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Mucoadhesive Fenretinide Patches for Site-specific Chemoprevention of Oral Cancer: Enhancement of Oral Mucosal Permeation of Fenretinide by Co-incorporation of Propylene Glycol and Menthol

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Abstract

The objective of this study was to enhance oral mucosal permeation of fenretinide by co-incorporation of propylene glycol (PG) and menthol in fenretinide/Eudragit® RL PO mucoadhesive patches. Fenretinide is an extremely hydrophobic chemopreventive compound with poor tissue permeability. Co-incorporation of 5-10 wt% PG (mean J_s = 16-23 μg cm^{-2} h^{-1}; 158-171 μg fenretinide/g tissue) or 1-10 wt% PG + 5 wt% menthol (mean J_s = 18-40 μg cm^{-2} h^{-1}; 172-241 μg fenretinide/g tissue) in fenretinide/Eudragit® RL PO patches led to significant *ex vivo* fenretinide permeation enhancement ($p < 0.001$). Addition of PG above 2.5 wt% in the patch resulted in significant cellular swelling in the buccal mucosal tissues. These alterations were ameliorated by combining both enhancers and reducing PG level. After buccal administration of patches in rabbits, *in vivo* permeation of fenretinide across the oral mucosa was greater (~43 μg fenretinide/g tissue) from patches that contained optimized permeation enhancer content (2.5 wt% PG + 5 wt% menthol) relative to permeation obtained from enhancer-free patch (~ 17 μg fenretinide/g tissue) ($p < 0.001$). *In vitro* and *in vivo* release of fenretinide from patch was not significantly increased by co-incorporation of permeation enhancers, indicating that mass transfer across the tissue, and not the patch, largely determined the permeation rate control *in vivo*. As a result of its improved permeation and its lack of deleterious local effects, the mucoadhesive fenretinide patch co-incorporated with 2.5 wt% PG + 5 wt% menthol represents an important step in the further preclinical evaluation of oral site-specific chemoprevention strategies with fenretinide.

Keywords

fenretinide; oral mucosa; local drug delivery; mucoadhesive patch; permeation enhancement; propylene glycol; menthol

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Supporting Information. A figure that shows the design of fenretinide mucoadhesive patch, method section of preparation of fenretinide mucoadhesive patches, figure that shows the effect of co-incorporation menthol on the appearance of drug-loaded films, and discussion section. This material is available free of charge via the Internet at http://pubs.acs.org.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), which is a world-wide health problem, will affect approximately 36,000 Americans with over 7,000 deaths this year.[1] Despite extensive research and introduction of therapeutic advances such as radiation-intensification, prognosis for persons with HNSCC remains among the lowest of all solid tumors.[2] Intervention with effective chemopreventive agents-to prevent progression or induce regression- at the pre-cancerous stage would greatly improve clinical outcomes.[3] Analogous to other surface origin malignancies, initiated head and neck epithelium undergoes progressive growth disturbances (grades of epithelial dysplasia) prior to conversion to overt carcinoma.[2-6] Furthermore, many of these dysplastic lesions arise in visible mucosa, making topical application and direct clinical monitoring of lesion progression feasible. Despite obtaining complete surgical excision, many of these dysplastic lesions recur; necessitating sequential surgeries and increasing patient anxieties regarding cancer development.[2-6]

Vitamin A and its derivatives including fenretinide have been the mainstay of many previous HNSCC chemopreventive trials.[3,7,8] Their preferential usage reflects the abilities of vitamin A compounds to therapeutically modulate epithelial cell growth by induction of terminal differentiation and apoptosis of cultured keratinocytes.[9,10] Despite promising *in vitro* results, systemic delivery of fenretinide in oral cancer chemoprevention trials was ineffective.[8,11] These negative data largely reflect dose-limiting toxicities in conjunction with an inability to achieve therapeutically relevant levels.[8,11-14] Local drug delivery, on the other hand, has proven to be highly effective in providing therapeutic drug concentration directly at the site of numerous cancers, thereby improving the therapeutic efficacy of the drug and patient compliance.[15-20] Furthermore, topical application of a bioadhesive gel that contained freeze dried black raspberries provided positive results[19,21] which served as the basis for our ongoing multicenter, placebo-controlled Phase 2 clinical trial. We have observed, however, that topical berry gel is insufficient to manage some patients' precancerous oral lesions.[22] To help address this unmet clinical need, we have designed a mucoadhesive patch to deliver a chemically and mechanistically distinct chemopreventive, fenretinide. We envision that combination therapy will provide additive or synergistic chemopreventive effects.

In our previous study,[23] we developed a unique and optimal mucoadhesive patch formulation that has the potential to provide site-specific continuous *in vitro* and *in vivo* release of extremely hydrophobic and low water soluble fenretinide. Poor tissue permeability of fenretinide,[13,14] however, remained as a potential concern. The ultimate dosing regimen anticipated for administration of fenretinide mucoadhesive patches to the oral cavity is not yet known. Furthermore, many dysplastic lesions arise at sites critical for function, e.g., lateral and ventral tongue, floor of mouth, soft palate and buccal mucosa, which may make extended administrations times difficult.[24] Therefore, in this study, we sought to make further advancement on mucoadhesive fenretinide patch by investigating the potential of co-incorporation of tissue enhancers (propylene glycol (PG) and menthol) in the patch to enhance the permeation and tissue levels of fenretinide. Permeation enhancement may also be helpful to optimize the time of patch application. We anticipate that mucoadhesive patch formulations will enable clinical re-introduction of an effective epithelial-relevant chemopreventive compound to treat head and neck dysplastic lesions.[3]

Menthol is a monocyclic terpene with a pleasant taste and odor.[25] It is an effective, safe, non-carcinogenic and widely used permeation enhancer in transdermal drug delivery.[25] Propylene glycol, on the other hand, is commonly used cosolvent and increases the permeability of hydrophobic drugs across biological membranes via solvent drag.[26-28] When PG is combined with another tissue enhancer, pronounced drug permeation can be expected

as it acts as a cosolvent for both permeant and enhancer (e.g., menthol) and may facilitate enhanced penetration of both the molecules.[28,29] Herein, we evaluated fenretinide-Eudragit® RL PO-solubilizer-containing patches with and without PG and menthol both *in vitro* and *in vivo*.

Fenretinide-Eudragit® RL PO-solubilizers patches with and without permeation enhancers were prepared as described previously.[23] The objective of this study was to enhance buccal mucosal permeation of fenretinide in vitro and in vivo by co-incorporation of PG and menthol in the patch formulation.

EXPERIMENTAL SECTION

Chemicals, Tissue, and Animals

Fenretinide was received as a gift sample from Merck & Co., Inc. (Whitehouse Station, NJ). Sodium deoxycholate, Tween® 80 and L-menthol were purchased from Sigma-Aldrich, Co. (St. Louis, MO). Noveon® AA-1 polycarbophil (PC), hydroxypropyl methylcellulose (HPMC) 4KM and Eudragit® RL-PO were all gifts from Lubrizol Corp. (Wickliffe, OH), Colorcon®, Inc., (West point, PA), and Evonik Degussa Corp. (Piscataway, NJ), respectively. Propylene glycol was purchased from MP Biomedicals, LLC (Solon, OH). Teflon® overlay was purchased from Scientific Commodities, Inc., (Lake Havasu City, AZ). Tegaderm™ roll was purchased from 3M Health Care (St. Paul, MN). Porcine buccal tissue was obtained from slaughter house (Dunbar Meat Packing Company, Milan, MI, USA). Rabbits were purchased from Harlan Laboratories (Indianapolis, IN, USA).

Preparation of Oral Mucoadhesive Patches for Enhanced Buccal Permeation of Fenretinide

Fenretinide/Eudragit® RL-PO/solubilizers patches with and without permeation enhancers (PG and menthol) were prepared by a solvent casting and assembly techniques as described previously (see detailed Methods in Supporting Information).[23] Three steps were involved in the preparation of fenretinide patch: formation of adhesive (hydroxypropyl methylcellulose and polycarbophil at a weight ratio of 3:1) and drug release (5 wt% fenretinide/Eudragit® RL-PO/40 wt% sodium deoxycholate/20wt% Tween® 80) layers, and assembly of adhesive and drug release layers onto backing layer (Tegaderm™ film) (see Figure S1 and Method in Supporting Information). In this study, drug release (fenretinide) layer consisted of permeation enhancer(s) in addition to the composition given above. Eudragit® RL-PO/5 wt % fenretinide/40 wt% sodium deoxycholate/20wt% Tween® 80 layer loaded with PG alone (5 and 10 wt%) or menthol alone (5 and 10 wt%) or in combination (1 wt% PG + 5wt% menthol, 2.5 wt% PG + 5wt% menthol, and 10 wt% PG + 5wt% menthol) were prepared in a similar manner and used in the current investigations.

Fenretinide HPLC Assay

HPLC assays were performed on a Waters 2695 alliance system (Milford, MA, USA) consisting of a 2996 Photodiode array detector and a personal computer with Empower 2 Software. A symmetry C18 column (4 μm, 150 mm × 4.6 mm) was used. Isocratic elution with acetonitrile: 0.1 % (v/v) phosphoric acid (67:33 v/v) was employed at a flow rate of 1.0 mL/min and detection wavelength was set at 365 nm. Standard curve of fenretinide was established in acetonitrile: ethanol (50:50) and concentration of unknown samples was calculated from the standard curve.

Determination of Fenretinide Solubility in Bovine Serum

A known quantity (0.9, 2.26, 3.97, 8.03, and 20.5 mg) of fenretinide was added to polypropylene tubes containing 15 mL fetal bovine serum. The samples were incubated at 37 °C under constant rotation using a rigged rotator and protection from light. At every 24 h

till 7 days, the samples were centrifuged at 8000 rpm for 10 minutes and 200 μL of supernatant was withdrawn. Withdrawn serum sample was replaced with fresh serum sample, mixed properly, and incubated again under similar conditions. To the withdrawn sample (200 μL), 2 mL of acetonitrile was added,[30,31] agitated overnight on a mechanical shaker with protection from light, passed through 0.45 μm PVDF filter units, and analyzed by HPLC.

Determination of Fenretinide Loading

Fenretinide/Eudragit® films were digested in acetonitrile: ethanol (50:50), passed through 0.45 μm PVDF filter units, and analyzed by HPLC after suitable dilution. The fenretinide loading was calculated as the percentage of the amount of fenretinide versus the total weight of the film mixture (i.e., fenretinide, Eudragit®, and other excipients).

Evaluation of *In Vitro* Release of Fenretinide from Oral Mucoadhesive Patches

Simulated saliva comprised of 14.4, 16.1, 1.3, 0.55, and 2 mM sodium chloride, potassium chloride, calcium chloride dihydrate, magnesium chloride hexahydrate, and dibasic potassium phosphate and the pH was adjusted to 6.8. *In vitro* release studies were conducted in simulated saliva containing 5% (w/v) sodium deoxycholate under perfect sink conditions. Mucoadhesive patches were placed in 50 mL tubes (separate tubes for each sampling interval) and 40 mL release medium was added to each tube. The tubes were placed in an incubator maintained at 37 °C and shaken at 100 RPM. At predetermined time intervals (0.5, 3, and 6 h), tubes were taken out and the patches were immediately freeze-dried. The amount of fenretinide remaining in the patch was determined as per the method described in loading assay. The cumulative amount of fenretinide released was calculated by subtracting the fraction remaining in the patches from the initial drug content.

Ex Vivo Permeation of Fenretinide across Porcine Buccal Mucosa

Ex vivo permeation of fenretinide across porcine buccal mucosa was conducted using side-by-side flow-through diffusion cells (donor and receiver chamber volume = 3 mL).[32] The diffusional interface was a spherical shape with a diameter of 1 cm. Porcine buccal tissue was obtained from a local slaughterhouse and used within 2 hours of slaughter. The tissue was stored in Krebs buffer at 4 °C upon removal. The epithelium was separated from the underlying connective tissue with a scalpel and mounted between the donor and the receiver chambers. Fenretinide patch was then attached to the buccal mucosa (adhesive layer facing mucosa and backing layer exposed to buffer) in donor chamber. Donor and receiver chambers were filled with 3 mL degassed phosphate buffered saline (PBS, pH = 7.4) containing 0.084% Tween® 80 (v/v) and simulated saliva (pH 6.8), respectively. Both the chambers were maintained at 37 °C by circulating the water from a thermostatically controlled water bath. The receiver chamber medium was stirred at 600 rpm. After specified duration (1, 2, 3, 4, 5, 6, 7, 8 and 12 h), 1 mL sample was withdrawn from the receiver chamber and immediately replaced with fresh medium. Fenretinide was quantified by HPLC. At the end of permeation study, phenol red at a concentration of 300 μg/mL was added to the donor chamber to check the integrity of buccal mucosa. Phenol red acts as a marker compound, which does not permeate through an intact porcine buccal membrane.[33] Upon the completion of *ex vivo* permeation study, porcine buccal tissue was removed and fenretinide level in the tissue was determined as described below.

Determination of Fenretinide Levels in Buccal Tissue

Treated porcine buccal tissue was cut into small pieces and placed in 4-mL polypropylene tubes. One milliliter of water was added to the tubes and homogenized for 1 minute. Then, 2 mL of acetonitrile was added to the tubes and vortexed for 1 hour. After 1 h, tubes were

centrifuged at 2600 g at 25 °C for 20 min and the supernatant was analyzed by HPLC to determine fenretinide content.

Haematoxylin and Eosin Staining

A portion of each tissue was fixed in buffered 10% formalin and embedded in paraffin wax. Then, 5 μm sections were placed on microscope slides, deparaffinized using xylene, and rehydrated using ethanol solutions in a gradient of 80% up to 100% and distilled water. The tissue slices were placed in 0.7% w/w haematoxylin solution, rinsed twice in acid ethanol (0.1 N HCl in 95% ethanol) to remove the excess stain. Subsequently, the tissue slices were placed in 0.1% w/w eosin solution and dehydrated using solutions of ethanol in a gradient of 80% up to 100% and then xylene.

Light Microscopy Analysis

Light microscopy was performed using Olympus BX51 microscope (Olympus, Tokyo, Japan) at 40 × magnification. Images of the sections were captured using a fitted camera (Olympus DP70 digital camera, Tokyo, Japan), and software (Olympus DP controller, Tokyo, Japan).

Evaluation of *In Vivo* Fenretinide Release and Permeation

Animal studies were approved by the Ohio State University Institutional Animal Care and Use Committee and adhered to National Institute of Health guidelines. Female New Zealand white rabbits (12 weeks old and weight ranging 2.7-3.1 kg) were anesthetized with isoflurane (5% v/v in oxygen) via inhalation for patch placement and removal. Six fenretinide oral mucoadhesive patches/time point were placed on the buccal mucosa of individual rabbit's oral cavity (drug + adhesive layers facing the mucosa). Slight pressure was applied to the backing layer of the patch for 1 minute to establish mucoadhesion with the rabbit buccal mucosa. After different attachment times (0.5, 3 and 6 h), the patches were carefully removed and remaining fenretinide in patches was determined by HPLC. The cumulative amount of fenretinide released was determined by subtracting the fraction remaining in the patches from the initial drug content. To determine *in vivo* permeation and tissue deposition of fenretinide, rabbits were sacrificed via intravenous potassium chloride injection after 0.5, 3, and 6 h of patch attachment and then the buccal tissue was harvested. Fenretinide levels in buccal tissue were then determined by assaying the tissue samples as per the method described in the determination of fenretinide level in buccal tissue.

Statistical Analysis

The results are expressed as mean ± SE (n = 3/4 (*in vitro*) or 5 (*ex vivo*) or 6 (*in vivo*)). An unpaired Student's t-test and one-way ANOVA were used to compare the means of *in vitro* and *in vivo* drug release, *ex vivo* porcine buccal mucosal permeation and tissue levels of fenretinide, *in vivo* tissue levels of fenretinide and assess statistical significance. Results were considered statistically significant if $p<0.001$.

RESULTS AND DISCUSSION

Mucoadhesive Fenretinide Patches with Enhanced Drug Permeability

In our previous study,[23] we developed a novel mucoadhesive patch formulation of fenretinide for site-specific chemoprevention of oral cancer. Solubilizer-free patches exhibited poor *in vitro* and *in vivo* drug release behavior. Co-incorporation of either single or mixed solubilizers (e.g., Tween® 20 and 80, sodium deoxycholate) in fenretinide/Eudragit® patches led to significantly improved continuous *in vitro* and *in vivo* fenretinide release.[23] The use of fenretinide in chemoprevention of oral cancer has been hindered by

several key limitations, e.g., poor solubility, biological membrane permeability and bioavailability, and rapid elimination of drug from the body. Undesired effects are rendered mainly by its extremely high hydrophobicity (log $P = 8.03$) and low water solubility (below detection limit).[11,13,14] In addition, preliminary *in vitro* and *in vivo* studies conducted in our labs also suggested the necessity of permeation enhancers as the content of fenretinide was higher on the outer surface of buccal mucosa compared to interior tissue (*data not shown*). Hence, we made further improvements in this study by rendering to fenretinide patch enhanced mucosal permeation of fenretinide.

Fenretinide-loaded Eudragit® RL PO layers with and without permeation enhancers were prepared by a solvent casting method with drug loading efficiency of 90-95 %, as seen in Table 1. The thickness of fenretinide and adhesive layers, and the Tegaderm™ adhesive film were measured to be ~ 0.28, 0.28, and 0.05 mm, respectively. After assembling drug and adhesive layers onto backing layer, the total thickness of the patch was measured to be ~ 0.33 mm.

Determination of Optimal Quantity of Surfactant to Maintain *Ex Vivo* Sink Condition for Fenretinide: Solubility of Fenretinide in Bovine Serum

Sink condition is one of the key features that govern *in vitro* release or *ex vivo* biological membrane permeability of hydrophobic drugs.[34,35] The process of *ex vivo* drug transport from the patch (donor compartment) to receiver medium (receiver compartment) involves release of drug from the patch to the buccal surface, permeation of drug into the buccal tissue, and release (after dissolution if necessary) of drug from the tissue into the receiver chamber medium. The patch we developed for extremely hydrophobic fenretinide comprises of effective solubilizers to facilitate continuous *in vitro* and *in vivo* fenretinide release and tissue permeation enhancers to improve fenretinide permeability across buccal mucosa. To maintain a perfect sink condition in the release/receiver chamber medium, appropriate quantity of suitable solubilizing agent is commonly incorporated.[35] In this study, the optimal quantity of non-ionic surfactant in the release media was selected by matching the drug solubility to that in bovine serum.

The solubility of fenretinide in bovine serum at different fenretinide concentrations (0.9, 2.26, 3.97, 8.03 and 20.5 mg) and incubation times (1-7 days) is shown in Figure 1. The solubility of fenretinide in bovine serum was found to be 21 ± 1 μg/mL (see Figure 1A). Bovine serum comprises numerous proteins namely albumin, lipoproteins and serum retinol-binding protein (RBP). Enhanced solubility of fenretinide in bovine serum can be attributed to protein-drug binding or complexation.[36-38]

As expected, the time taken by fenretinide to reach equilibrium with bovine serum was affected by the amount of fenretinide added in bovine serum. For example, when the amount of fenretinide was increased from 0.9 to 8.03 mg, the time required to achieve equilibrium was reduced from 7 to 4 days (see Figure 1B). Further increases in fenretinide quantity did not reduce the time required for equilibration, thereby suggesting the necessity of minimum ~ 8 mg of fenretinide and 4 days incubation time to reach equilibrium state with 15 mL serum. A concentration of 0.084% Tween® 80 required to reach equivalent solubility of fenretinide (21 μg/mL in bovine serum) in test medium (receiver chamber medium i.e. PBS, pH 7.4) was then determined from the perfect linear relationship of fenretinide solubility in PBS versus Tween® 80 concentration above the surfactant critical micelle concentration.[39] Hence, PBS + 0.084% Tween® 80 was then used to mimic physiological solubilization/sink condition in the *ex vivo* drug permeation studies.

Enhanced *Ex Vivo* Porcine Buccal Mucosal Permeation of Fenretinide by Co-incorporation of Propylene Glycol and Menthol in Fenretinide/Eudragit® RL-PO Patches

The effect of co-incorporation of single (5 and 10 wt% PG or menthol) and mixed (1 wt% PG + 5 wt% menthol, 2.5 wt% PG + 5 wt% menthol or 10 wt% PG + 5 wt% menthol) permeation enhancers in fenretinide/Eudragit® RL PO mucoadhesive patches on *ex vivo* porcine buccal mucosal permeation of fenretinide is shown in Figure 2. *Ex vivo* permeation of fenretinide increased steadily over a period of 8 h and then reached a plateau thereafter (see Figure 2). The both the flux (J_s) at steady state and the enhancement factor (EF = J_s with enhancer/J_s without enhancer) were calculated.

The fraction of drug permeated across buccal mucosa and deposited in the buccal tissue, and values of J_s and EF are given in Table 2. Co-incorporation of single (see Figure 2A and B) or mixed (see Figure 2C) permeation enhancers in the patch led to significant enhancement ($p < 0.001$) in the rate and extent of fenretinide permeation across porcine buccal mucosa (see Table 2). For example, the flux for permeation enhancer-free patch was found to be ~ 10 μg cm^{-2} h^{-2}. After co-incorporation of 10 wt% PG or 10 wt% PG + 5 wt% menthol, the flux was increased to ~ 23 (EF = 2.3) and 40 (EF = 4) μg cm^{-2} h^{-2}, respectively. In contrast, a slight increase in the flux was observed with menthol patch formulations (J_s = ~ 13 μg cm^{-2} h^{-2}). The levels of drug in tissue were in agreement with the values of flux (see Table 2). Fenretinide content in buccal tissue after 12 h of *ex vivo* permeation with permeation enhancer-free patch was found to be ~ 44 μg/g. Co-incorporation of PG or PG + menthol led significantly high amount of fenretinide recovery from the buccal tissue (~ 171 and 241 μg fenretinide/g tissue with 10 wt% PG and 10 wt% PG + 5 wt% menthol formulation, respectively), thereby indicating increased tissue localization/penetration of fenretinide in the presence of PG or PG + menthol. A moderate enhancement effect was exhibited by menthol alone.

Propylene glycol exerts its permeation enhancement effect by competing for the solvation sites of the polar head groups of the lipid bilayers and occupying the hydrogen bonding sites, thereby increasing the solubility of this site for the permeant.[26-28] It has also been hypothesized that PG may increase the lipid fluidity which in turn facilitates enhanced drug permeation.[26-28] Enhanced permeation of fenretinide in the presence of PG can be attributed to one or both of these mechanisms. Menthol, on the other hand, has the ability to modify the drug diffusivity and/or partitioning by disrupting the conformational order of the intercellular lipids in bilayers.[40] Menthol alone it did not provide significant permeation enhancement of fenretinide ($p > 0.001$). This result can be attributed at least in part to non-homogeneous distribution of menthol in fenretinide/Eudragit® RL PO matrix (see Figure S2 and Discussion in Supporting Information) due to crystallization and aggregation of menthol during solvent evaporation.[41] Interestingly, when PG was combined with menthol, this issue was overcome (see Figure S2 in Supporting Information) and pronounced fenretinide permeation enhancement was observed relative to menthol alone (see Figure 2C and Table 2). Better fenretinide permeation observed with mixed permeation enhancers (PG + menthol) can be attributed to synergistic effect between menthol and PG.[25,26,28,29,40] These findings are in good agreement with report of Yamane *et al.*[29] In their study, synergistic enhancement of the ability of terpenes was observed in the presence of PG.

Although the specific oral mucosal permeation mechanism of fenretinide is not clear from our *ex vivo* and *in vivo* permeation data, this data was beyond the scope of the manuscript. Our goal was to apply known permeation enhancers and solubilizers toward delivery of very important chemopreventive fenretinide, and to optimize their formulation composition to enhance drug permeation *in vitro* and *in vivo*, while minimizing any histological changes. We have further conducted pharmacokinetic and pharmacologic evaluation of fenretinide mucoadhesive patches in rabbits and this data will be published elsewhere. In the future we

hope to (a) delineate experimentally the expected contributions of enhanced permeation, including solvation effects, fluidization of membrane lipid bilayers, and disruption of the conformational order of the intercellular lipids in bilayers facilitating transcellular or paracellular or combined pathways for fenretinide permeation enhancement, and (b) to further rule out potential toxicity associated with the proposed low content of menthol and PG in the fenretinide patch formulation.

Morphological and Histological Characteristics

Photomicrographs of the sections of porcine buccal tissue after 8 h buccal mucosal attachment of permeation enhancer-free and permeation enhancers-loaded fenretinide mucoadhesive patches are shown in Figure 3. The porcine buccal mucosa, similar to human buccal mucosa,[42] consists of an outermost layer of keratinized stratified squamous epithelium, below which lies a basement membrane, a lamina propria followed by the submucosa containing the buccinator muscle as the innermost layer.[43] Regardless of patch application, all sections showed an appropriately maturing stratified squamous epithelium. Scattered mitotic figures were restricted to the basilar layers, and the outermost granular and corneal layers showed appropriate terminal differentiation as reflected by surface parakeratin production. No evidence of changes consistent with extensive epithelial perturbations attributable to a contact mucositis e.g. hydropic degeneration of the basal cell layer or acantholysis were noted.

Basal epithelial cells are tightly bound together in the control (no patch attachment) sample (see Figure 3A). Noticeable morphological changes (e.g., prickle cells) in the underlying layers and significant loss of superficial cell layers were not apparent after attachment of 5 (Figure 3B) and 10 (see Figure 3C) wt% PG loaded patches. An increase in intercellular edema and swelling of buccal epithelium are visible, however, in Figure 3B and C when the loading of PG is above 5 wt%.

The photomicrographs of buccal epithelium after treatment with 5 and 10 wt% menthol loaded patches are respectively shown in Figure 3D and E. It is visible that the epithelium layers were intact in both the samples. In addition, there was no sign of cellular swelling and significant histological and ultrastructural changes. Similar results were observed in samples treated with 1 wt% PG + 5 wt% menthol (see Figure 3F) and 2.5 wt% PG + 5 wt% menthol (see Figure 3G) loaded patches. In contrast, tissue exposed to the 10 wt% PG + 5% menthol loaded patch showed a moderate increases in intracellular space and intercellular edema (see Figure 3H). Since menthol did not cause any epithelial cell alteration, it is likely that higher (10 wt%) loading of PG in the patch resulted in increased intracellular space and intercellular edema.

The histological changes (e.g., increases in intracellular space and intercellular edema) observed in the tissues treated with 5 and 10 wt% PG loaded patches (see Figure 3B, C and H) are indicative of diffusion of PG into individual keratinocytes as well as the intercellular spaces.[26,44,45] Upon penetration and accumulation in cells, it is likely that PG interacted with intercellular or membrane lipids,[26,28] thereby increasing the permeability of fenretinide through epithelium. These findings and hypotheses are in agreement with other reports.[26,45,46] Senel *et al.*[45] investigated the potential of dihydroxy and trihydroxy bile salts to enhance buccal penetration of fluorescein isothiocyanate. The findings obtained from freeze-fracture electron microscopy suggested presence of bile salts in the cytoplasmic space domain.[45] Since 2.5 wt% PG + 5 wt% menthol loaded patches exhibited optimal drug permeation enhancement with no morphological and histological changes, this formulation was selected and used to further evaluate *in vitro* and *in vivo* release, and *in vivo* permeation and tissue deposition kinetics of fenretinide, as described below.

In Vitro and *In Vivo* Release Characteristics of Permeation Enhancers-Loaded Fenretinide/ Eudragit® RL-PO Patches

Evaluation of *in vitro* and *in vivo* drug release characteristics of optimized fenretinide patch formulations (permeation enhancer-free and 2.5 wt% PG + 5 wt% menthol-loaded fenretinide/Eudragit® RL-PO patches) was conducted in simulated saliva and rabbits, respectively. To determine the cumulative amount of fenretinide released *in vitro/in vivo*, drug fraction remaining in the patches after 0.5, 3, and 6 h of *in vitro* incubation or *in vivo* attachment was determined and then subtracted from the initial drug content (see Figure 4 for *in vitro* and *in vivo* fenretinide release curves). Both the patch formulations provided continuous *in vitro* and *in vivo* fenretinide release from Eudragit® polymeric matrices, and the addition of PG and menthol did not significantly affect the release kinetics, indicating further fenretinide solubilization[26,28] and/or changes to the patch swelling behavior. Therefore, the patch release characteristics were largely determined by sodium deoxycholate and Tween® 80, which served as the effective solubilization role in the patch formulation.

Interestingly, there was a significant difference ($p < 0.001$) between *in vitro* and *in vivo* fenretinide release characteristics of permeation enhancer-free and 2.5 wt% PG + 5 wt% menthol-loaded fenretinide/Eudragit® RL-PO patches (see Figure 4), although the continuous release trend was the same. This difference can be linked to dissimilarity in test conditions (e.g., *in vitro* drug release in simulated saliva vs. *in vivo* drug release followed by permeation across buccal mucosal membrane). The finding observed in the current study was in agreement with the report of Junginger *et al.*[46] Junginger *et al.* also found significant differences between *in vitro* and *in vivo* permeation of FITC-labeled dextran across pig buccal mucosa, and the permeability of this compound increased in the presence of a penetration enhancer, sodium glycodexycholate.[46]

Enhanced *In Vivo* Rabbit Buccal Mucosal Permeation and Deposition of Fenretinide by Co-incorporation of Propylene Glycol and Menthol in Fenretinide/Eudragit® RL-PO Patches

In vivo tissue levels of fenretinide after 0.5, 3, and 6 h of buccal administration of permeation enhancer-free and 2.5 wt% PG + 5 wt% menthol-loaded fenretinide/Eudragit® RL-PO patches in rabbits is shown in Figure 5. The level of fenretinide in rabbit buccal tissue increased steadily as function of attachment time of both the patch (permeation enhancer-free and permeation enhancers-loaded patches) formulations (see Figure 5), thereby indicating excellent efficacy novel patch formulations to provide continuous *in vivo* fenretinide permeation across the rabbit buccal mucosa. However, extent of fenretinide permeation and tissue deposition provided by 2.5 wt% PG + 5 wt% menthol-loaded patches was significantly higher (43.0 ± 7.7 μg fenretinide/g tissue after 6 h of attachment) than that of permeation enhancer-free patch (17.3 ± 0.3 μg fenretinide/g tissue after 6 h of attachment) (see Figure 5). These results indicate excellent effectiveness of co-incorporation of PG and menthol to obtain improved oral mucosal permeation and tissue levels of fenretinide. Different permeation and tissue deposition kinetics of fenretinide obtained with *ex vivo* and *in vivo* studies can be attributed to dissimilarity in key test conditions (e.g., porcine vs. rabbit buccal mucosas, *ex vivo* vs. *in vivo* sink conditions).

Importantly, these data demonstrate the therapeutic advantage imparted by mucoadhesive patch local delivery of fenretinide, i.e., obtaining pharmacologically active levels in the target tissue. *In vitro* fenretinide concentrations between 1 and 10 μM have been established as inducing desirable chemopreventive effects, e.g., cellular terminal differentiation (<3 μM) and apoptosis (>5 μM).[47] As per previously published intra-tissue fenretinide mass to molar conversions, the levels of fenretinide delivered to rabbit buccal mucosa from permeation enhancers loaded patch ranged from 7.75 μg/g (0.5 hour; 19.8 μM) to 42.36 μg/g (6 hours; 108.2 μM).[48] Therefore, short duration patch application (i.e., less than 30

minutes) will provide therapeutically relevant concentrations in the targeted oral epithelium, and due to the decreased treatment time, should facilitate patient compliance.

CONCLUSIONS

The objective of this study was to further enhance intraoral site-specific fenretinide delivery by developing mucoadhesive patches that can provide enhanced buccal mucosal permeation and tissue levels of fenretinide. This hypothesis was tested by co-incorporating suitable permeation enhancers (PG and menthol) in fenretinide/Eudragit® RL-PO patches. Mucoadhesive patches containing well-designed drug delivery (fenretinide + solubilizers + permeation enhancers), adhesive, and backing layers were prepared by solvent casting and assembling techniques. Co-incorporation of PG or PG + menthol in patches led to significant *ex vivo* and *in vivo* buccal mucosal permeation and tissue deposition of fenretinide, a extremely hydrophobic and poorly tissue permeable chemopreventive agent. Mucoadhesive patch co-incorporated with 2.5 wt% PG + 5 wt% menthol was found to be an optimal fenretinide patch formulation for its oral mucosal permeation enhancement without significantly affecting the observed histology of the oral mucosa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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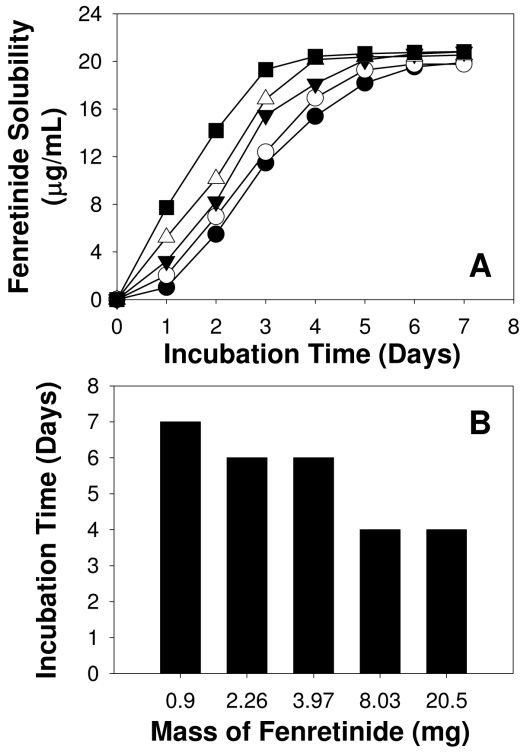

Figure 1.

Solubilization of fenretinide in bovine serum. The effect of quantity (0.9 (•), 2.26 (○), 3.97 (▼), 8.03 (△), and 20.05 (■)) of fenretinide added in 15-mL bovine serum and incubation time on the solubility of fenretinide (A) and the relationship between the quantity of fenretinide added and time required to reach equilibration (B). Solubility study was conducted at 37 °C under the protection from light.

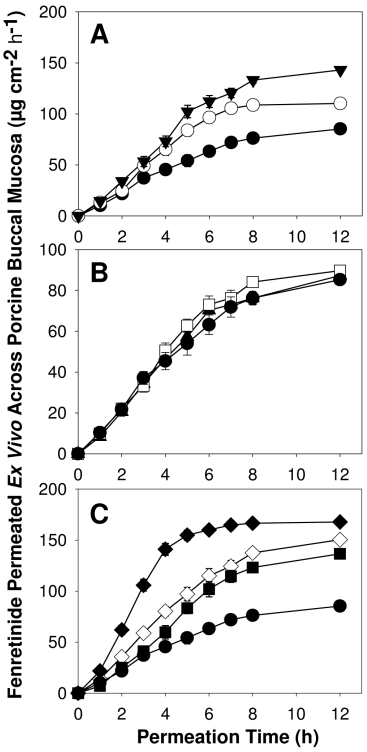

Figure 2.

Co-incorporation of propylene glycol (PG) or PG + menthol in fenretinide/Eudragit® RL PO patches significantly enhance fenretinide permeation across porcine buccal mucosa. The effect of co-incorporation of 0 (•), 5 (○) and 10 (▼) wt% PG (A), 5 (▲) and 10 (□) wt% menthol (B), and 1 wt% PG + 5 wt% menthol (■), 2.5 wt% PG + 5 wt% menthol (◇) and 10 wt% PG + 5 wt% menthol (◆) (C) in patches on *ex vivo* permeation of fenretinide across porcine buccal mucosa. *Ex vivo* permeation studies were conducted using side-by-side flow-through diffusion cells at 37 °C. Permeation enhancer-free patch comprised of 5 wt% fenretinide, 20 wt% Tween® 80, and 40 wt% sodium deoxycholate. Symbols represent mean ± SE, $n = 5$.

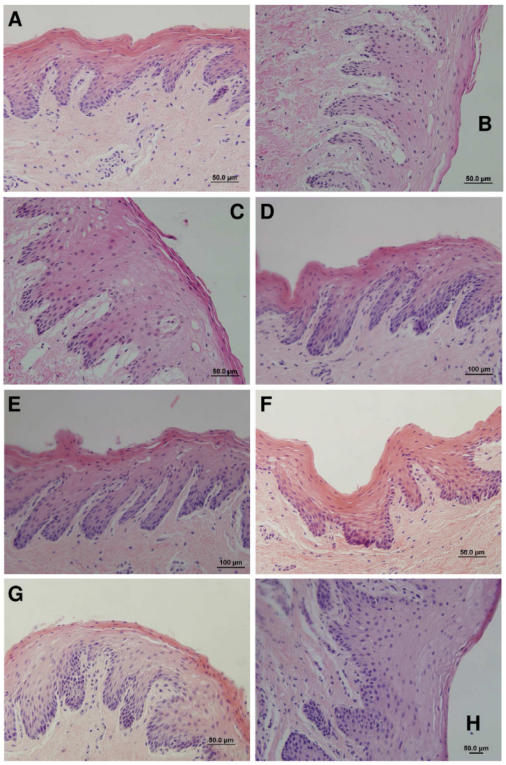

Figure 3.
Histological examination of porcine buccal tissue after 8 h buccal mucosal attachment of permeation enhancer-free and permeation enhancers-loaded fenretinide mucoadhesive patches. The effect of co-incorporation of 0 (A), 5 (B) or 10 (C) wt% propylene glycol (PG), 5 (D) or 10 (E) wt% menthol, and 1 wt% PG + 5 wt% menthol (F) or 2.5 wt% PG + 5 wt% menthol (G) or 10 wt% PG + 5 wt% menthol (H) in fenretinide/Eudragit RL PO mucoadhesive patches on histological changes of porcine buccal tissue. As is apparent from these photomicrographs, patch application (with or without permeation enhancers) did not dramatically perturb porcine buccal mucosa. All sections demonstrate a preserved basement membrane and basal cell layer, an intact stratified squamous surface epithelium with an overlying parakeratotic layer. Notably, no evidence of changes consistent with extensive epithelial damage e.g. hydropic degeneration of the basal cell layer or acantholysis were observed in multiple sections. The evidence of increased intracellular and intercellular edema observed in epithelia exposed to increased levels PG (C and H.) likely reflects diffusion of PG into individual keratinocytes as well as the intercellular spaces. Images were taken by a light microscope. Permeation enhancer-free patch comprised of 5 wt% fenretinide, 20 wt% Tween® 80, and 40 wt% sodium deoxycholate.

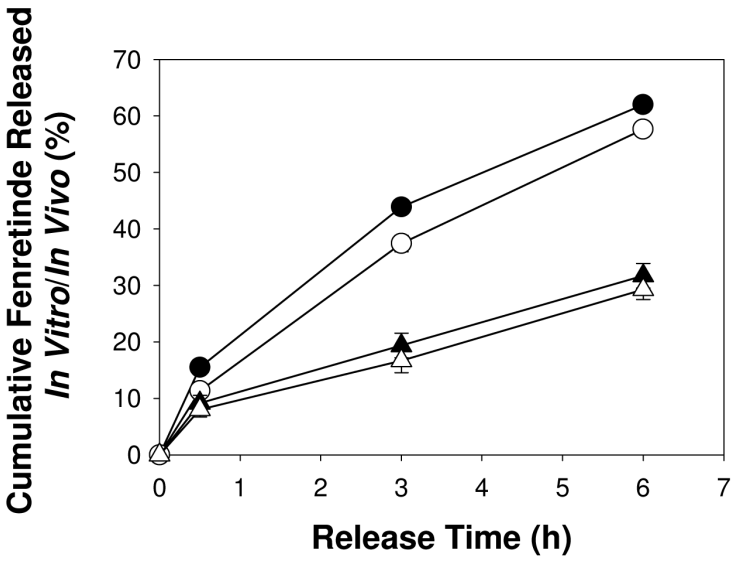

Figure 4.

In vitro and *in vivo* release characteristics of permeation enhancers-free and permeation enhancers-loaded fenretinide/Eudragit® RL PO mucoadhesive patches. Cumulative amount of fenretinide released *in vitro*/*in vivo* from permeation enhancers-free (○: *in vitro*; △: *in vivo*) and permeation enhancers (2.5 wt% propylene glycol + 5 wt% menthol)-loaded (•: *in vitro*; ▲: *in vivo*) patches as a function of time. *In vitro* and *in vivo* release studies were conducted in simulated saliva containing 5% w/v sodium deoxycholate (pH 6.8) at 37 °C and rabbits, respectively. Permeation enhancer-free patch comprised of 5 wt% fenretinide, 20 wt% Tween® 80, and 40 wt% sodium deoxycholate. Symbols represent mean ± SE, n = 4 (*in vitro*) or 6 (*in vivo*).

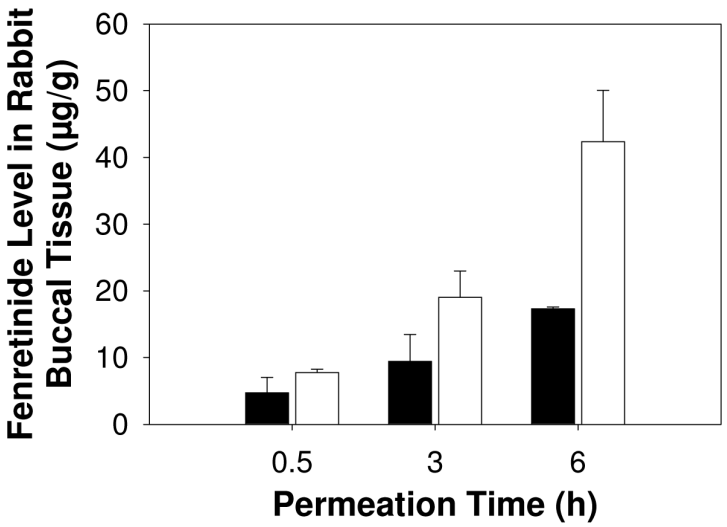

Figure 5.

Co-incorporation of permeation enhancers (2.5 wt% propylene glycol + 5 wt% menthol) in fenretinide/Eudragit® RL PO patch enhances *in vivo* buccal mucosal permeation of fenretinide. Tissue levels of fenretinide as a function of buccal administration time of permeation enhancer-free (*filled bars*) and permeation enhancers-loaded (*open bars*) patches in rabbits. Permeation enhancer-free patch comprised of 5 wt% fenretinide, 20 wt% Tween® 80, and 40 wt% sodium deoxycholate. Bars represent mean ± SE, $n = 6$).

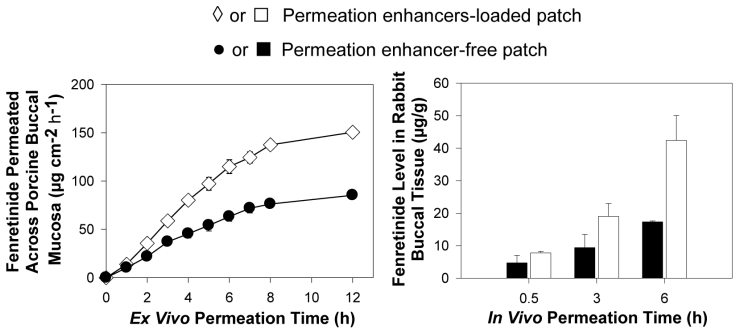

Figure 6.

Table 1

Evaluation of Microencapsulation of Fenretinide in Permeation Enhancer-Free and Permeation Enhancers-Loaded Eudragit® RL-PO Films.

Patch formulation	Fenretinide loading (wt%)		Loading efficiency (%)*
Theoretical[a]	Actual*		
Permeation enhancer-free	5.26	4.76 ± 0.06	90.44 ± 1.14
5 wt% PG	5.00	4.59 ± 0.07	91.81 ± 1.40
10 wt% PG	4.76	4.52 ± 0.12	94.94 ± 2.52
5 wt% Menthol	5.00	4.62 ± 0.06	92.40 ± 1.20
10 wt% Menthol	4.76	4.51 ± 0.11	94.71 ± 2.31
1 wt% PG + 5 wt% Menthol	4.95	4.48 ± 0.13	90.50 ± 2.63
2.5 wt% PG + 5 wt% Menthol	4.88	4.53 ± 0.11	92.87 ± 2.26
10 wt% PG + 5 wt% Menthol	4.54	4.14 ± 0.05	91.10 ± 1.10

*Mean ± SE, $n = 3$;

[a]Based on polymer + excipients weight

Table 2

Evaluation of Potential of Co-incorporation of Permeation Enhancers (Propylene Glycol (PG), Menthol or PG + Menthol) in Fenretinide/Eudragit® RL PO Patches to Enhance Porcine Buccal Mucosal Permeation of Fenretinide *Ex Vivo.*

Patch formulation	Flux (J_s) (μg cm^{-2} h^{-1})[a]	Fenretinide in the receptor medium (μg/mL)	Fenretinide in the tissue (μg/g)	EF[b]
Permeation enhancer-free	10.0 ± 0.5	22.3 ± 0.5	43.8 ± 6.1	1.0
5 wt% PG	16.2 ± 0.9	28.8 ± 0.8	158.5 ± 4.7	1.6
10 wt% PG	22.8 ± 1.3	37.4 ± 0.5	170.7 ± 5.3	2.3
5 wt% Menthol	12.4 ± 0.6	22.8 ± 0.5	61.7 ± 5.1	1.2
10 wt% Menthol	12.7 ± 0.7	23.5 ± 0.7	65.3 ± 4.7	1.3
1 wt% PG + 5 wt% Menthol	17.9 ± 0.6	35.8 ± 1.0	172.1 ± 7.6	1.8
2.5 wt% PG + 5 wt% Menthol	20.2 ± 0.8	39.4 ± 0.8	175.6 ± 7.0	2.0
10 wt% PG + 5 wt% Menthol	39.8 ± 0.9	43.9 ± 0.6	241.1 ± 9.8	4.0

[a] J_S Steady state flux was calculated from linear regression of cumulative amount permeated vs. time (linear portion of the permeated amount of fenretinide vs. time profile);

[b] Enhancement factor (EF) = J_S in the presence of permeation enhancer/J_S in the absence of permeation enhancer; Values represent mean ± SE, n = 5.