jarho et al 1996a). Lower CD concentrations would have led to an incomplete dissolution of the lipophilic parent compounds, while higher concentrations would have produced excessive complexation of the compounds, thereby reducing the amounts of free compounds available for absorption. In this context, the results obtained with these phosphate ester prodrugs are promising.

In the permeation experiments, no phosphate esters were detected on the receiver side, indicating that these prodrugs released the parent compound just prior to, or during, the absorption. Enzymatic hydrolysis experiments of prodrugs were performed to confirm hydrolysis of the prodrugs by corneal enzymes. All phosphate esters **1b**, **2b** and **3b** were hydrolysed in the 4% cornea homogenate ($t_{1/2} = 20.2 \pm 0.2$, 14.6 ± 0.3 and 18.1 ± 1.4 min, respectively), and they quantitatively released the corresponding parent compounds (Figure 3). The possible hydrolysis of phosphate ester prodrugs can occur just before penetration on the surface of the cornea, during the penetration or after the prodrug has crossed the cornea. Histochemical studies of bovine and rabbit corneas have revealed that alkaline phosphatase is found in the corneal epithelium, endothelium and also in keratocytes (Lojda et al 1976).

The effect of inhibiting alkaline phosphatase for the in-vitro corneal permeation of **3b** was investigated in order to study the importance of enzymatic hydrolysis on the flux of phosphate ester prodrugs. Corneal alkaline phosphatase was inhibited simply by using phosphate buffer instead of Tris buffer in the permeation experiments. Inhibition of alkaline phosphatase by phosphate was confirmed by observing phosphate ester hydrolysis in an alkaline phosphatase-containing solution, in both Tris ($t_{1/2} = 4.5$ min) and phosphate ($t_{1/2} = 231.5$ min) buffers (Figure 4). When the permeability experiment with **3b** was made using phosphate

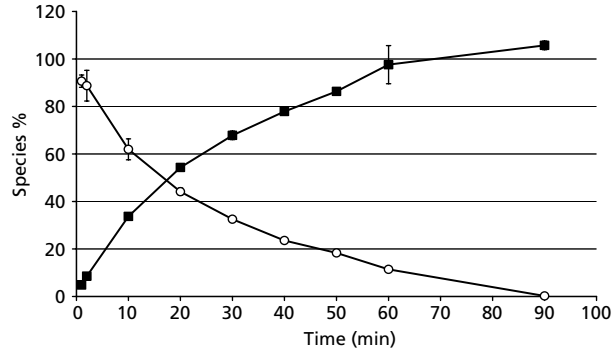

Figure 3 Time courses (mean ± s.d.; n = 2) for AEA phosphate ester **1b** (○) and AEA **1a** (■) during hydrolysis of the prodrug in 4% cornea homogenate (pH 7.65, 37°C).

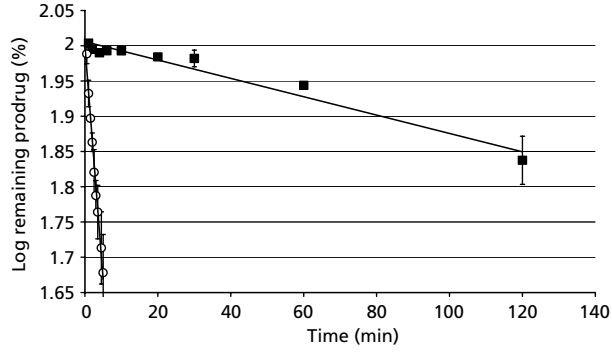

Figure 4 Pseudo first-order plots (mean ± s.d.; n = 2) for the hydrolysis of **3b** in an alkaline phosphatase-containing solution in Tris (○) and phosphate buffers (■), showing the inhibitory effect of phosphate buffer (pH 7.65, 37°C).

buffer, which is a known inhibitor of alkaline phosphatase, the flux was reduced by 70% to 4.3 ± 0.7 nmol $(cm^2 \cdot h)^{-1}$. The decreased flux with phosphate buffer suggests that these phosphate ester prodrugs might be enzymatically cleaved to release the lipophilic parent compound on the corneal surface, just prior to absorption.

The advantage of phosphate esters over other water-soluble prodrug strategies is in their chemical stability (Juntunen et al 2003a, b), which of course is of high importance in the development of solution formulations like eye-drops. Another possible advantage is their ability to form micellar aggregates, which can solubilize the lipophilic parent compound at higher concentrations than its intrinsic solubility, and thus reduce the risk of precipitation, which is an obvious stability-related problem with solution formulations for water-soluble prodrugs of lipophilic parent compounds (i.e. minor amounts of lipophilic parent compound will be released during storage as a function of time).

Conclusions

Although the most effective corneal permeation was achieved with the lipophilic parent compounds administered with HP-β-CD, the results obtained with cannabinoid phosphate esters suggest that the aqueous phosphate esters prodrug approach is a potential alternative to oil-based or cyclodextrin formulations in ophthalmic applications to overcome the delivery/formulation problem caused by poor aqueous solubility. Phosphate esters of these cannabinoids have adequate aqueous solubility, and they are enzymatically hydrolysed by alkaline phosphatase to their lipophilic parent compounds, which subsequently permeate the cornea.

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