Pharmacokinetic Evaluation of a Mucoadhesive Fenretinide Patch for Local Intraoral Delivery: A Strategy to Re-introduce Fenretinide for Oral Cancer Chemoprevention

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By
Maynard Prescott Phelps, DMD
Graduate Program in Dentistry

The Ohio State University
2012

Thesis Committee:
Dr. Susan R. Mallery, Adviser
Dr. Peter E. Larsen
Dr. Henry W. Fields
Abstract

Systemic delivery of fenretinide in oral cancer chemoprevention trials has been largely unsuccessful due to dose-limiting toxicities and suspected sub-therapeutic intra-epithelial drug levels. Local drug delivery, however, provides site-specific therapeutically-relevant levels while minimizing systemic exposure. These studies evaluated the pharmacokinetic and growth-modulatory parameters of a fenretinide mucoadhesive patch application on rabbit buccal mucosa. Fenretinide and blank-control patches were placed on right/left buccal mucosa, respectively, in 8 rabbits (30 minutes, q.d., 10-days). No clinical or histological deleterious effects occurred. LC-MS/MS analyses of post-treatment samples revealed a delivery gradient with highest fenretinide levels achieved at the patch-mucosal interface (no metabolites), pharmacologically-active levels in fenretinide-treated oral mucosa (mean: 5.65μM; trace amounts of 4-oxo-4-HPR), and undetectable sera levels. Epithelial markers for cell proliferation-Ki67, terminal differentiation-transglutaminase1, and glucuronidation-UGT1A1 exhibited fenretinide concentration-specific relationships (elevated transglutaminase1 and UGT1A1 levels <5μM, reduced Ki-67 indices >5μM) relative to blank-treated epithelium. All fenretinide-treated tissues showed significantly increased intraepithelial TUNEL positivity, implying activation of intersecting apoptotic and differentiation pathways. Human oral mucosal correlative studies showed substantial inter-donor variations in levels of the enzyme (CYP3A4) responsible for conversion of fenretinide to its highly active metabolite, 4-oxo-4-HPR. Complementary in vitro assays in human oral
keratinocytes delineated optimal differentiation dosages (1-3μM) as shown by increased levels of transglutaminase1 and involucrin. These cell studies also revealed fenretinide's preferential suppression of DNA-synthesis in dysplastic as opposed to normal oral keratinocytes. Collectively, these data show that mucoadhesive patch-mediated fenretinide delivery is a viable strategy to re-introduce a compound known to induce keratinocyte differentiation to human oral cancer chemoprevention trials.
Dedication

Dedicated to

my wife, Joy,

my children, Mylan, Tyson, and Lauren

and

my parents, Patrick and Oksun Phelps
Acknowledgements

First I would like to thank God for providing me good health and opening opportunities for me to achieve my goals. I thank my wife and children for giving me a life away from the rigors of residency while coping with my absence without protest. I would also like to thank my parents and sister for their unwavering love and support throughout my life.

I thank my advisor, Dr. Susan Mallery, for her incredible support and guidance. She has always been encouraging, supportive, and available throughout my training. I would like to thank Andrew Holpuch for his work, organization, creativity, and dependability, making a project of this magnitude possible. I am of course thankful of all the people involved in this project, including the staff and students of Dr. Mallery’s Lab, my thesis committee members and coinvestigators, Drs. Peter E. Larsen and Henry W. Fields, and our collaborators, Drs. Steven P. Schwendeman, Kashappa-Goud H. Desai, Gary D. Stoner, and Zhongfa Liu.

Thank you to my oral and maxillofacial surgery faculty for their patience, guidance, and sacrifice over the course of their careers, including during my training. I appreciate Dr. Larsen’s efforts to maintain this residency program’s excellent reputation while serving as not only division chair but also at times as program director. Dr. Ness has brought with him enthusiasm and leadership and has expanded our depth of surgical training as our new program director. Drs. Kennedy, Evans, and Kaye have always been approachable and sincere in their desire to teach residents. I appreciate
the instruction that I received from Drs. Prior, Weaver, Ganzberg, Allen, Kalmar, and our numerous adjunct faculty. Finally, I would like to thank Dr. Chacon for giving me the opportunity to train in this respected residency program.
VITA

June 1998 .............................. Martin Luther King, Jr High School
               Detroit, Michigan
August 2002 ............................ Bachelor of Science, Plant Biology
               University of Michigan - Ann Arbor,
               Michigan
June 2006 ............................... Doctor of Dental Medicine
               Harvard School of Dental Medicine
               Boston, Massachusetts
2008 – present .......................... Resident, Oral and Maxillofacial Surgery
               The Ohio State University
               Columbus, Ohio

Publications

Wu X, Desai KG, Mallery SR, Holpuch AS, Phelps MP, Schwendeman SP:
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. Molecular Pharmaceutics, 2012

Fields of Study

Major field: Dentistry
Specialization: Oral and Maxillofacial Surgery
# Table of Contents

Abstract ........................................................................................................................................... ii
Dedication ....................................................................................................................................... iv
Acknowledgments....................................................................................................................... v
Vita ................................................................................................................................................. vii
List of Tables .................................................................................................................................. x
List of Figures ............................................................................................................................... xi
Chapter 1: Introduction/Review of Literature .............................................................................. 1
Chapter 2: Detailed Methodology ............................................................................................... 22
Chapter 3: Original Manuscripts ................................................................................................. 35
Chapter 4: Integrated Conclusions ............................................................................................. 101
References ...................................................................................................................................... 111
List of Tables

Table 1. .................................................................................................................9
Table 2. .............................................................................................................12
Table 3. ............................................................................................................76
Table 4. ..........................................................................................................82
List of Figures

Figure 1. ......................................................................................................................... 4
Figure 2. .......................................................................................................................... 24
Figure 3. .......................................................................................................................... 26
Figure 4............................................................................................................................ 57
Figure 5............................................................................................................................ 58
Figure 6............................................................................................................................ 59
Figure 7............................................................................................................................ 60
Figure 8............................................................................................................................ 61
Figure 9............................................................................................................................ 62
Figure 10......................................................................................................................... 78
Figure 11......................................................................................................................... 80
Figure 12......................................................................................................................... 85
Figure 13......................................................................................................................... 88
Figure 14......................................................................................................................... 90
Chapter 1: Introduction/Review of Literature

Statement of the problem

In 2011, there were an estimated 40,250 new cases of oral and pharyngeal cancer in the U.S., with an estimated 7,850 deaths attributable to this disease\(^1\). Cancers limited to the oral cavity comprised half of all cancers of the head and neck\(^1\). In the U.S., the age adjusted incidence of oropharyngeal cancer in men and women is 15.1% and 6.3%, respectively, with black males representing the group of highest risk\(^2\). While oropharyngeal cancer ranks 8\(^{\text{th}}\) in new cancer cases amongst American males, it makes up 25% of new cancer cases in southern Asian countries such as Pakistan, India, Sri Lanka, and Bangladesh. Approximately 90% of all cancers of the oral cavity are squamous cell carcinomas (OSCC). The overall 5-year survival rate of patients with SCC of the head and neck hovers around 50-60%, which hasn’t changed considerably over the last few decades\(^2\). Therapies range from local excision to potentially disfiguring tumor resection with neck dissection followed by radiation therapy. Mortality is often from recurrence or de novo development of additional cancerous lesions. The repetition of treatments aimed at treating the disease and subsequent restoring of function and esthetics increases the associated cost to the patient and to society. Lang et al. estimated the excess annual healthcare cost of patients in the U.S. with squamous cell carcinoma of the head and neck over their cohorts to be $12,000, exceeding that of other solid tumors such as breast, prostate, colon, and rectal cancers\(^3\). In many cases,
OSCC arises from identifiable precursor lesions, namely leukoplakia and erythroplakia. Treatments for these lesions range from local excision to observation with routine biopsy. This exposes the patient to multiple surgeries and mental anguish without ensuring a halt to cancer progression. Non-invasive local intervention, specifically topical chemoprevention, aimed at these precursor lesions may improve overall patient outcomes.

Risk Factors

Two of the most significant risk factors for oropharyngeal cancer in the U.S. include tobacco smoking and heavy alcohol consumption, with both habits acting synergistically to increase overall risk. Both alcohol and tobacco together account for 75% of all OSCC. Other established risk factors include chewing tobacco, snuff dipping, and betel quid use. Several other possible etiologic factors have been investigated, although the evidence is not as convincing as for the above mentioned factors. A hospital controlled case study by Petridou et al. found that consumption of fruits and vegetables was inversely associated with oral cancer, while meat was positively associated. Dietary deficiencies in vitamins and minerals has also been proposed to increase the risk of developing oral SCC. An international multicenter case control study by Herrero et al. found that antibodies to HPV16 E6 or E7 were risk factors for cancer of the oral cavity (OR = 2.9) and the oropharynx (OR = 9.2). Not surprisingly, some studies have even found associations between sexual behaviors and oral cancer. Other predisposing factors include EBV, HIV, previous head and neck radiation, and occupational exposure.
Molecular Perturbations/Alterations

See Figure 1 below for a summary of the proposed molecular changes involved in the development of OSCC\textsuperscript{12}. The molecular changes that lead to tumorigenesis typically effect two important gene classes: tumor suppressor genes and oncogenes\textsuperscript{13}. Shin et al. looked at p53 (tumor suppressor) mutations in oral dysplastic and squamous cell lesions. Using in situ hybridization with probes specific for chromosomes 9 and 17, they found that lesions with dysregulated p53 expression showed nearly 2–4-fold increased levels of chromosome polysomy when compared to histologically normal tissue which did not display a p53 mutation\textsuperscript{14}. Jiang et al. looked at loss of heterozygosity (LOH) in 13 cases of leukoplakia with foci of cancerization within the same lesions, and found identical LOH in both the cancer foci and leukoplakia in 11 of the 13 cases\textsuperscript{15}. Their study found a high incidence of LOH at the 9p loci (66.7%). The tumor suppressor p16 gene is located on 9p21. In a separate study, a similar percentage of 72% LOH at 9p was found in both dysplastic and cancerous lesions\textsuperscript{16}. Mao et al. investigated 37 leukoplakia lesions for LOH at the 9p21 and the 3p14 foci, and found that 37% of those with LOH at either loci went on to develop head and neck squamous cell carcinoma (HNSCC), while only 6% of those without LOH at these foci went on to transform over the median follow-up period of 63 months\textsuperscript{17}. Overexpressions of cyclooxygenase-2 (COX-2) and phospho-epidermal growth factor receptor (pEGFR) are also considered important in the development of OSCC\textsuperscript{12,18}. In addition, several genetic disorders in which tumor suppressor genes are defective have been associated with increased risk of OSCC, such as xeroderma pigmentosum, ataxia telangiectasia,
Bloom syndrome and Fanconi's anaemia\textsuperscript{19,20}. However, aside from the above mentioned genetic disorders, OSCC has no known heritable genetic profile, unlike other common cancers such as BRCA1 and BRCA2 mutations in breast cancer\textsuperscript{21}.

Figure 1. Molecular progression model of multistep oral carcinogenesis. Copied from Lippman et al.\textsuperscript{12}

Field Cancerization

The definition of field cancerization proposed by Braakhuis et al. is the presence of one or more areas consisting of epithelial cells that have genetic alterations\textsuperscript{22}. Field cancerization is a theory in which a cell develops a genetic alteration that provides a reproductive advantage, allowing a clone of daughter cells, or field lesion, to expand
laterally within the epithelium. This initial clone may appear both macro and microscopically normal. A field lesion has a monoclonal origin, and does not show invasive growth or metastatic behavior, a hallmark criteria of cancer. However, as cells incur additional mutations, they form smaller subclones of greater aberrancy, eventually leading to invasive carcinoma. Based on this theory, even if the primary tumor is removed with clean microscopic margins, a portion of the original clone may be left in the field, leading to a 'second primary tumor' or recurrence.

Evidence to support this theory have been found in several organ systems, including the oral cavity, oropharynx and larynx, lung, esophagus, vulva, cervix, colon, breast, bladder, and skin\textsuperscript{22}. Tabor et al. found that in 10 of 28 patients treated for HNSCC, LOH was found in macroscopically normal tissue adjacent to the tumor\textsuperscript{23}. In 7 of these 10 patients, LOH indices found at the surgical margins corresponded to the biopsy margin of adjacent macroscopically normal tissue, suggesting a common precancerous field. Using a highly sensitive TP53 plaque assay to detect specific p53 mutations, Brennan and colleagues found that in just over 50% of patients with microscopically normal surgical margins, cells with identical p53 mutations to that of the tumor were present in the ‘normal’ tissue\textsuperscript{24}. These studies lend support to the presence of a field of normal appearing but genetically altered cells related to the primary tumor. This field of altered cells is difficult to detect clinically and histologically, thus creating difficulty in obtaining true clear surgical margins. Thus, for OSCC, targeting the altered field with topical chemopreventive agents may serve as a way to reduce recurrence without more extensive and disfiguring surgery, especially since obtaining molecularly clear margins may be impossible or impractical.
In addition, stem cells within the basal layer may harbor the genetic perturbations common within the field lesion\textsuperscript{26}. Stem cells are long-lived cells that, following an injury to the epithelium, will undergo mitosis to produce transient amplifying cells. The transient amplifying cells will then provide the next population of epithelial cells during the recovery period. Because of the retention of the genetic perturbation within the long-lived stem cell population and their ability to proliferate later in life, constant vigilance is required of both the practitioner and patient to monitor the epithelium for changes potentially for the lifetime of the patient.

**Precursor Lesions**

Identifiable precursor lesions for oral squamous cell carcinoma have been well studied, and due to their ease of accessibility for evaluation and biopsy, offer an ideal model for chemoprevention study. The literature supports the clinical diagnoses of leukoplakia, erythroleukoplakia, and erythroplakia as potential premalignant lesions that may progress to invasive carcinoma\textsuperscript{26}. These lesions share the same risk factors as OSCC. Oral erythroplakia has no sex predilection and is most common in the 6\textsuperscript{th} and 7\textsuperscript{th} decades of life. Oral leukoplakia, however, occurs more commonly in the 4\textsuperscript{th} and 5\textsuperscript{th} decades of life\textsuperscript{27}. At the time of biopsy of these clinical lesions, histologic diagnoses can range from atypia to carcinoma in situ. Several studies have demonstrated the close association of leukoplakia and OSCC\textsuperscript{15,18,26,28}. Leukoplakia has an overall annual transformation rate of 1\%, which can be higher in patients with the following risk factors: female gender, long duration of lesion, non-smoker, size >200mm\textsuperscript{2}, non-homogenous type, and presence of epithelial dysplasia\textsuperscript{26}. In a study by Silverman et. al., 17.5\% of
patients with leukoplakia progressed to OSCC within an average study period of 8 years\textsuperscript{28}. Lesions with a red component, or erythroleukoplakia, demonstrated a 4 fold increase in transformation risk. The prognosis for erythroplakia is significantly worse. In one study of 65 biopsy specimens of erythroplakia, 33 were invasive carcinoma, 26 were carcinoma in situ or severe epithelial dysplasia, and only 6 were mild to moderate dysplasia\textsuperscript{27}.

**Chemoprevention**

Cancer chemoprevention is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer\textsuperscript{29}. Chemoprevention is used for patients without a known cancerous lesion, in contrast to chemotherapy. This distinction is important as patient tolerance of adverse effects is likely far greater when faced with the morbidity and mortality of known cancer versus the potential for cancer. Thus, an ideal chemopreventive agent is well tolerated and has no adverse effects. Unlike many current cancer therapies such as radiation and chemotherapy which are known to be potentially mutagenic, a mutagenic chemopreventive would be counter productive in preventing cancer based on the theory of multi-step carcinogenesis.

*Systemic Delivery Strategies*

Given the fact that OSCC often develops from identifiable precursor lesions as stated above, these lesions are perfect targets for chemoprevention. Not surprisingly,
there are numerous OSCC chemopreventive studies which have been conducted, many of which used systemic delivery. These studies were summarized by Holpuch et al. (our lab) and are shown in table 1. Limitations of these studies include no requirement for smoking cessation (the most important risk factor for OSCC), no measurement of the chemopreventive agents within the target tissues (with few exceptions), and frequent systemic adverse effects. However, many of these studies showed at least mixed results, with improvement in appearance and/or histologic grade of leukoplakia in a subset of patients following the administration of high dose β-carotene, fenretinide, a combination of isotretinoin, oral alpha-tocopherol, and sub-Q interferon, and even green tea extract. Systemic delivery, while very familiar to patients in terms of ease of application, carries several limitations, including the effects of first pass metabolism, exposure of multiple organ systems to the chemopreventive agent, and potential lack of sufficient delivery to the target site.
<table>
<thead>
<tr>
<th>Therapeutic Agent</th>
<th>Year</th>
<th>Delivery Method, Schedule, &amp; Duration</th>
<th>Biopsies &amp; Microscopic Diagnosis</th>
<th>Side Effects</th>
<th>Therapeutic Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isotretinoin</strong> (13-cis-retinoic acid)</td>
<td>1986</td>
<td>Oral capsule, 1-2 mg/kg q.d. Duration: 3 months with 6-month follow-up</td>
<td>Pre- and post-treatment biopsy-confirmed dysplastic and atypical hyperplastic lesions (n=44)</td>
<td>Mild to Moderate</td>
<td>Isotretinoin Group (n=24): 54% of lesions showed regression of dysplasia. Notably, lesion recurrence was exhibited in 9 of 16 evaluable patients. Placebo Group (n=20): 10% of lesions showed regression of dysplasia (p=0.01 relative to isotretinoin group). Alcohol/tobacco cessation not required</td>
</tr>
<tr>
<td><strong>Isotretinoin and β-carotene</strong></td>
<td>1993</td>
<td>Induction Phase: isotretinoin (oral capsule, 1.5 mg/kg q.d.) Duration: 3 months Maintenance Phase: isotretinoin (oral capsule, 0.5 mg/kg q.d.) or β-carotene (oral capsule, 30 mg q.d.) Duration: 9 months</td>
<td>Pre- and post-treatment biopsy-confirmed dysplastic and hyperplastic NOS lesions (n=70)</td>
<td>Mild to Severe</td>
<td>Induction Phase (n=70): 55% of lesions exhibited complete or partial regression, while 35% demonstrated stable disease. Notably, 18 of 42 patients with dysplasia had a decreased histological grade, and 7 patients experienced disease progression. Maintenance Phase (n=53): β-carotene (n=29): 45% of patients exhibited complete/partial or stable response, while 55% demonstrated disease progression. Isotretinoin (n=24): 92% of patients demonstrated complete/partial or stable response, while 8% experienced disease progression. Alcohol/tobacco cessation not required</td>
</tr>
<tr>
<td><strong>β-carotene</strong></td>
<td>1999</td>
<td>Induction Phase: β-carotene (oral capsule, 30 mg b.i.d.) Duration: 6 months Maintenance Phase: β-carotene (oral capsule, 30 mg b.i.d.) or placebo Duration: 12 months</td>
<td>None reported</td>
<td>Induction Phase (n=50): 52% of lesions demonstrated complete or partial response, while 40% exhibited stable disease and 8% progressed. Notably, a 39% histological response rate was observed in dysplastic lesions. Maintenance Phase (n=23): β-carotene (n=11): 82% of the lesions remained stable, 18% progressed. Placebo (n=12): 83% of the lesions remained stable, 17% progressed. *Quantified both plasma and buccal cell β-carotene concentrations Alcohol/tobacco cessation not required</td>
<td></td>
</tr>
<tr>
<td><strong>Isotretinoin, α-tocopherol, &amp; interferon-α cocktail</strong></td>
<td>1999</td>
<td>Isotretinoin (oral capsule, 100 mg/m² q.d.) α-tocopherol (oral capsule, 1200 IU, q.d.) interferon-α (SubQ, 3 MU/m², twice/week) Duration: 12 months with 6-month follow-up</td>
<td>Pre- and post-treatment biopsies *19 of 50 lesions dysplastic</td>
<td>6-month examination (n=10): 50% of histologically evaluated lesions demonstrated complete/partial response, while 20% exhibited stable disease and 30% progressed. 12-month examination (n=7): 14% of histologically evaluated lesions demonstrated partial response, while 43% exhibited stable disease and 43% progressed. Notably, 4 patients eventually progressed to cancer. Alcohol/tobacco cessation not required</td>
<td></td>
</tr>
<tr>
<td><strong>Fenretidine</strong></td>
<td>2005</td>
<td>Oral capsule, 100 mg b.i.d. versus no treatment Duration: 12 months with 5-year follow-up</td>
<td>Surgically excised benign hyperkeratotic and dysplastic lesions (n=170) *3-4% of lesions were dysplasia</td>
<td>Mild to Severe</td>
<td>Fenretidine Group (n=84): 15 patients exhibited lesional recurrences, 4 patients developed new leukoplakic lesions, and 1 patient developed oral cancer. No Treatment Group (n=86): 15 patients exhibited lesional recurrences, 10 patients developed new leukoplakic lesions, and 2 patients developed oral cancer. Alcohol/tobacco cessation not required</td>
</tr>
<tr>
<td><strong>Fenretidine</strong></td>
<td>2006</td>
<td>Oral capsule, 200 mg q.d. Duration: 3 months with 9-month follow-up</td>
<td>Retinoid resistant &quot;oral intraepithelial-neoplasia&quot; *10 of 35 lesions dysplastic</td>
<td>Mild to Moderate</td>
<td>3-month examination (n=35): 34% of patients exhibited clinical partial responses, while 43% of patients demonstrated stable disease and 23% progressed. Notably, 9 of 12 partial responders progressed within 9 months of trial completion. Alcohol/tobacco cessation not required</td>
</tr>
</tbody>
</table>

Table 1. Summary of trials that used systemic chemopreventive agent delivery (from Holpuch et al.30)
Local Delivery Strategies

Local (topical) delivery offers the potential advantage of delivering high concentrations of chemopreventive agents to the epithelium and immediate surrounding structures while minimizing systemic effects. The mouth allows for the use of patient applied topical delivery systems, which have a long history of application in dentistry (toothpaste, mouthrinse, analgesic gels, etc). Furthermore, the health professional can quickly identify treatment success or failure and implement a new treatment modality as soon as it is needed. Chemoprevention can not only be applied to the precursor lesions that lead to primary tumors, but also to the surrounding tissues after ablative surgery, since death from SCC is often from recurrence or the development of a new primary.

To date there have been numerous OSCC chemoprevention trials employing local delivery. Rudin et al. studied an attenuated adenovirus, ONYX-015, which is preferentially cytotoxic to cells with defective p53 signaling. The virus was applied to biopsy confirmed oral dysplasia via a mouthrinse. While 7 of 19 patients showed complete histologic response to therapy, many of these complete responders and even partial responders later demonstrated recurrent disease. Epstein et al. studied topical bleomycin for treatment of dysplastic oral leukoplakia, and among 19 participants, found a mean histologic improvement of 2 stages. Unfortunately, 15.8% progressed to OSCC at later follow-ups, and smoking and alcohol cessation were not required for participants. It is of note that in all of these topical studies, adverse effects were few and well tolerated by patients. Common amongst almost all chemopreventive trials of oral dysplasia, however, are two main problems: 1) there are subsets of patients that
show remarkable improvements and those that do not, and 2) even patients who demonstrate significant improvement often show later disease progression or recurrence following cessation of treatment. Because in most of these studies the main risk factor for OSCC, smoking, continued during the trials, the true effectiveness and longevity of the improvements seen remain unknown. Other local delivery approaches will be discussed in the following text, especially the use of retinoids and natural products. Holpuch et al. summarized the OSCC chemoprevention trials to date using topical delivery, shown in table 2\(^{30}\).
<table>
<thead>
<tr>
<th>Therapeutic Agent</th>
<th>Year</th>
<th>Delivery Method, Schedule, &amp; Duration</th>
<th>Biopsies &amp; Microscopic Diagnosis</th>
<th>Side Effects</th>
<th>Therapeutic Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotretinoin (13-cis-retinoic acid)</td>
<td>1993</td>
<td>1 mg oral troche (3 mg/day, 5 mg/day, 10 mg/day) Duration: 6 months with 11-month follow-up</td>
<td>Pre- and post-treatment biopsies of oral leukoplakia (n=16)</td>
<td>Mild</td>
<td>Of 11 evaluable patients, 3 demonstrated complete regression and 6 partial regression. Post-treatment lesion recurrence was observed in 2 of 3 patients with complete regression, and 1 partial responder regressed to complete. Alcohol/tobacco cessation not required</td>
</tr>
<tr>
<td>Isotretinoin (13-cis-retinoic acid)</td>
<td>1999</td>
<td>0.1% isotretinoin gel or placebo applied t.i.d. Duration: 4 months Following 4 month study completion, placebo patients completed additional 4 months with 0.1% isotretinoin gel.</td>
<td>Pre- and post-treatment biopsy-confirmed oral leukoplakia (n=10)</td>
<td>None</td>
<td>Isotretinoin gel treatment group demonstrated lesional regression. Placebo group exhibited stable disease, but when treated with isotretinoin gel they exhibited lesional regression. (1 complete response and 8 partial responses). Post-treatment lesion recurrence not reported. Alcohol/tobacco cessation not required</td>
</tr>
<tr>
<td>Fenretide</td>
<td>1994</td>
<td>Topical application of capsule contents (100 mg b.i.d.) Duration: 2 months</td>
<td>Pre- and post-treatment biopsies of oral leukoplakia (n=6) and lichen planus (n=2). *Histology not confirmed.</td>
<td>None</td>
<td>Clinical lesion regression in 2 of 3 patients at 2 months, but did not specify whether the lesions were leukoplakia or lichen planus. Topical application was well-tolerated by oral mucosa. Low systemic levels of fenretinide were detected. Post-treatment lesion recurrence not reported. Alcohol/tobacco cessation not reported</td>
</tr>
<tr>
<td>Tretinoin (all-trans retinoic acid)</td>
<td>1999</td>
<td>0.05% tretinoin gel applied q.i.d. Duration: average of 1.5 years in biopsy-confirmed dysplasia</td>
<td>Pre- and post-treatment biopsy-confirmed oral dysplasia in 9 of 26 patients</td>
<td>None reported</td>
<td>Of patients with confirmed dysplasia, 55% exhibited lesional regression or stable disease, and 45% had lesions that progressed. Notably, of the 26 total trial participants (i.e., including benign lesions), 7 patients demonstrated complete remission. 3 of those 7 experienced post-treatment clinical lesion recurrences (i.e., not biopsy-confirmed). Alcohol/tobacco cessation not required</td>
</tr>
<tr>
<td>Acitretin</td>
<td>2000</td>
<td>Oral mucoadhesive tablet, 10 mg b.i.d. Duration: 4 weeks</td>
<td>Pre- and post-treatment biopsies of oral leukoplakia (n=21) *6 of 21 lesions dysplastic</td>
<td>None</td>
<td>10 of the 14 patients (2 of 6 dysplastic lesions) in the acitretin groups demonstrated lesional regression, while 0 of 7 in the placebo group exhibited lesional improvement. Pharmacokinetic analyses demonstrated sustained acitretin salivary levels for up to 9 hours (4.9-43 mg/ml), and low intra-lesional and systemic levels (&lt;50 ng/ml). Alcohol/tobacco cessation not reported.</td>
</tr>
<tr>
<td>ONYX-015 Adenovirus</td>
<td>2003</td>
<td>Oral rinse, 15 ml for 30 minutes q.d. at various intervals Duration: up to 48 weeks with 2-year follow-up</td>
<td>Pre- and post-treatment biopsy-confirmed oral dysplasia (n=19)</td>
<td>Moderate</td>
<td>7 of the 19 dysplastic lesions demonstrated histological regression (37%), but 4 of these patients exhibited dysplastic recurrences. Side effects: Circulating adenoviral antibodies/virus replication Alcohol/tobacco cessation not reported</td>
</tr>
<tr>
<td>Wild-type p53 Adenoviral Vector</td>
<td>2009</td>
<td>Intraoral injection on days 1, 4, 7, 10, and 13 Duration: 15 days with 6-month to 2-year follow-up</td>
<td>Pre- and post-treatment biopsy-confirmed oral dysplasia (n=18)</td>
<td>Moderate</td>
<td>4 patients exhibited complete regression without recurrence at 6 months, 12 patients demonstrated partial reduction/stable disease, and 2 patients progressed to cancer. Side effects: Injection-site tissue necrosis, fever, elevated white blood cell count, and flu-like symptoms Alcohol/tobacco cessation not reported</td>
</tr>
</tbody>
</table>

Table 2. Summary of trials that used local chemopreventive agent delivery (from Holpuch et al.30)
Our labs have conducted several groundbreaking studies for chemoprevention of SCC via topical approach. Rodrigo et al. performed in vitro studies using freeze dried black raspberry extract on 5 different oral SCC cell lines\textsuperscript{37}. These data showed dose dependent suppression of OSCC cell proliferation with retention of viability. They also found decreases in cell mediators that are typically over-expressed in tumor cell lines, such as nitric oxide synthase (NOS) and VEGF. Ugalde et al. tested topical application of a concentrated gel containing 10% black raspberry extract on healthy human volunteers to determine levels achievable in saliva, tissue, and plasma\textsuperscript{38}. While they found significant but variable concentrations of anthocyanins achieved in tissue and saliva, levels in plasma were universally undetectable. Mallery et al. tested the 10% black raspberry gel (BRB) on human subjects with biopsy diagnosed premalignant lesions\textsuperscript{39}. They found a greater than 95% compliance rate among study participants. Histologic improvement in grade was found in 41% of subjects, although there was wide variation between individuals attributable to individual variations in enzyme profiles. They also found a decrease in LOH at most loci, implicating a genetic improvement within the cell population\textsuperscript{62}. Given the successful outcomes of this initial pilot study, a placebo controlled multicenter trial is currently in progress. Participants in these studies refrained from smoking during the study periods.

**History of Vitamin A in Chemoprevention and Cancer Therapy**

Retinoids are involved in vision, cell proliferation, cell differentiation, immune function, neural function, and the establishment of the body plan during early development\textsuperscript{40}. Retinol (vitamin A) is converted to retinal, then to retinoid acid (RA), the
latter step being irreversible\textsuperscript{41}. RA is later broken down by the cytochrome P450 pathway. Nuclear retinoic acid receptors (RARs) include $\alpha$, $\beta$, and $\gamma$ receptors and the 9-cis retinoic acid receptors $\alpha$, $\beta$, and $\gamma$\textsuperscript{42}. Binding of the ligands all-trans retinoic acid and 9-cis retinoic acid to the RARs controls gene expression. Vitamin A and its resultant activity through RARs is critical for epithelial maintenance and maturation, and a diet deficient in vitamin A can lead to squamous metaplasia of the epithelium\textsuperscript{42}. In fact, loss of RAR function often occurs during cancinogenesis, with many benign papillomas maintaining receptor expression while less differentiated cancers show loss of expression\textsuperscript{40,41}. RA acts via multiple convergent pathways to arrest the cell cycle, including effects on CDKs and cyclins\textsuperscript{43}. These properties of RA led to its experimental application in various chemopreventive studies involving epithelium, including OSCC.

**Retinoid Chemopreventive Trials**

Due to the known effects that RA has on epithelium, numerous chemopreventive investigations have been performed on the oral cavity. Papadimitrakopoulou et al. performed a chemopreventive clinical trial in which a cocktail of oral isotretinoin, oral alpha-tocopherol, and interferon alpha was given to patients with biopsy-proven epithelial dysplasia of the head and neck for a period of 12 months. While 50\% (7/14) of the laryngeal lesions demonstrated complete response, 0\% (0/7) of the oral lesions showed a complete response. Also of note, toxicities from therapy ranged from dryness of the skin and mucous membranes to severe eye dryness not relieved by artificial tears and hypertriglyceridemia greater than 5 times the normal level\textsuperscript{33}. In another chemopreventive study using topical vitamin A on oral leukoplakia lesions confirmed by
biopsy as epithelial dysplasia, 7 out of 24 patients showed complete clinical remission, although post-treatment biopsies were not performed\textsuperscript{44}. Ten of the 24 patients (not the same as the clinical responders) had a post treatment biopsy which on average showed no change is histologic grade. A study by Poveda-Rova et al. tested the effects of systemic vs. topical retinoid therapy on proliferative verrucous leukoplakia (PVL), another precancerous condition of the oral cavity\textsuperscript{45}. In their study, 5 patients were given two daily applications of 0.1% 13-cis-retinoic acid in orabase for 6 months, and 11 patients took systemic acitretin for 5 months, and one patient had both treatments. Overall, the same number of patients in each treatment group showed regression of PVL as showed progression, but all of the patients who took systemic retinoids showed adverse reactions, which included desquamation, pruritus, alopecia, dyslipidemia, fragile nails, hot flashes, and blurry vision, while only one patient given the topical retinoid developed an adverse reaction. In summary, most of the above mentioned retinoids at best had mixed results in chemopreventive therapy, but the topical therapies had minimal side effects.

\textbf{Fenretinide (N-(4-hydroxyphenyl)retinamide), also known as 4HPR}

Fenretinide, or N-(4-hydroxyphenyl)retinamide, is a synthetic analog of all-trans retinoic acid (ATRA) made in the late 1960s, which has showed considerable anti-tumor properties in vitro, but mixed in vivo results in human and animal studies\textsuperscript{46,47,48}. Its lack of genotoxicity\textsuperscript{49} makes it a useful adjunct in chemoprevention, allowing for regular application without the risk of causing additional deleterious genetic alterations. It has been found to be effective as a chemopreventive by decreasing both the rate of new
occurrences and the rate of recurrences of leukoplakia following surgical removal of the original leukoplakia lesion\textsuperscript{48}. It's also shown promise as a chemopreventive agent in human studies of breast and ovary cancers\textsuperscript{50}. However, regardless of the daily oral dose, human subjects often develop side effects to fenretinide, most commonly dry skin, but also decreases in night vision due to decreased serum retinol. Overall, it's side effects are significantly less toxic than those of other retinoids\textsuperscript{49,51}. It is a highly hydrophobic compound (as is Vitamin A), and likely requires similar transport aids in serum. However, once at its target tissue, fenretinide is able to accumulate in cell lipid bilayers\textsuperscript{52}.

**Mechanism and metabolism**

Unlike other retinoids which promote cell differentiation and/or cytostasis via RARs, fenretinide also promotes apoptosis of transformed cells via reactive oxygen species (ROS), while sparing normal cells\textsuperscript{47}. This action is RAR receptor independent. Coadministration with an antioxidant such as Vitamin C inhibits apoptosis, while apoptosis is induced even in RAR knockout cells\textsuperscript{47}. However, the effects of fenretinide are dose dependent, with apoptosis being induced at higher concentrations, between 3 and 10 μmol/L, while cell differentiation via RAR is induced at 1 μmol/L\textsuperscript{53}. Unfortunately, achieving serum and tissue levels within the apoptosis range has been elusive in human OSCC studies so far, as discussed in the following ‘Clinical Trials’.

At least 6 different metabolites of fenretinide have been identified, of which at least two common metabolites, 4'-oxo 4-HPR and 4-HPR-O-glucuronide, are active, while the most common metabolite, 4-MPR, is inactive\textsuperscript{54,55,56}. The primary metabolic
enzymes responsible for the metabolism of fenretinide include various individual human cytochrome P450s (CYPs), such as 2C8, 3A4 and 3A5. Ketoconazole, a CYP3A4 inhibitor, when added to human and mouse liver microsomes, increased fenretinide concentrations by over 2 fold, implicating not only the importance of CYP3A4, but also the significance of first pass metabolism for ingested fenretinide. Individual variations in CYP enzymes have been shown to lead to significantly different serum concentrations of metabolites. The uridine 5′-diphospho-glucuronosyl transferases (UGTs) 1A1, 1A3 and 1A6 produced the 4-HPR glucuronide metabolite. Differences in enzymatic metabolism and the importance of CYP’s and UGT’s in first pass metabolism serve as significant obstacles to oral fenretinide therapies.

In Vitro Studies

Scher et al. performed an in vitro study using various concentrations of fenretinide, from 0.1 to 10μmol/L, for durations of 24-96 hours, on 4 different head and neck squamous cell cancer (HNSCC) cell lines. For 2 cell lines, JHU-011-SCC and JHU-020-SCC, only 1 μmol/L over 72 hours achieved a significant decrease in cell viability, whereas 7.5-10 μmol/L of fenretinide was required over 96 hours to achieve reduced viability in their most resistant cell line, FaDu. Fenretinide treatment resulted in increased DNA fragmentation and thus increased apoptosis for most of the cell lines except FaDu. Based on the article, however, it does not appear that the FaDu lines were routinely exposed for 96 hours at 10 μmol/L for the apoptosis and DNA fragmentation experiments. This may have been guided by the fact that the clinical trials
cited in their discussion have only achieved serum concentrations of 2.5 μmol/L. They also did not study normal squamous epithelial cells.

Eicher and Lotan compared the efficacy of β-all-trans-retinoic acid (RA) and fenretinide on the 1483 oral head and neck squamous cell carcinoma cell line\textsuperscript{34}. The cells were treated for 9 days with 10 μmol/L of either RA or fenretinide. Efficacy was assessed by cell number and by detection of apoptosis via in situ DNA end-labeling technique. They found that fenretinide suppressed growth by 83.2%, versus 32.1% for RA. They also found qualitatively more cells in the fenretinide group with DNA end-labeling versus the control, and no increased DNA end-labeling for the RA group. However, no non-dysplasia epithelial cells were studied.

**Clinical Trials**

Two recent clinical trials using fenretinide to treat oral leukoplakia demonstrate the difficulty of achieving therapeutically relevant concentrations of fenretinide via systemic administration. The first study by a M.D. Anderson group consisted of 38 participants with leukoplakia that was resistant to other therapeutic retinoids\textsuperscript{32}. Each patient was given 200 mg tablets of fenretinide by mouth daily for 3 months. Mean serum levels of fenretinide at the end of the 3 month study period were 0.23 μM, and only 34% of patients showed a positive response to treatment, while 29% progressed. Based on their in vitro studies, a fenretinide tissue concentration of 3-5 μM is needed to induce apoptosis via the RAR independent mechanism. They followed up this study with a second clinical trial in which patients took 900 mg of fenretinide twice daily. Serum levels averaged 0.122 μM, again far below the 3-5 μM required to produce apoptosis\textsuperscript{53}. 
This study was prematurely terminated due to lack of efficacy. Importantly, none of the M.D. Anderson studies assessed fenretinide levels at the target tissue. Both studies included protocols to adjust dosing as needed if a study participant was to develop unwanted effects, and many participants in these studies required these adjustments.

In a randomized fenretinide chemoprevention trial by Chiesa et al., 170 patients with non-cancerous oral leukoplakias were followed for 5 years. The initial leukoplakia lesions were treated by laser ablation following biopsy. They found that 200mg of oral fenretinide was protective for the first 25 months of the study period against first events, including leukoplakia recurrence and cancer. However, they did not measure serum or tissue levels of fenretinide. A total of 14 patients withdrew from treatment due to side effects, which included hematologic toxicity (triglyceride abnormalities), cutaneous toxicity (dry skin and mucous membranes, and dermatitis) and gastric toxicities. Ironically, none reported visual side effects, such as decreased night vision.

In a pilot study by Tradati et al., 8 patients with either lichen planus (2) or leukoplakia (6) were treated with topical fenretinide for a minimum of 1 month. Treatment consisted of the patient breaking open a 100mg capsule of fenretinide, then applying it’s contents to the oral mucosa, twice a day, after toothbrushing. All study patients demonstrated a favorable clinical response, and none reported any side effects. The study does not give any details of the serum or target tissue concentrations reached, the criteria to which improvements were judged, or criteria for patient compliance and adverse effects. This is the only previous study using topical fenretinide for leukoplakia, and unfortunately it has severe limitations.

**Patch Delivery**
Patch delivery of drugs via the oral mucosa is far from a novel idea. Several investigators have designed mucoadhesive patches for drug delivery\textsuperscript{54,59,60}. Tanabe et al. designed an ethyl cellulose mucoadhesive patch to successfully deliver the NSAID indomethacin systemically\textsuperscript{54}. Other groups have studied mucoadhesive patch delivery of antibiotics\textsuperscript{59}. While these studies differ from ours in that the goal was to produce systemic effects, they do prove that the oral mucosa is permeable enough to allow for effective drug delivery. In contrast, DentiPatch is a mucoadhesive patch developed to deliver lidocaine topically to the oral mucosa\textsuperscript{60} to provide site specific analgesia in an effort to decrease the need for traditional infiltration, which carries a greater risk of systemic exposure. However, despite the success of these formulations, there are no studies to date which investigated oral mucoadhesive patches for local delivery of chemopreventives, especially fenretinide.

As stated previously, none of the previous chemoprevention trials using fenretinide measured the levels of drug achieved at the target tissue. We feel that systemic delivery of fenretinide is fraught with challenges. First, ingested hydrophobic drug must enter the blood serum, where it likely requires transport proteins similar to Vitamin A. It must then survive first pass metabolism. Because the epithelium is avascular, fenretinide in serum must diffuse through several layers of cells to reach its target tissue. Our goal was to bypass these challenges via a topical delivery approach.

Because fenretinide is hydrophobic, it doesn’t lend itself to a gel formulation as readily as black raspberry extract. Also due to its lipophilic nature, a topical approach required intimate contact between fenretinide and the oral mucosa to prevent an intervening salivary/moist layer from forming, which is why a mucoadhesive patch with
an impermeable backing was developed. For this approach to be effective, fenretinide would need to diffuse through the cornified outer layer of epithelium, then through the remainder of the epithelium to at least the basal cell layer. Thus, the mucoadhesive patch had to employ permeation enhancers to facilitate penetration of the mucosa. Finally, the patch had to deliver fenretinide at therapeutically relevant concentrations to the target tissue without deleterious effects.

**Specific Aim**

The specific aim of this study was to determine the tissue, patch-mucosa interface, and plasma levels of fenretinide following application of a novel mucoadhesive fenretinide patch to normal rabbit oral mucosa, and to evaluate the pharmacokinetic and growth-modulatory parameters of treated mucosa.

**Null Hypothesis**

There will be minimal uptake of fenretinide into rabbit oral mucosa following patch placement, and no subsequent changes in pharmacokinetic and growth-modulatory parameters.
Chapter 2: Materials and Methods

The following chapter is an expanded version of the 'Materials and Methods' section from the original manuscript. The author of this thesis was directly involved with all animals studies, and less so with the laboratory studies, as reflected in the more detailed description of the former.

Animal Subject Approval

Rabbit studies were conducted with approval from The Ohio State University's Institutional Animal Care and Use Committee (Animal Protocol Number 2009A0078)

Rational for use of New Zealand White rabbits

Rabbits were selected for this study for a multitude of reasons. They are large enough to provide an adequate mucosal area for patch placement while small enough to be less costly in terms of housing, veterinary care, and quantity of medications administered. Their oral tissues are histologically similar to that of humans, allowing for appropriate comparison. Female rabbits do not menstruate, so cyclical hormonal influences, if present, should be comparatively minimal. Rabbits have easily identifiable ear vasculature which assists with phlebotomy. Rabbits are not able to vomit, limiting
aspiration risk and possible contamination of the study field throughout non-intubated general anesthesia. Working around an endotracheal tube would have potentially made the study technically more difficult. Finally, rabbits are docile and relatively easy to keep, handle, and transport.

Sample Size Determination

For the sample size estimation analysis, significance (α) was established at the 0.05 level and the beta error at 0.10. Using a conservative estimate, i.e. requisite sample size if the difference between the means of control versus treated tissues was 1/3 the difference obtained from our previously conducted animal local delivery pharmacokinetic analyses\(^6\), an n=8 was determined.

Mucoadhesive Fenretinide Patch

Development of a mucoadhesive patch capable of sustained delivery of fenretinide to the oral mucosa was done primarily by Kashappa-Goud H. Desai, Ph.D., under the guidance of Steven P. Schwendeman, Ph.D., with Susan Mallery, D.D.S., Ph.D. and Andrew Holpuch as collaborators\(^5\). Challenges unique to development of a mucoadhesive fenretinide patch included determining a formulation to stabilize and deliver the highly hydrophobic compound to a moist mucosal layer, making this patch adhesive to oral mucosa, and limiting exposure of non-target sites to fenretinide. Eudragit® RL PO/RS PO copolymers, derived from esters of acrylic and methacrylic acid, are proven to not only allow for manipulation of drug release parameters for
hydrophobic agents, but have also been shown to be mucoadhesive as well. Desai et al. proved that the use of solubilizers, namely Tween® 80+40 wt% sodium deoxycholate, enhanced sustained delivery of fenretinide significantly over the use of Eudragit copolymers alone.

Figure 2 shows both a photographic and illustrative diagram of the mucoadhesive fenretinide patch. Fenretinide loaded Eudragit® RL PO/RS PO copolymer with solubilizers constituted the center of the patch. The adhesive layer consisted of a blend of hydroxypropyl methylcellulose 4KM and polycarbophil at a weight ratio of 3:1, prepared by a casting method. The impermeable backing was made from Tegaderm adhesive film. In addition to being adhesive and flexible, the patch was thin, measuring only 0.33mm in thickness.

![Figure 2. Mucoadhesive fenretinide patch](image-url)
Animal Protocol

Eight female New Zealand white rabbits (Harlan Laboratories, Indianapolis, IN, USA) weighing 2.71±0.18 kg and 12 weeks of age were used for analysis of intraoral fenretinide delivery from mucoadhesive patches. Upon arrival, rabbits were acclimated for 7 days and monitored for signs of distress. Prior to general anesthesia for patch placement, each rabbit was sedated with 2 mg/ml acepromazine (Butler Schein Animal Health, Dublin, OH, USA) injected into the nape of the neck. Induction and maintenance of general anesthesia was performed using a nose cone for isoflurane inhalation (5% v/v in oxygen). Rabbits were placed over an electric warmer and their heart rate, respiratory rate, and temperature were monitored throughout the procedure. Once the animal was unconscious and prior to patch placement, 0.5cc of blood was collected from the central ear artery. A fenretinide-loaded mucoadhesive patch was placed on the right buccal mucosa and a blank control patch placed on the left buccal mucosa, both immediately posterior to the oral commissure (Figure 3).
Figure 3. Patch placement on the buccal mucosa just posterior to the oral commissure

Patches were left in place for 30 minutes. An additional blood sample was collected at 30 minutes, and the patches were removed. 200 µl of 1x phosphate buffered saline (PBS) was rinsed over the site of the fenretinide patch and immediately collected (patch-mucosa interface sample). The rabbits were then awakened from general anesthesia, and observed for at least an additional 30 minutes. After final recovery vital signs were obtained, the rabbits were immediately transported to their housing facility. No adverse events or deaths occurred. This procedure was conducted every day for 10 consecutive days. Following treatment on day 10, rabbits were sacrificed via intravenous potassium chloride injection while under general anesthesia, and oral biopsies of the fenretinide-treated and blank-treated mucosal sites were harvested. All analyses performed compared intra-rabbit blank-treated versus
fenretinide-treated mucosal specimens. These oral biopsies were cut in two halves: one section for LC-MS/MS analysis (stored in stabilizing buffer: pH 6.5, 1x PBS + 9 mM EDTA + 25 mM sodium ascorbate + 21 mM sodium sulfate) and the other section for protein analysis (half for immunohistochemistry: 10% formalin, half for Western blot: RNALater). All tissue and patch-mucosa interface LC-MS/MS samples were immediately frozen and stored at -80°C until analysis. Blood samples were clotted then centrifuged at 10,000xg, followed by collection and storage of the serum supernate at -80°C until analysis.

**Laboratory Techniques**

*Preparation of rabbit patch-mucosa interface, oral mucosa homogenate and serum samples for LC-MS/MS analysis.*

Due to the high concentration of fenretinide present in the patch-mucosa interface samples, they were pre-diluted prior to analysis (i.e., serially diluted in 1x PBS 200- and 2000-fold). 100 µl of the 200- or 2000-fold diluted samples were mixed with 10 µl of internal standard (hesperetin, 10 µg/µl). Samples were extracted with 800 µl ethyl acetate for 30 minutes by mechanical shaking. For mucosa homogenate samples, both fenretinide and blank-treated samples were grossly dissected to remove excess amounts of underlying connective tissue. Oral epithelium was homogenized in 1x PBS for 3x5 minutes on a bead mill, and 260 µl of the homogenate was spiked with 10 µl of internal standard and 300 µl of lysis buffer. Samples were incubated on ice for 5 minutes, centrifuged at 11000xg for 1 minute and the supernatant was extracted with 1
ml ethyl acetate for 30 minutes. To process rabbit serum samples, 100 µl of each rabbit serum sample was spiked with 10 µl of internal standard followed by extraction using 800 µl ethyl acetate for 30 minutes. After the extractions, all the samples were centrifuged at 11000×g for 1 minute, and ethyl acetate fraction was collected and evaporated to dryness under nitrogen stream. The residue was reconstituted in 100 µl of 50% methanol containing 0.2% formic acid and subsequently subjected to LC-MS/MS for analysis.

**LC-MS/MS analysis.**

The LC-MS/MS analysis was conducted on a Thermo TSQ Quantum triple quadrupole mass spectrometer equipped with an electrospray ionization source, Shimadzu LC-20AD HPLC pump, and LC-20AC autosampler. The samples were analyzed on a Thermo Betabasic 8 column (50×2.1 mm, 5 µ), of which the mobile phase (85% methanol with 0.2% formic acid) was used in isocratic mode with 0.2 ml/min flow rate. The analysis was conducted in positive mode. The transfer line temperature was 325ºC. The MRM transitions selected for the analysis of fenretinide, 4-oxo-HPR, 4-methoxy-HPR and the internal standard were 392>283, 406>297, 406>283 and 303>177 m/z, respectively.

**Conversion of fenretinide mass quantities to molar equivalents**

In order to compare oral mucosal fenretinide levels (ng fenretinide/gm tissue) to previously published therapeutic values (i.e., 1-10 µM), mass quantities were converted
to micromolar concentrations, by standards previously\textsuperscript{63}. Evaluation and quantification of chemoprevention-associated markers and metabolic enzyme distribution within the rabbit surface epithelium was performed by immunoblotting and immunohistochemical staining.

**Histologic analysis of buccal mucosa**

The presence/absence of microscopic deleterious side effects within the fenretinide-treated and blank-treated rabbit oral mucosa was assessed by standard hematoxylin and eosin staining of paraffin-embedded tissues. Rabbit mucosal tissues were first screened by immunoblotting to determine the presence or absence of proteins associated with or responsive to fenretinide.

**IHC**

Matched rabbit oral mucosal specimens were also analyzed by immunohistochemistry (IHC) to elucidate levels of immunoblot-confirmed metabolic enzymes and chemopreventive endpoints within the target oral epithelia. The antibodies employed for Western blot and IHC staining were: goat polyclonal UGT1A1 (WB: 1:100, IHC: not conducted), goat polyclonal UGT1A6 (WB: 1:100, IHC: not conducted), mouse monoclonal CYP3A4 (WB: 1:250, IHC: not conducted), mouse monoclonal CYP26A1 (WB: 1:250, IHC: not conducted), goat polyclonal CYP2C8/9/18/19 (WB: 1:500, IHC: not conducted), mouse monoclonal Ki-67, mouse monoclonal keratinocyte-specific transglutaminase 1 (TGase 1; WB: 1:100, IHC: 1:50 and 1:300) antibodies (Santa Cruz
Biotechnology, Santa Cruz, CA). Blocking buffer (negative control) was used in place of the primary antibody, and all tissue sections (including negative control) were incubated with their respective biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA). Vectastain ABC reagent (Vector Laboratories) was applied, and identically timed immunoreactions were conducted with DAB substrate and hematoxylin counterstain. Evaluation of apoptotic indices in fenretinide-treated versus blank-treated rabbit epithelium was assessed via an immunofluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Indianapolis, IN, USA). Immunohistochemical and immunofluorescent images were obtained with identical magnification and exposure limits using a Nikon DS-Fi-1 high-resolution digital camera and analyzed via Image-Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD, USA).

Image analysis and quantification was conducted on the positive reacted antibodies: Ki-67, TGase 1, TUNEL, and UGT1A1 tissue sections. Quantification of positive Ki-67 nuclear staining was conducted by selecting the basal epithelial layer of cells as the area of interest, setting limits of staining intensity considered to be positive (identical for intra-rabbit tissues), and quantifying the positive nuclear indices relative to basement membrane length (i.e., Ki-67 positive nuclei/length of epithelial basement membrane). Similarly, quantification of the TGase 1, TUNEL, and UGT1A1 tissue specimens involved selection of the positive staining intensity limits (identical for intra-rabbit tissues) within the area of interest (i.e., entire oral epithelium excluding the non-staining superficial orthokeratin layer), and were quantified by area of positive staining relative to total epithelial area of interest. Notably, these methods of
immunohistochemistry quantification are restrictive to intra-rabbit comparisons (i.e.,
inter-rabbit comparisons are not accurate).

**Evaluation of metabolic enzyme heterogeneity in human oral mucosa by Western
blot.**

Eight clinically and histologically normal human oral mucosal tissues were
collected from patients undergoing elective oral surgical procedures. Human subject
participation was in accordance with Ohio State University’s Institutional Review Board
approval and followed the tenets of the Declaration of Helsinki 1964. Tissues were
collected and immediately bisected for Western blot (mammalian protein extraction
reagent; subsequent homogenization, protein extraction, and Bradford protein
quantification) or immunohistochemical (10% formalin; subsequent standard tissue
processing) analyses. Western blot analyses were conducted using the iBlot Western
blot system (Invitrogen, Carlsbad, CA, USA) on 4-12% Bis-Tris gels (Invitrogen). Thirty
micrograms of human oral mucosal protein were loaded in each well, pooled human liver
microsomes (BD Biosciences, San Jose, CA, USA) were loaded as a positive control,
separated by SDS-PAGE, and blotted by the standard iBlot protocol (i.e., nitrocellulose
stacks and 7 minute transfer time). Blots were incubated in 5% non-fat milk blocking
buffer overnight at 4°C with the following primary antibodies: CYP3A4 (1:250), CYP26A1
(1:250), CYP2C8 (1:500), UGT1A1 (1:250), UGT1A6 (1:250), amine N-
methyltransferase (rabbit polyclonal; INMT, 1:1000; Sigma-Aldrich, Saint Louis, MO,
USA), and Beta-actin loading control (1:20,000, Santa Cruz). Each blot was
subsequently incubated at room temperature for 1 hour with the respective horseradish
peroxidase secondary antibody: goat anti-mouse, goat anti-rabbit, or donkey anti-goat (1:1000, Santa Cruz) in blocking buffer. Following secondary antibody incubation blots were incubated with the ECL Plus Western blot detection system (Amersham GE Healthcare Life Sciences, Buckinghamshire, UK) and exposed on CL-Xposure films (Kodak, Rochester, NY, USA). Quantification of positive immunoreactive bands (i.e., CYP3A4) were assessed via densitometry analysis (Kodak 1D3 image analysis software; Kodak) and results normalized relative to the expression of endogenous β-actin.

**Determination of site-specificity of metabolic enzymes in human oral mucosa by immunohistochemical analyses.**

Although Western blots demonstrate relative levels of the protein of interest within the full-thickness oral mucosa specimens, they do not specify relative levels of tissue distribution in the targeted treatment site, i.e., oral epithelium. In order to identify the site-specific distribution of protein levels in oral mucosa, immunohistochemical analyses were therefore conducted for CYP3A4 (1:100) by the methods previously described.

**Assessment of cell proliferation and modulation of chemoprevention-relevant parameters following fenretinide treatment of human oral keratinocytes in vitro.**

Normal human oral keratinocytes (HOK) were cultured in oral keratinocyte growth medium with oral keratinocyte growth supplement and penicillin/streptomycin
(ScienCell, Carlsbad, CA, USA). A dysplastic cell strain was developed through stable transduction of the human papillomavirus E6/E7 genes in the HOKs, as per previously published methods. HOK E6/E7 transduction was confirmed via PCR and Western blot.

Evaluation of the effects of fenretinide (4-HPR) and its bioactive metabolite 4-oxo-4-HPR on cell proliferation was conducted using the BrdU cell proliferation ELISA (Roche). Briefly, log growth HOK or HOK E6/E7 cells were seeded at 1x10^5 cells per well in 24-well plates. Cells were treated with vehicle (0.01% DMSO), 1, 5, or 10 μM 4-HPR or 4-oxo-4-HPR for 24-, 48-, and 72-hour time points. Subsequent detection of BrdU incorporation followed previously published protocol guidelines, and was detected on a FLUOstar Omega plate reader (BMG Labtech, Cary, NC, USA).

Additional HOK studies assessed the modulation of chemoprevention-related parameters. Ninety-percent confluent normal HOK cells were treated with vehicle (0.01% DMSO), 1, 3, or 5 μM fenretinide for 24 hours. Notably, these fenretinide concentrations correspond to both previously published differentiation-inducing levels and concentrations obtained in optimally responsive rabbit oral mucosal specimens. Following 24-hour treatment, total protein was directly harvested via mammalian protein extraction reagent, and quantified by the Bradford protein assay. Western blots, which were normalized to GAPDH protein (1:200, Santa Cruz), were conducted to determine the presence of UGT1A1 (1:100), TGase 1 (1:100), and involucrin (1:100; mouse monoclonal, Santa Cruz). The Western blot protocol and quantification were conducted as previously described.
Statistical analysis

Normality of data determined whether parametric or nonparametric statistics were used. Fenretinide-treated versus blank-treated rabbit mucosal specimens were compared using the Wilcoxon matched-pairs signed-ranks test. Inter-rabbit variations of mean 10-day fenretinide levels at the patch-mucosal interface were evaluated with the Kruskal-Wallis nonparametric analysis of variance. Fenretinide-treated and blank-treated immunohistochemical sections were compared using the Wilcoxon matched-pairs signed-ranks test (TGase 1 and Ki-67) or paired t-test (TUNEL and UGT1A1). BrdU incorporation in HOK and HOK E6/E7 cells following 4-HPR and 4-oxo-4-HPR was evaluated using a Kruskal-Wallis nonparametric analysis of variance followed by a Dunn’s multiple comparisons post-test.
Chapter 3: Original Manuscripts

Evaluation of a Mucoadhesive Fenretinide Patch for Local Intraoral Delivery: A Strategy to Re-introduce Fenretinide for Oral Cancer Chemoprevention

Andrew S. Holpuch¹, Maynard P. Phelps¹, Kashappa-Goud H. Desai², Wei Chen⁴,⁵, George M. Koutras¹, Byungdo Han¹, Blake M. Warner¹,⁶, Ping Pei¹, Garrett A. Seghi¹, Meng Tong¹, Michael B. Border¹, Henry W. Fields⁷, Gary D. Stoner⁸, Peter E. Larsen¹, Zhongfa Liu³,⁴, Steven P. Schwendeman², Susan R. Mallery¹,³

¹Division of Oral Maxillofacial Surgery, Pathology & Anesthesiology, College of Dentistry, The Ohio State University, Columbus, OH, 43210.

²Department of Pharmaceutical Sciences, University of Michigan, Ann Arbor, MI 48109.

³The Ohio State University Comprehensive Cancer Center.

⁴College of Pharmacy, The Ohio State University.

⁵Zhejiang Cancer Research Institute, Zhejiang Cancer Hospital, Hangzhou, China 310022.

⁶College of Public Health, The Ohio State University.

⁷Division of Orthodontics, College of Dentistry, The Ohio State University.

⁸Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, 53226.
Financial Support: NIH grants: R01 CA129609, RC2 CA148099, R21 CA132138, F30 DE020992, T32 DE14320; Additional Funding: Fanconi Anemia Research Fund and National Center for Research Resources UL1RR025755, Pelotonia Fellowship Program, AADR Student Research Fellowship, Ohio Division of the American Cancer Society Fellowship

Abstract

Systemic delivery of fenretinide in oral cancer chemoprevention trials has been largely unsuccessful due to dose-limiting toxicities and sub-therapeutic intra-oral drug levels. Local drug delivery, however, provides site-specific therapeutically-relevant levels while minimizing systemic exposure. These studies evaluated the pharmacokinetic and growth-modulatory parameters of fenretinide mucoadhesive patch application on rabbit buccal mucosa. Fenretinide and blank-control patches were placed on right/left buccal mucosa, respectively, in 8 rabbits (30 minutes, q.d., 10-days). No clinical or histological deleterious effects occurred. LC-MS/MS analyses of post-treatment samples revealed a delivery gradient with highest fenretinide levels achieved at the patch-mucosal interface (no metabolites), pharmacologically-active levels in fenretinide-treated oral mucosa (mean: 5.65μM; trace amounts of 4-oxo-4-HPR), and undetectable sera levels. Epithelial markers for cell proliferation (Ki-67), terminal differentiation (transglutaminase 1 – TGase1), and glucuronidation (UDP-glucuronosyltransferase1A1 – UGT1A1) exhibited fenretinide concentration-specific relationships (elevated TGase1 and UGT1A1 levels <5μM, reduced Ki-67 indices >5μM) relative to blank-treated epithelium. All fenretinide-treated tissues showed significantly
increased intraepithelial apoptosis (TUNEL) positivity, implying activation of intersecting apoptotic and differentiation pathways. Human oral mucosal correlative studies showed substantial inter-donor variations in levels of the enzyme (cytochrome P450 3A4 – CYP3A4) responsible for conversion of fenretinide to its highly active metabolite, 4-oxo-4-HPR. Complementary *in vitro* assays in human oral keratinocytes revealed fenretinide and 4-oxo-4-HPR’s preferential suppression of DNA-synthesis in dysplastic as opposed to normal oral keratinocytes. Collectively, these data showed that mucoadhesive patch-mediated fenretinide delivery is a viable strategy to re-introduce a compound known to induce keratinocyte differentiation to human oral cancer chemoprevention trials.

**Introduction**

Over the past several decades, many promising cancer-preventing compounds have been evaluated in patients with oral premalignant lesions [reviewed in 1]. Systemic delivery trials which predominantly relied on oral capsule administration induced dose-limiting systemic toxicities and lacked initial and/or sustained efficacy [1]. Formulations used in local delivery trials, e.g., mucoadhesive gels [2-4] and rinses, demonstrated a range of therapeutic efficacies largely without deleterious side effects [1]. Notably, only three of the nineteen reviewed trials (i.e., two local and one systemic) quantified compound levels achieved at the target site [1]. This lack of data precludes determination of whether the compounds evaluated were pharmacologically ineffective or failed to reach therapeutic levels in lesional tissues (i.e., oral epithelium).

Many of these oral cancer chemopreventive studies evaluated vitamin A, its precursors, and analogs (retinoids) [1]. *In vitro*, fenretinide (a synthetic analog of all-
trans retinoic acid) has shown exceptional capacity to promote keratinocyte terminal differentiation or apoptosis in a dose-dependent fashion [5]. Furthermore, fenretinide exhibits a reduced toxicity-induction profile (i.e., decreased gastrointestinal distress and nyctalopia), and thus has been an agent of choice for recent oral cancer chemoprevention clinical trials [6-9]. Fenretinide trials in patients with oral dysplastic lesions, however, have been largely unsuccessful [7-9]. Notably, these studies investigated systemic delivery of fenretinide (oral capsule) at varying concentrations (low-dose: 100 mg b.i.d. or 200 mg q.d., high-dose: 900 mg b.i.d.), which resulted in minimal therapeutic efficacy accompanied by dose-limiting toxicities [7-9]. Sera levels, which were used as a surrogate marker for target tissue levels, never achieved therapeutic concentrations [7-9]. Issues such as first pass metabolism in the liver and reliance upon perfusion from the underlying vasculature to overlying target surface epithelium likely compromised levels achieved from systemic delivery. In contrast, a pilot local delivery trial in which patients placed fenretinide capsule contents on a variety of reactive and preneoplastic oral lesions did not demonstrate any local or systemic deleterious effects [10].

Our labs obtained promising results from a local delivery strategy that evaluated the effects of a 10% freeze-dried black raspberry (BRB) gel in patients with oral dysplastic lesions [2,3]. Results from these and additional recent studies showed: local gel delivery provided a pharmacologic advantage [4], a subset of patients responded favorably to local BRB gel application [2], and differential bioactivation and retention of chemopreventive compounds in human oral mucosa [11]. These data, which implied that BRB was insufficient to induce regression in some patients’ dysplastic oral lesions, served as the impetus for the current study. Considering these clinical implications, our
laboratories recently developed a novel mucoadhesive patch which provided improved site-specific intraoral delivery of fenretinide [12]. The goal of the current study was to determine if mucoadhesive patches delivered therapeutically relevant fenretinide levels to oral mucosa. Subsequent evaluations of fenretinide-treated and blank-treated rabbit oral tissues assessed the modulation of chemopreventive and metabolic parameters. Corresponding studies of human oral tissues and cultured oral keratinocytes recapitulated the rabbit data and provided human clinical relevance.

**Methods and Materials**

*Intraoral mucoadhesive fenretinide patch pharmacokinetic analyses in New Zealand white rabbits*

Rabbit studies were conducted with approval from The Ohio State University's Institutional Animal Care and Use Committee. Eight female New Zealand white rabbits (Harlan Laboratories, Indianapolis, IN, USA) weighing 2.71±0.18 kg were used for analysis of intraoral fenretinide delivery from mucoadhesive patches [12]. Upon arrival, rabbits were acclimated for 7 days and monitored for signs of distress. Following acclimation, each rabbit was sedated with a subcutaneous injection (0.2cc) of acepromazine (2 mg/ml, Butler Schein Animal Health, Dublin, OH, USA) and placed under general anesthesia via isoflurane inhalation (2-3 v/v%) for the entire 30-minute patch application procedure. Once unconscious and prior to patch placement, 0.5cc of blood was collected from the central ear artery. A fenretinide-loaded mucoadhesive patch (0.5 mg fenretinide/patch, i.e., 400- to 3,600-fold less than daily systemic
administrations in recent clinical trials [7-9]) was attached to the right buccal mucosa and a blank control patch attached to the left buccal mucosa, both immediately posterior to the intraoral commissure of the upper and lower lips (Figure 4). Patches were left in place for 30 minutes and an additional blood sample collected at 30 minutes. The patches were then removed, fenretinide-treated underlying surface epithelium lavaged with 200 µl of 1x phosphate buffered saline (PBS), and patch-mucosa interface sample immediately collected. This procedure was conducted q.d. for 10 consecutive days.

Following treatment on day 10, rabbits were sacrificed via intravenous potassium chloride injection, and oral biopsies of the fenretinide-treated and blank-treated mucosal sites were harvested. Modulation of chemopreventive and metabolic parameters relative to intra-mucosal fenretinide concentrations were compared in intra-rabbit blank-treated versus fenretinide-treated mucosal specimens, while patch-mucosal interface samples provided inter-rabbit comparisons of daily patch delivery efficacy. Notably, these oral biopsies were cut in two pieces: half for LC-MS/MS analysis (stored in stabilizing buffer: pH 6.5, 1x PBS + 9 mM EDTA + 25 mM sodium ascorbate + 21 mM sodium sulfate) and the other half for protein analysis (half for immunohistochemistry: 10% formalin, half for Western blot: RNALater). All tissue and patch-mucosa interface LC-MS/MS samples were immediately frozen and stored at -80°C until analysis. Blood samples were clotted, centrifuged at 10,000xg, serum supernatant collected and stored at -80°C until analysis.

Preparation of rabbit patch-mucosa interface, oral mucosa homogenate and serum samples for LC-MS/MS analysis
Due to the high concentration of fenretinide present in the patch-mucosa interface samples, they were pre-diluted prior to analysis (i.e., serially diluted in 1x PBS 200- and 2,000-fold). 100 µl of the 200- or 2,000-fold diluted samples were mixed with 10 µl of internal standard (hesperetin, 10 µg/µl). Samples were extracted with 800 µl ethyl acetate for 30 minutes by mechanical shaking. For mucosa homogenate samples, both fenretinide and blank-treated samples were grossly dissected to remove excess amounts of underlying connective tissue. Oral epithelium was homogenized in 1x PBS for 3x5 minutes on a bead mill, and 260 µl of the homogenate was spiked with 10 µl of internal standard and 300 µl of lysis buffer. Samples were incubated on ice for 5 minutes, centrifuged at 11,000×g for 1 minute and the supernatant was extracted with 1 ml ethyl acetate for 30 minutes. To process rabbit serum samples, 100 µl of each rabbit serum sample was spiked with 10 µl of internal standard followed by extraction using 800 µl ethyl acetate for 30 minutes. After the extractions, all the samples were centrifuged at 11,000×g for 1 minute and ethyl acetate fraction was collected and evaporated to dryness under nitrogen stream. The residue was reconstituted in 100 µl of 50% methanol containing 0.2% formic acid and subsequently subjected to LC-MS/MS for analysis.

**LC-MS/MS analysis**

The LC-MS/MS analysis was conducted on a Thermo TSQ Quantum triple quadrupole mass spectrometer equipped with an electrospray ionization source, Shimadzu LC-20AD HPLC pump, and LC-20AC autosampler. The samples were analyzed on a Thermo Betabasic C8 column (50×2.1 mm, 5 µm), of which the mobile
phase (85% methanol with 0.2% formic acid) was used in isocratic mode with 0.2 ml/min flow rate. The analysis was conducted in positive mode. The transfer line temperature was 325ºC. The multiple reaction monitoring (MRM) transitions selected (based on parameters established by fenretinide/metabolite-spiked blank rabbit oral mucosal tissues and sera) for the analysis of fenretinide, 4-oxo-HPR, 4-MPR and the internal standard were 392>283, 406>297, 406>283 and 303>177 m/z, respectively.

Conversion of fenretinide mass quantities to molar equivalents

In order to compare oral mucosal fenretinide levels (ng fenretinide/gm tissue) to previously published therapeutic values (i.e., 1-10 μM), mass quantities were converted to micromolar concentrations, by standards previously published [13].

Evaluation and quantification of chemoprevention-associated markers and metabolic enzyme distribution within the rabbit surface epithelium by immunoblotting and immunohistochemical staining

The presence/absence of deleterious side effects within the fenretinide-treated and blank-treated rabbit oral mucosa was assessed by standard hematoxylin and eosin staining of paraffin-embedded tissues. Rabbit mucosal tissues were first screened by immunoblotting to determine the presence or absence of proteins associated with or responsive to fenretinide. Matched rabbit oral mucosal specimens were also analyzed by immunohistochemistry (IHC) to elucidate levels of immunoblot-confirmed metabolic enzymes and chemopreventive endpoints within the target oral epithelia. The antibodies
employed for Western blot and IHC staining were: goat polyclonal UGT1A1 (WB: 1:100, IHC: 1:300), goat polyclonal UGT1A6 (WB: 1:100, IHC: not conducted), mouse monoclonal CYP3A4 (WB: 1:250, IHC: not conducted), mouse monoclonal CYP26A1 (WB: 1:250, IHC: not conducted), goat polyclonal CYP2C8/9/18/19 (WB: 1:500, IHC: not conducted), mouse monoclonal Ki-67 (WB: not conducted – highly specific in IHC applications [14], IHC: 1:150), mouse monoclonal keratinocyte-specific transglutaminase 1 (TGase 1; WB: 1:100, IHC: 1:50 and 1:300) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blocking buffer (negative control) was used in place of the primary antibody, and all tissue sections (including negative control) were incubated with their respective biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA). Vectastain ABC reagent (Vector Laboratories) was applied, and identically timed immunoreactions were conducted with DAB substrate and hematoxylin counterstain. Evaluation of apoptotic indices in fenretinide-treated versus blank-treated rabbit epithelium was assessed via an immunofluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Indianapolis, IN, USA). Immunohistochemical and immunofluorescent images were obtained with identical magnification and exposure limits using a Nikon DS-Fi-1 high-resolution digital camera and analyzed via Image-Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD, USA).

Image analysis and quantification was conducted on the positive reacted antibodies: Ki-67, TGase 1, TUNEL, and UGT1A1 tissue sections. Quantification of positive Ki-67 nuclear staining was conducted by selecting the basal epithelial layer of cells as the area of interest, setting limits of staining intensity considered to be positive (identical for intra-rabbit tissues), and quantifying the positive nuclear indices relative to
basement membrane length (i.e., Ki-67 positive nuclei/length of epithelial basement membrane). Similarly, quantification of the TGase 1, TUNEL, and UGT1A1 tissue specimens involved selection of the positive staining intensity limits (identical for intra-rabbit tissues) within the area of interest (i.e., entire oral epithelium excluding the non-staining superficial orthokeratin layer) and quantification by area of positive staining relative to total epithelial area of interest. Notably, these methods of immunohistochemistry quantification are restrictive to intra-rabbit comparisons (i.e., inter-rabbit comparisons are not appropriate due to variation of the selected inter-rabbit positivity limits).

**Evaluation of metabolic enzyme heterogeneity in human oral mucosa by Western blot**

Eight clinically and histologically normal human oral mucosal tissues were collected from patients undergoing elective oral surgical procedures. Human subject participation was approved by Ohio State University’s Institutional Review Board and followed the tenets of the Declaration of Helsinki 1964. Tissues were collected and immediately bisected for Western blot (mammalian protein extraction reagent; subsequent homogenization, protein extraction, and Bradford protein quantification) or immunohistochemical (10% formalin; subsequent standard tissue processing) analyses. Western blot analyses were conducted using the iBlot Western blot system (Invitrogen, Carlsbad, CA, USA) on 4-12% Bis-Tris gels (Invitrogen). Thirty micrograms of human oral mucosal protein was loaded in each well, pooled human liver microsomes (BD Biosciences, San Jose, CA, USA) were loaded as a positive control, separated by SDS-PAGE, and blotted by the standard iBlot protocol (i.e., nitrocellulose stacks and 7 minute
transfer time). Blots were incubated in 5% non-fat milk blocking buffer overnight at 4°C with the following primary antibodies: CYP3A4 (1:250), CYP26A1 (1:250), CYP2C8 (1:500), UGT1A1 (1:250), UGT1A6 (1:250), indolethylamine N-methyltransferase (rabbit polyclonal; INMT, 1:1000; Sigma-Aldrich, Saint Louis, MO, USA), and Beta-actin loading control (1:20,000, Santa Cruz). Each blot was subsequently incubated at room temperature for 1 hour with the respective horseradish peroxidase secondary antibody: goat anti-mouse, goat anti-rabbit, or donkey anti-goat (1:1000, Santa Cruz) in blocking buffer. Following secondary antibody incubation blots were incubated with the ECL Plus Western blot detection system (Amersham GE Healthcare Life Sciences, Buckinghamshire, UK) and exposed on CL-Xposure films (Kodak, Rochester, NY, USA). Quantification of positive immunoreactive bands (i.e., CYP3A4) were assessed via densitometry analysis (Kodak 1D3 image analysis software; Kodak) and results normalized relative to the expression of endogenous β-actin.

*Determination of site-specificity of metabolic enzymes in human oral mucosa by immunohistochemical analyses*

Although Western blots demonstrate relative levels of the protein of interest within the full-thickness oral mucosa specimens, they do not specify relative levels of tissue distribution in the targeted treatment site, i.e., oral epithelium. To identify the site-specific distribution of protein levels in oral mucosa, immunohistochemical analyses were therefore conducted for CYP3A4 (1:100) by the methods previously described.
Assessment of cell proliferation following fenretinide and 4-oxo-4-HPR treatments of human oral keratinocytes in vitro

Normal human oral keratinocytes (HOK) were cultured in oral keratinocyte growth medium with oral keratinocyte growth supplement and penicillin/streptomycin (ScienCell, Carlsbad, CA, USA). A dysplastic cell strain was developed through stable transduction of the human papillomavirus E6/E7 genes in the HOKs, as per previously published methods [15]. HOK E6/E7 transduction was confirmed via PCR and Western blot (data not shown).

Evaluation of the effects of fenretinide (4-HPR) and its bioactive metabolite 4-oxo-4-HPR on cell proliferation was conducted using the BrdU cell proliferation ELISA (Roche). Briefly, log growth HOK or HOK E6/E7 cells were seeded at 1x10^4 cells per well in 24-well plates. Cells were treated daily with vehicle (0.01% DMSO), 1, 5, or 10 μM 4-HPR or 4-oxo-4-HPR for 24, 48, and 72-hour time points. BrdU (10 μM) was added 24-hours prior to the respective cell harvesting time point, where subsequent detection (absorbance: 370 nm) of BrdU incorporation followed the standard protocol (Roche) on a FLUOstar Omega plate reader (BMG Labtech, Cary, NC, USA).

Statistical analysis

Normality of data was assessed using the Shapiro-Wilks test to determine the appropriate use of parametric or nonparametric statistical tests. Fenretinide-treated versus blank-treated rabbit mucosal specimens were compared using the Wilcoxon matched-pairs signed-ranks test. Inter-rabbit variations of mean 10-day fenretinide
levels at the patch-mucosal interface were evaluated with the Kruskal-Wallis nonparametric analysis of variance. Fenretinide-treated and blank-treated immunohistochemical sections were compared using the Wilcoxon matched-pairs signed-ranks test (TGase 1 and Ki-67) and paired t-test (TUNEL and UGT1A1). BrdU incorporation in HOK and HOK E6/E7 cells following 4-HPR and 4-oxo-4-HPR was evaluated using a Kruskal-Wallis nonparametric analysis of variance followed by a Dunn’s multiple comparisons post-test.

Results

*Therapeutically relevant fenretinide levels are achieved in rabbit oral mucosa without deleterious side effects*

Intraoral fenretinide mucoadhesive patch application delivered a fenretinide gradient with the highest levels achieved at the patch-mucosal interface, pharmacologically active levels in treated oral mucosa, and undetectable sera levels (Figure 5A). Levels of fenretinide achieved at the fenretinide-treated patch-mucosal interface (mean±SEM: 11513.9±391.2 ng/mg protein) were comparable in all animals throughout the 10-day treatment period (p>0.5, n=8). Furthermore, levels in fenretinide-treated oral mucosa were significantly greater than their rabbit-matched blank-treated sites (fenretinide-treated mean±SEM: 2210.3±986.2 ng/gm tissue [5.65±2.52μM] versus blank-treated mean±SEM: 9.0±0.08 ng/gm tissue [0.02±0.00μM], p<0.01, n=8). Despite achieving intra-mucosal fenretinide concentrations greater than the *in vitro*-established therapeutic range (i.e., 1-10 μM) in rabbits VII (12.98 μM) and VIII (19.96 μM) and the
presence of a natural fenretinide reservoir, i.e., buccal fat pad, both clinical and histopathological assessments of treatment sites revealed normal oral mucosa, Figure 5B. In addition, the inactive fenretinide metabolite 4-MPR was not detected in the patch-mucosal interface, oral mucosa, or serum samples, while trace amounts (i.e., below the linear level of quantification) of 4-oxo-4-HPR were observed in the fenretinide-treated oral mucosal samples of rabbits III through VIII.

*Fenretinide modulates oral epithelial growth state parameters*

Immunohistochemical analyses of fenretinide-treated tissue relative to matched blank-treated tissue exhibited fenretinide concentration-dependent patterns with regard to cell proliferation (Ki-67), terminal differentiation (TGase 1), and apoptosis (TUNEL). Although the Ki-67 findings were not statistically significant, moderate decreases in cell proliferation were observed in oral mucosal tissues containing greater than 5 μM fenretinide (i.e., rabbits VII and VIII, Figure 6A-B). Similarly, TGase 1 levels were increased in oral mucosal tissues with levels of fenretinide in the range of 0.1-5 μM (i.e., rabbits I-VI), while TGase 1 levels were decreased in tissues containing greater than 5 μM (i.e., rabbits VII and VIII, Figure 6A). This relationship was further demonstrated in the representative photomicrographs in Figure 6B, in which rabbit IV (2.22 μM) exhibited a 54.0% increase of TGase 1 levels in the fenretinide-treated relative to the blank-treated epithelium and rabbit VIII (19.96 μM) exhibited a 24.0% decrease of TGase 1 levels in the fenretinide-treated relative to blank-treated epithelium. Notably, the TGase 1 staining pattern in rabbit IV (differentiation-inducing levels) extended from the basal to the granular layers and was strongest in the keratinocyte cytosol. In contrast, the
fenretinide-treated tissue from rabbit VIII (apoptosis-inducing levels) exhibited strong nuclear staining in the basal layer, which also extended to the spinous and granular layers. Furthermore, all of the fenretinide-treated oral epithelial tissues showed significantly increased apoptotic indices (Figure 6A, p<0.01), which tended to be elevated in tissues with greater than 3 μM fenretinide, as demonstrated in Figure 6B. Notably, inset photomicrographs in Figure 6B represent positive indices (red highlighted areas – Ki-67 and TGase 1) or negative control (TUNEL – due to inherent auto-fluorescence in paraffin-embedded tissue sections).

**Induction of UGT1A1 levels in fenretinide-treated rabbit oral epithelium**

Immunohistochemical analysis of fenretinide-related phase I/II metabolic enzymes identified significantly increased UGT1A1 expression in fenretinide-treated relative to blank-treated rabbit oral mucosal tissues (p<0.01, Figure 7B). Notably, the percent of UGT1A1 induction was greatest in the mucosal samples with less than 5 μM fenretinide (i.e., rabbit I-VI, Figure 7B). Representative photomicrographs demonstrated a dose-dependent effect of fenretinide on phase II enzyme induction (Figure 7C). Additional fenretinide-specific metabolic enzymes (i.e., CYP3A4, CYP2C8, CYP26A1, and INMT) were not detected in rabbit oral mucosa (data not shown).

_Human oral mucosa contained fenretinide-related metabolic enzymes capable of generating the bioactive 4-oxo-4-HPR metabolite and also exhibits appreciable inter-individual heterogeneity_
In contrast to rabbit oral mucosa, human oral epithelia possessed CYP3A4 (Figure 8). As demonstrated by immunoblotting, considerable inter-patient heterogeneity of protein levels was exhibited (i.e., 16-fold difference of CYP3A4 in samples 2 and 5; Figure 8). These levels of protein expression were not associated with the amount of epithelium in each sample, and therefore, reflected actual inter-patient variations. In addition, the fenretinide-related metabolic enzymes CYP2C8, CYP26A1, and INMT were not detected in human oral mucosa, while results from UGT1A1 and UGT1A6 immunoblots were inconclusive (data not shown).

_Fenretinide and its bioactive metabolite 4-oxo-4-HPR modulated the growth state of cultured normal human oral keratinocytes_

Evaluation of 4-HPR and its oxidized, bioactive metabolite 4-oxo-4-HPR treatment effects on BrdU incorporation in normal and dysplastic oral keratinocytes showed a preferential suppression of DNA synthesis in dysplastic as opposed to normal keratinocytes. Dose, duration and treatment compound all impacted BrdU incorporation (p<0.05, Figure 9). Although 4-HPR treatment did not significantly decrease DNA synthesis in dysplastic relative to normal cells at 24-hours, subsequent 48- and 72-hour time points showed significant inhibition in the 5 µM treated oral dysplastic cells (p<0.05). 4-oxo-4-HPR treatment resulted in significantly decreased BrdU incorporation in dysplastic cells relative to normal at all time points (24-hours: 10 µM, 48-hours: 10 µM, 72-hours: 5 µM; p<0.05).
Discussion

Despite its favorable chemopreventive profile \textit{in vitro}, systemic administration of fenretinide in oral cancer chemoprevention clinical trials has demonstrated limited therapeutic efficacy and dose-limiting side effects [7-9]. In contrast, local delivery strategies can circumvent first pass liver metabolism, minimize systemic exposure, and deliver therapeutically-relevant drug levels to the target tissues. Location, however, is paramount when considering use of local delivery formulations. The oral cavity is an optimal site for local delivery as it is amenable to direct visualization, which facilitates both agent placement and clinical monitoring. The basis for this current study arose from our familiarity with local delivery formulations in conjunction with our enthusiasm to re-introduce fenretinide for clinical oral cancer chemoprevention trials.

These pharmacokinetic studies confirmed the therapeutic advantage imparted by intra-oral fenretinide mucoadhesive patch application, i.e., delivery of pharmacologically active levels of fenretinide to the rabbit oral mucosa (i.e., 5.65 \( \mu \text{M} \) average) while negating systemic exposure and toxicities. Our data also showed undetectable levels of the inactive metabolite 4-MPR and trace amounts of the potent metabolite 4-oxo-4-HPR in the fenretinide-treated rabbit oral mucosal samples. Accompanying Western blots did not show the presence of the major metabolic enzymes responsible for conversion of fenretinide to 4-MPR (amine N-methyltransferases, such as INMT) or 4-oxo-4-HPR (CYP3A4, CYP2C8, CYP26A1) in rabbit oral mucosa [16,17]. The trace amount of 4-oxo-4-HPR detected could therefore reflect 4-HPR metabolism by other cytochrome P450 enzymes or oral microflora. While technical challenges prevented LC-MS/MS detection of 4-HPR-O-glucuronide, the marked induction of UGT1A1 levels in rabbits I
through VI suggested concomitant fenretinide metabolism to the more potent 4-HPR-O-glucuronide [18]. This finding was limited to the percent increase relative to the blank-treated control tissues (i.e., not total epithelial levels of UGT1A1), and therefore assumed increased metabolism with enzyme induction. Notably, the marked induction of UGT1A1 in rabbits I through VI was coupled with low baseline enzyme levels in the blank-treated control tissues, while rabbits VII and VIII demonstrated elevated baseline levels and minimal induction. Similar to previous studies demonstrating maximal Phase II enzyme expression [19], these findings also suggested that UGT1A1 is maximally expressed in rabbits VII and VIII.

Also observed in these studies was a delivery gradient, in which highest fenretinide levels were consistently achieved at the patch-mucosal interface, followed by second highest, variable levels within the targeted epithelium. Ideally, highest fenretinide levels should be achieved at the pivotal basal layer cells that direct keratinocyte growth and differentiation. The constant inter-rabbit levels at the patch-mucosal interface suggested effective patch delivery, while the range of intra-mucosal fenretinide concentrations was likely attributed to inter-rabbit epithelial permeability variations. This permeability issue was addressed in a recent study by our laboratories, which evaluated a fenretinide mucoadhesive patch formulated with the permeability enhancers propylene glycol and menthol [20]. This permeability-enhanced patch demonstrated consistent fenretinide penetration of rabbit oral mucosa following a single 30-minute patch application, which achieved comparable intra-mucosal levels to those observed in the current 10-day study [20]. Furthermore, these collective findings (i.e., similar intra-mucosal fenretinide levels after a single 30-minute patch application and 30-minute application q.d. for 10-days) suggested that therapeutic levels of fenretinide were
achieved after each 30-minute patch application and were subsequently metabolized and cleared from the treatment site prior to subsequent patch application 24-hours later. Similarly, these observations indicated that transient fenretinide levels affect keratinocyte protein translation in a therapeutic fashion.

A distinct rabbit versus human species variation was seen with regard to oral mucosal tissues’ fenretinide metabolic profile. The present and previous studies by our laboratory confirm the presence of highly variable levels of UGT1A and CYP3A4 enzymes in normal human oral epithelium, implying the probable metabolism of fenretinide to its active metabolites 4-HPR-O-glucuronide and 4-oxo-4-HPR in human applications [11]. Additionally, while the absence of INMT in human oral mucosa suggests the inability to form the inactive metabolite 4-MPR, other members of the amine N-methyltransferase family could also inactivate fenretinide [16]. Collectively, the human fenretinide-related metabolic enzyme profile favors production of bioactive metabolites, and exhibits the capacity for increased fenretinide retention at the treatment site via local enteric recycling (i.e., 4-HPR-O-glucuronide recycling by bacterial β-glucuronidases [11]). The large inter-patient heterogeneity, however, will likely necessitate individual metabolic enzyme profiling to determine optimal duration and frequency of patch placement to achieve the desired therapeutic effect (i.e., patients with an elevated metabolic capacity could benefit from multiple doses throughout the day to effectively increase the drug levels at the target site).

The observed dose-dependent modulation of the keratinocyte growth state recapitulated previously published in vitro differentiation-associated concentrations and also confirmed that patch-delivered fenretinide retained its bioactive properties [5]. Although not statistically significant, levels of the enzyme responsible for cornified
envelope formation, i.e., TGase 1, increased in all oral mucosa tissues containing 0.1 to 5 μM fenretinide (i.e., previously established differentiation range). In contrast, tissues with fenretinide levels greater than 10 μM (i.e., apoptotic range [5]) contained reduced TGase 1 levels. Interestingly, apoptotic indices were not drastically increased in the oral tissues with greater than 10 μM fenretinide, and did not inversely correlate with the TGase 1 data. These preliminary findings could reflect the complexity and interaction of the pathways responsible for the keratinocyte transitioning from a proliferative growth state. These somewhat paradoxical results are supported by the concept that epithelial differentiation is a specialized form of apoptosis [21,22] and, key to the chemopreventive aspect, both pathways result in the keratinocyte leaving the proliferative pool. Furthermore, several studies have shown nuclear translocation of the other transglutaminase isoform (TGase 2) resulted in cross-linking of transcription factors and subsequent induction of apoptosis [23,24]. Although the relationship between TGase 1 nuclear staining and TUNEL positivity was not definitive, the prospect that TGase 1 fulfills a similar role in keratinocytes is highly probable. Ongoing studies in our labs are investigating these interactions.

Our data show that the Ki-67 proliferation indices in normal rabbit oral epithelia were not affected by fenretinide patch application. As preservation of an intact oral mucosal surface is essential for defense, these findings are favorable. Similarly, normal oral keratinocyte proliferation, as assessed by BrdU incorporation, was not perturbed by the addition of either fenretinide or 4-oxo-4-HPR. Treatment of dysplastic oral keratinocytes, however, showed both fenretinide and 4-oxo-4-HPR significantly suppressed DNA synthesis; suggesting the prospect for preferential targeting of dysplastic relative to normal keratinocytes. Similarly, if future studies demonstrate that
4-oxo-4-HPR provides greater therapeutic efficacy than fenretinide, subsequent patch formulations would deliver the bioactive metabolite, 4-oxo-4-HPR.

Lesions of oral epithelial dysplasia are molecularly and biochemically diverse [25]. It is therefore reasonable to predict that combinations as opposed to a single category agent may be necessary for chemoprevention of some dysplastic lesions. Indeed, our previous BRB gel chemoprevention trial, in which a subset of patients’ lesions did not respond to topical gel application, emphasizes this point. In addition, complimentary local delivery strategies (e.g., mucoadhesive patch and rinse) would provide both site-specific and field coverage components to aid in the prevention of initial and second primary oral dysplastic lesions. A locally deliverable fenretinide formulation re-introduces a potent keratinocyte differentiation-inducing agent to the oral chemoprevention battery and initiates the prospect for anthocyanin-retinoid based combination therapy.

Funding

This work was supported by the National Institute of Health’s National Cancer Institute (R01 CA129609, RC2 CA148099, R21 CA132138 to S.R.M.), National Institute of Dental and Craniofacial Research (F30 DE020992 and T32 DE14320 to A.S.H.), and National Heart, Lung, and Blood Institute (R01 HL68345 to S.P.S.). Additional funding was provided by the Fanconi Anemia Research Fund and National Center for Research Resources UL1RR025755 (to S.R.M.), Pelotonia Fellowship Program (to G.M.K.; any opinions, findings, and conclusions expressed in this material are those of the authors and do not necessarily reflect those of the Pelotonia Fellowship Program), the American
Association for Dental Research Student Research Fellowship (to A.S.H.), and the Ohio Division of the American Cancer Society Fellowship (to M.P.P.).

**Acknowledgements**

Please see original paper for complete list of referenced papers. The authors wish to thank Mary Lloyd and Mary Marin for their assistance with tissue processing, and Drs. Laura Gallaugher and Michelle Creamer for providing expert advice throughout the rabbit studies. See page 111 for thesis references.
Figures

Figure 4. *Mucoadhesive patch attachment on rabbit oral mucosa.* Fenretinide mucoadhesive patches were attached (q.d., 30 minutes for 10 consecutive days) to the right buccal mucosa (blank patches on left buccal mucosa) immediately posterior to the intra-oral commissure of the upper and lower lips. Pre- and post-treatment blood specimens and patch-mucosal interface samples were collected daily, and fenretinide-treated and blank-treated oral mucosal biopsies were harvested upon completion of the 10-day study. All samples were analyzed via LC-MS/MS to detect the levels of fenretinide and its metabolites (4-oxo-4-HPR and 4-MPR).
Figure 5. *Intra-oral fenretinide patch application delivered pharmacological intra-oral levels and did not induce any deleterious side-effects.*

**A.** Patch application delivered a fenretinide gradient with highest levels achieved at the patch-mucosal interface, therapeutically-relevant levels in the treated oral mucosa (mean±SEM: 5.65±2.52 μM, n=8), and undetectable levels in sera (linear limit of quantification: 1 ng/ml analyte).

**B.** Histopathological evaluation of hematoxylin and eosin stained blank-treated and fenretinide-treated rabbit tissues revealed histologically normal oral mucosa. No evidence of either intra-epithelial or superficial connective tissue contact mucositis or inflammation was observed.
Figure 6. Quantified immunohistochemical analyses showed fenretinide’s dose-specific effects on treated rabbit oral epithelia growth state. A. Results of these studies showed that lower fenretinide levels (<5 μM) elicited differentiation effects. While Ki-67 positivity was modestly reduced in epithelia of rabbits I through VI (<5 μM), epithelial proliferation was markedly decreased at higher fenretinide levels (>5 μM, rabbits VII and VIII). Similarly, TGase 1 levels (indicator of terminal differentiation) showed higher induction at lower fenretinide levels (<5 μM). Apoptosis (TUNEL indices) was significantly increased in fenretinide-treated relative to blank-treated mucosal epithelia (n=8). These data, however, did not demonstrate an apparent dose-dependent relationship.

B. IHC images were quantified (blank-treated relative to fenretinide-treated epithelia) by designating the stratum basale (Ki-67) or full-thickness epithelia (excluding orthokeratinized layer - TGase 1 and TUNEL) as the area of interest. Positive indices (denoted by red highlighted areas in the inset photomicrographs – Ki-67 and TGase 1) were divided by the total area of interest (TGase 1 and TUNEL) or by the basement membrane length (Ki-67; yellow line in the inset photomicrograph).
Figure 7. Patch-delivered fenretinide effects intra-epithelial metabolic enzyme profile.
A. Human oral mucosa fenretinide metabolic pathways can include generation of the inactive metabolite (4-MPR), active metabolites (4-oxo-4-HPR and 4-HPR-O-glucuronide), and the functionally unknown metabolite (4-HPR-O-sulfate). Abbreviations and intra-oral locations are as follows: LPH, lactase phlorizin hydrolase (oral microflora); β-Gluc, β-Glucuronidase (human saliva); SULT, sulfotransferase (absent in human oral cavity); UGT1A, UDP-glucuronosyltransferase 1A (human oral epithelium); CYP3A4, cytochrome P450 3A4 (human oral epithelium); INMT, indolethylamine N-methyltransferase (absent in human oral cavity). B. Differentiation-inducing levels of fenretinide (<5 μM) increased intra-epithelial levels of UGT1A1 relative to blank-treated tissues. C. Positive staining in the red highlighted area (area of interest) was used to quantify UGT1A1 levels.
Figure 8. Immunoblots of normal human oral mucosa revealed considerable inter-donor heterogeneity in a fenretinide bioactivating enzyme, CYP3A4. Similar to other intra-epithelial metabolic enzymes, CYP3A4 was primarily distributed in the lower epithelial layers (i.e., basilar and spinous), with decreased levels in the increasingly differentiated granular and cornified layers. Notably, levels of additional fenretinide-activating enzymes (i.e., CYP2C8 and CYP26A1) and the fenretinide-inactivating/eliminating enzyme (i.e., INMT) were not detected in the human oral epithelia. Although previous studies have demonstrated the presence of UGT1A enzymes in human oral epithelia [11], UGT1A1 and UGT1A6 immunoblots were indeterminate (data not shown).
Figure 9. Fenretinide and its bioactive metabolite 4-oxo-4-HPR demonstrated preferential growth suppression towards premalignant (dysplastic) oral keratinocytes. Treatment of normal and dysplastic oral keratinocytes with doses of fenretinide or 4-oxo-4-HPR that are achievable via mucoadhesive patch delivery significantly decreased DNA synthesis in a dose, cell strain, and time-dependent fashion. Dysplastic keratinocytes were markedly more susceptible to suppression of DNA synthesis. These data convey the promise for mucoadhesive fenretinide patch application in oral cancer chemoprevention.
Mucoadhesive Fenretinide Patches for Site-Specific Chemoprevention of Oral Cancer: Enhancement of Oral Mucosal Permeation of Fenretinide by Coincorporation of Propylene Glycol and Menthol

Xiao Wu, Kashappa-Goud H. Desai, Susan R. Mallery‡, Andrew S. Holpuch‡, Maynard P. Phelps‡, and Steven P. Schwendeman*†
† Department of Pharmaceutical Sciences, University of Michigan, Ann Arbor, Michigan
‡ Department of Oral Maxillofacial Surgery and Pathology, College of Dentistry and the Comprehensive Cancer Center and Solove Research Institute, The Ohio State University, Columbus, Ohio

DOI: 10.1021/mp200655k
Publication Date (Web): January 26, 2012
Copyright © 2012 American Chemical Society

*College of Pharmacy, University of Michigan, 428 Church St., Ann Arbor, MI 48109. Phone: 734-647-8339. Fax: 734-615-6162. E-mail: schwende@umich.edu.

Abstract

The objective of this study was to enhance oral mucosal permeation of fenretinide by coincorporation of propylene glycol (PG) and menthol in fenretinide/Eudragit RL PO mucoadhesive patches. Fenretinide is an extremely hydrophobic chemopreventive compound with poor tissue permeability. Coincorporation of 5–10 wt % PG (mean Js = 16–23 μg cm−2 h−1; 158–171 μg of fenretinide/g of tissue)
or 1–10 wt % PG + 5 wt % menthol (mean Js = 18–40 μg cm⁻² h⁻¹; 172–241 μg of fenretinide/g of 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 coincorporation 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 coincorporated 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.

**Introduction**

Head and neck squamous cell carcinoma (HNSCC), which is a worldwide 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 precancerous 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 coincorporation 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 reintroduction 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, noncarcinogenic and widely used permeation enhancer in transdermal drug delivery.(25) Propylene glycol, on the other hand, is a 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 coincorporation of PG and menthol in the patch formulation.

Experimental Section

Chemicals, Tissue, and Animals

Fenretinide was received as a gift sample from National Cancer Institute (USA). 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 a slaughterhouse (Dunbar Meat Packing Company, Milan, MI, USA). Rabbits were purchased from Harlan Laboratories (Indianapolis, IN, USA).
Preparation of Mucoadhesive Patches for Enhanced Oral Mucosal Permeation of Fenretinide

Fenretinide/Eudragit RL-PO/solubilizer patches with and without permeation enhancers (PG and menthol) were prepared by 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/20 wt % 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, the 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/20 wt % Tween 80 layers loaded with PG alone (5 and 10 wt %) or menthol alone (5 and 10 wt %) or in combination (1 wt % PG + 5 wt % menthol, 2.5 wt % PG + 5 wt % menthol, and 10 wt % PG + 5 wt % 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 the detection wavelength was set at 365 nm. A standard curve of fenretinide was established in acetonitrile:ethanol (50:50), and the 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 of 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 until 7 days, the samples were centrifuged at 8000 rpm for 10 min 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) and the sample was 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 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 of 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). 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 h 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 the donor chamber. Donor and receiver chambers were filled with 3 mL of 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), a 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 min. Then, 2 mL of acetonitrile was added to the tubes and vortexed for 1 h. After 1 h, tubes were centrifuged at 2600g at 25 °C for 20 min and the supernatant was analyzed by HPLC to determine fenretinide content.
**Hematoxylin 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 hematoxylin solution and 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 an 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 Institutes 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 an individual rabbit’s oral cavity (drug + adhesive layers facing the mucosa). Slight pressure was applied to the backing layer of the patch for 1 min 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. Coincorporation 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 laboratories 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 3. The thickness of fenretinide and adhesive layers and the Tegaderm adhesive film was 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.
Table 3. Evaluation of Microencapsulation of Fenretinide in Permeation Enhancer-Free and Permeation Enhancer-Loaded Eudragit RL-PO Films

<table>
<thead>
<tr>
<th>Patch formulation</th>
<th>Fenretinide loading (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>theora</td>
</tr>
<tr>
<td>permeation enhancer-free</td>
<td>5.26</td>
</tr>
<tr>
<td>5 wt % PG</td>
<td>5.00</td>
</tr>
<tr>
<td>10 wt % PG</td>
<td>4.76</td>
</tr>
<tr>
<td>5 wt % menthol</td>
<td>5.00</td>
</tr>
<tr>
<td>10 wt % menthol</td>
<td>4.76</td>
</tr>
<tr>
<td>1 wt % PG + 5 wt % menthol</td>
<td>4.95</td>
</tr>
<tr>
<td>2.5 wt % PG + 5 wt % menthol</td>
<td>4.88</td>
</tr>
<tr>
<td>10 wt % PG + 5 wt % menthol</td>
<td>4.54</td>
</tr>
</tbody>
</table>

\(^a\) Based on polymer + excipient weight.

\(^b\) Mean ± SE, n = 3.
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 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, an appropriate quantity of suitable solubilizing agent is commonly incorporated.\(^{(35)}\) In this study, the optimal quantity of nonionic 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 10. The solubility of fenretinide in bovine serum was found to be 21 ± 1 μg/mL (see Figure 10A). Bovine serum comprises of 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.\(^{(36-38)}\)
Figure 10. Solubilization of fenretinide in bovine serum. The effect of quantity (0.9 (●), 2.26 (*), 3.97 (▼), 8.03 (Δ), and 20.05 (■)) of fenretinide added in 15 mL of 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 protection from light.

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 10B). 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 of 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. 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 Coincorporation of Propylene Glycol and Menthol in Fenretinide/Eudragit RL-PO Patches**

The effect of coincorporation 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 11. Ex vivo permeation of fenretinide increased steadily over a period of 8 h and then reached a plateau thereafter (see Figure 11). Both the flux (Js) at steady state and the enhancement factor (EF = Js with enhancer/Js without enhancer) were calculated.
Figure 11. Coincorporation of propylene glycol (PG) or PG + menthol in fenretinide/Eudragit RL PO patches significantly enhance fenretinide permeation across porcine buccal mucosa. The effect of coincorporation 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 (▲) 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 5 wt % fenretinide, 20 wt % Tween 80, and 40 wt % sodium deoxycholate. Symbols represent mean ± SE, n = 5.
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 4. Coincorporation of single (see Figure 11A,B) or mixed (see Figure 11C) 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 4). For example, the flux for permeation enhancer-free patch was found to be $\sim 10 \, \mu g \, cm^{-2} \, h^{-1}$. After coincorporation of 10 wt % PG or 10 wt % PG + 5 wt % menthol, the flux was increased to $\sim 23$ (EF = 2.3) and 40 (EF = 4) $\mu g \, cm^{-2} \, h^{-1}$, respectively. In contrast, a slight increase in the flux was observed with menthol patch formulations ($J_s = \sim 13 \, \mu g \, cm^{-2} \, h^{-1}$). The levels of drug in tissue were in agreement with the values of flux (see Table 4). Fenretinide content in buccal tissue after 12 h of ex vivo permeation with permeation enhancer-free patch was found to be $\sim 44 \, \mu g/g$. Coincorporation of PG or PG + menthol led to a significantly high amount of fenretinide recovery from the buccal tissue ($\sim 171$ and 241 $\mu g$ 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.
<table>
<thead>
<tr>
<th>Patch Formulation</th>
<th>Fluxa (Js) (μg cm⁻² h⁻¹) in the receptor medium (μg/mL)</th>
<th>Fluxa (Js) in the tissue (μg/g)</th>
<th>Enhancement Factor (EFb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permeation enhancer-free</td>
<td>10.0 ± 0.5</td>
<td>22.3 ± 0.5</td>
<td>43.8 ± 6.1</td>
</tr>
<tr>
<td>5 wt % PG</td>
<td>16.2 ± 0.9</td>
<td>28.8 ± 0.8</td>
<td>158.5 ± 4.7</td>
</tr>
<tr>
<td>10 wt % PG</td>
<td>22.8 ± 1.3</td>
<td>37.4 ± 0.5</td>
<td>170.7 ± 5.3</td>
</tr>
<tr>
<td>5 wt % menthol</td>
<td>12.4 ± 0.6</td>
<td>22.8 ± 0.5</td>
<td>61.7 ± 5.1</td>
</tr>
<tr>
<td>10 wt % menthol</td>
<td>12.7 ± 0.7</td>
<td>23.5 ± 0.7</td>
<td>65.3 ± 4.7</td>
</tr>
<tr>
<td>1 wt % PG + 5 wt % menthol</td>
<td>17.9 ± 0.6</td>
<td>35.8 ± 1.0</td>
<td>172.1 ± 7.6</td>
</tr>
<tr>
<td>2.5 wt % PG + 5 wt % menthol</td>
<td>20.2 ± 0.8</td>
<td>39.4 ± 0.8</td>
<td>175.6 ± 7.0</td>
</tr>
<tr>
<td>10 wt % PG + 5 wt % menthol</td>
<td>39.8 ± 0.9</td>
<td>43.9 ± 0.6</td>
<td>241.1 ± 9.8</td>
</tr>
</tbody>
</table>

Table 4. Evaluation of Potential of Coincorporation 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

a - Js 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) = Js in the presence of permeation enhancer/Js in the absence of permeation enhancer; values represent mean ± SE, n = 5.
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 did not provide significant permeation enhancement of fenretinide (p > 0.001). This result can be attributed at least in part to nonhomogeneous distribution of menthol in fenretinide/Eudragit RL PO matrix (see Figure S2 and Discussion in the 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 11C and Table 4). Better fenretinide permeation observed with mixed permeation enhancers (PG + menthol) can be attributed to a 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 (a) to 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 enhancer-loaded fenretinide mucoadhesive patches are shown in Figure 12. 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, was noted.

Figure 12. Histological examination of porcine buccal tissue after 8 h buccal mucosal attachment of permeation enhancer-free and permeation enhancer-loaded fenretinide mucoadhesive patches. The effect of coincorporation 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.
Basal epithelial cells are tightly bound together in the control (no patch attachment) sample (see Figure 12A). 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 12B) and 10 (see Figure 12C) wt % PG loaded patches. An increase in intercellular edema and swelling of buccal epithelium are visible, however, in Figure 12B,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 12D and Figure 12E. 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 12F) and 2.5 wt % PG + 5 wt % menthol (see Figure 12G) loaded patches. In contrast, tissue exposed to the 10 wt % PG + 5% menthol loaded patch showed moderate increases in intracellular space and intercellular edema (see Figure 12H). 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 12B,C,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 Enhancer-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 13 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.
Figure 13. In vitro and in vivo release characteristics of permeation enhancer-free and permeation enhancer-loaded fenretinide/Eudragit RL PO mucoadhesive patches. Cumulative amount of fenretinide released in vitro/in vivo from permeation enhancer-free (○, in vitro; △, in vivo) and permeation enhancer (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 5 wt % fenretinide, 20 wt % Tween 80, and 40 wt % sodium deoxycholate. Symbols represent mean ± SE, n = 4 (in vitro) or 6 (in vivo).
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 13), 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 glycodeoxycholate. (46)

Enhanced In Vivo Rabbit Buccal Mucosal Permeation and Deposition of Fenretinide by Coincorporation 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 are shown in Figure 14. The level of fenretinide in rabbit buccal tissue increased steadily as a function of attachment time of both the patch (permeation enhancer-free and permeation enhancer-loaded patches) formulations (see Figure 14), thereby indicating excellent efficacy of 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 of fenretinide/g of tissue after 6 h of attachment) than that of permeation enhancer-free patch (17.3 ± 0.3 μg of fenretinide/g of tissue after 6 h of attachment) (see Figure 14). These results indicate excellent effectiveness of coincorporation 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).

Figure 14. Coincorporation 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 enhancer-loaded (open bars) patches in rabbits. Permeation enhancer-free patch comprised 5 wt % fenretinide, 20 wt % Tween 80, and 40 wt % sodium deoxycholate. Bars represent mean ± SE, n = 6).
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 intratissue 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 h; 19.8 μM) to 42.36 μg/g (6 h; 108.2 μM).(48) Therefore, short duration patch application (i.e., less than 30 min) 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 coincorporating 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. Coincorporation of PG or PG + menthol in patches led to significant ex vivo and in vivo buccal mucosal permeation and tissue deposition of fenretinide, an extremely hydrophobic and poorly
tissue permeable chemopreventive agent. Mucoadhesive patch coincorporated 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.

Acknowledgment

Please see original paper for complete list of referenced papers. This study was funded by Fanconi Anemia Research Fund and NIH grants: R01 CA129609, RC2 CA148099, R21 CA132138 to the laboratories of S.R.M. and/or S.P.S., and F30 DE020992 and T32 DE14320 to A.S.H. See page 111 for thesis references.
Chapter 4: Integrated Conclusions

Discussion

The purpose of our study was to determine if a fenretinide patch could deliver therapeutically relevant concentrations of fenretinide to oral epithelium. Our study is distinguished from previous studies in several ways. First, we employed a mucoadhesive patch for delivery which, while definitely not unique in itself, is the first reported use for fenretinide. The only in vivo human trial conducted using topical fenretinide consisted of breaking open a fenretinide capsule and applying the contents to the mucosa without a specific way to control for release duration at the site or to limit systemic exposure. With the help of our collaborators, we developed a method for stabilization and sustained release of fenretinide, including an impermeable Tegaderm backing to limit the amount of agent released into the saliva. We measured the tissue concentration of fenretinide, along with serum concentration, allowing for direct comparison to in vitro studies. While it remains to be proved that the patch will impart a therapeutic benefit to human patients with oral premalignant lesions, we achieved our goal of development and evaluation of a mucoadhesive patch to deliver fenretinide to the oral mucosa to achieve therapeutic concentrations without systemic or local adverse effects. Our studies also show changes in metabolic parameters at the tissue level in response to fenretinide. Thus, we disproved our null hypothesis.
Effective Drug Delivery to the Target Site

Our approach to OSCC chemoprevention, topical application, presents several challenges. The oral epithelium, while thin compared to the skin, functions as a natural barrier to substances, bacteria, viruses, evaporation, etc. Aside from designing a chemopreventive patch capable of stabilizing and releasing fenretinide, we also had to ensure that the released fenretinide would penetrate the oral mucosa. The oral mucosa is a moist environment, courtesy of numerous minor salivary glands present throughout the mouth. Fenretinide is highly hydrophobic, making the oral mucosa an unlikely place for it to be absorbed. Our patch utilized Tween and deoxycholate solubilizers to increase the release of fenretinide to the mucosa. In fact, our new study conducted with our collaborators showed even greater fenretinide release when permeation enhancers (2.5 wt% propylene glycol and 5 wt% menthol) were incorporated into the mucoadhesive patch\textsuperscript{64}. Once introduced to the oral epithelium, fenretinide has been shown to be absorbed into the cell membrane, which is more lipophilic. Other mechanisms for delivery which could be the target of future investigations include the use of resorbable implants and nano-particles\textsuperscript{65}.

Modulation of Surface Oral Epithelium

While our study did not use an animal cancer model, we did analyze the treated normal mucosa for changes in several growth parameters following 10 days of patch application. First, we did not notice any microscopic changes in the architecture of the oral epithelium, and importantly, no adverse effects such as atrophy, blistering,
ulceration, or immune response were noted. Ki-67 IHC staining, a marker used to show cell proliferation, showed decreases in 7 of 8 animals, especially in the animals with >5 μM concentration of fenretinide. However, these differences were not significant. Terminal differentiation (TGase 1) was increased in all animals with < 5 μM fenretinide, but decreased in tissue with > 5 μM fenretinide, consistent with fenretinide’s induction of cell maturation at low concentrations. Apoptotic indices (TUNEL) were significantly increased in all treated tissue. Previous studies have shown that fenretinide’s effects are more pronounced with dysplastic cells versus normal cells. We may extrapolate that the effects achieved on normal tissue in this study will be more significant in dysplastic tissue.

**Benefits of Local Versus Systemic Metabolism for Oral Cancer Chemoprevention**

One of the primary obstacles that we hope to overcome with patch delivery of fenretinide is first pass metabolism. Chemopreventives absorbed in most of the gastrointestinal tract will enter the hepatic portal system, where they are metabolized prior to entering the circulatory system for delivery to the oral mucosa. All of the enzymes identified thus far that metabolize fenretinide are present in the liver, suggesting a significant ‘first pass effect’ from ingested forms of fenretinide\(^{56}\). Furthermore, the most common metabolite of fenretinide is an inactive one, 4-MPR. Thus, first pass effect has the potential to significantly limit the concentration of fenretinide and perhaps its active metabolites in serum. Villablanca et al., in a study of children with high risk solid tumors, was able to show that at the maximal tolerated dosage of fenretinide (as high as 5,709 mg equivalent adult daily dose), the average
serum concentration of fenretinide following one week of therapy was 9.9 μmol/L. However, side-effects were far beyond those found in the much smaller doses used in oral fenretinide chemoprevention trials, and included Grade 3 and 4 AST and ALT toxicities, hypoalbuminemia, nausea, vomiting, and even pseudotumor cerebri. While an average serum concentration of 9.9 μmol/L was achieved after the first week, the protocol called for a 2 week rest period to minimize side effects, with an unreported serum concentration at the end of the 3 week period. The authors failed to provide evidence that the serum concentrations of fenretinide achieved correlated with target tissue concentrations.

Because our patch delivers fenretinide directly to the mucosa, metabolism in the liver is inconsequential. However, metabolism at the level of the mucosa is not avoided with this approach. Therefore, we looked for various enzymes which target fenretinide in human mucosa. The enzyme CYP3A4 was detected in humans, with significant inter-patient variation. This enzyme converts fenretinide to 4-oxo-4-HPR, an active metabolite with even greater activity than the parent compound. Other enzymes responsible for fenretinide metabolism, namely CYP2C8, CYP26A1, and INMT, were not found. Based on our study, we hypothesize that enzymatic activity on fenretinide at the mucosa level does not decrease the effectiveness of fenretinide, and may actually enhance its activity.
Future Investigations using Mucoadhesive Fenretinide Patch

Clinical Applications

The goal of our labs is to improve patient outcomes related to OSCC and its precursors via a broad, multifocal approach. Specifically, we are interested in preventing the progression of precancerous lesions or cancer recurrence through topical therapies to minimize systemic effects. Realizing that not only is OSCC a heterogeneous process, but also that each patient has a distinct metabolic profile, the focus of future research should center on multiple chemopreventive agents rather than a single agent, thus giving the impetus to investigate fenretinide. To address the accepted theory of field cancerization, we have conducted initial pilot studies using oral rinses containing black raspberry extract, which have produced even higher levels in saliva than when using a BRB gel alone. This rinse could potentially complement the use of a fenretinide patch.

Our lab completed a pilot study to determine the effectiveness of a topical bioadhesive black raspberry (BRB) gel in preventing progression of biopsy confirmed oral epithelial dysplastic lesions in 30 patient. We were able to show that, following 6 weeks of topical therapy, histologic and clinical regression of lesions as well as significant reductions in COX-2 levels and underlying angiogenesis were achieved. Perhaps even more compelling was the significant reduction in lesional LOH at tumor suppressor gene loci, revealing an actual change in the genetic structure of the lesional cell population. Topical BRB, however, was not able to elicit lesional regression in 30% of subjects despite > 90% compliance. For these patients, an additional agent and/or mode of delivery may prove beneficial, motivating our lab to perform the current
study. It is of note that none of the study participants in the BRB pilot study developed adverse effects, and our lab is currently conducting a multicenter, placebo controlled trial using topical BRB.

There are several chemopreventive agents and delivery methods that have been investigated, including the use of various vitamin A analogs (both systemic and topical delivery), use of ONYX-15 virus to induce apoptosis of transformed cells, topical NSAIDs, and of course BRB extract. Because of fenretinide’s proven in vitro efficacy, and safety in human clinical trials, we chose to investigate it’s topical application as a treatment adjunct to the use of topical BRB. The anthocyanins in BRB are thought to act in part by reducing reactive oxygen species (ROS) mediated cell signaling. However, fenretinide is thought to mediate RAR independent apoptosis of transformed cells via the production of ROS. Despite this possible conflict, our labs feel that both agents show promise as chemopreventives, and whether their interaction is synergistic or antagonist when applied topically will be the goal of future investigations.

**Future Human Trials**

Because of fenretinide’s established history of use in human clinical trials, a phase 0 human clinical trial will be our next goal in investigating the efficacy of a fenretinide mucoadhesive patch. Initial studies would investigate the patch itself, without fenretinide, to determine if there are any deleterious effects from the patch and/or its constituents. Because the patch components have a history of safe human application, we are optimistic that these trials will confirm the patch’s safety. Tegaderm is widely available for topical use, and Eudragit has a history of safe human application including
ingestible forms. Our next trial would then test a fenretinide patch on normal human mucosa. Again, similar to the current rabbit study, our goals would be to not only look at the concentration of fenretinide achieved within human epithelium, but also to determine if there are any adverse effects. Mucositis or reactive hyperkeratinization would be more than an inconvenience or unwanted effect; these adverse effects may hamper monitoring of dysplastic lesions. However, if the patch shows no adverse effects, perhaps we would then consider a pilot human study, as was successfully performed by our lab using BRB gel.

Given that fenretinide is a lipophilic molecule, we may look for possible drug reservoirs within the oropharynx to provide sustained site specific delivery. The cell membranes of the oral mucosa can store fenretinide within the liquid bilayer, although the storage capacity hasn’t been quantified. The bilateral buccal fat pads may serve as a potential reservoir for fenretinide, particularly for posterior precursor lesions. While not a focus of this investigation, if fat is proven to substantially increase the length of delivery and total drug delivered, there are options for delivery to other oral sites. The buccal fat pad is frequently used as a pedicled graft when reconstructing oral defects, showing good longevity following grafting\(^6^8\). Autologous adipose tissue transplantation is frequently performed for cosmetic augmentation, including the lips, and theoretically could be used for chemoprevention purposes if needed. Based on Phase 0 studies, progression to a Phase IIb trial is planned.
Potential Limitations

Patient Compliance

As with any treatment modality, we expect challenges with patient compliance. While oral topical delivery of pharmaceutical agents may be familiar to most patients, such as toothpaste, mouth rinses, analgesic gels, etc., mucoadhesive patches are not. To this end, we designed our patch to be thin, only 0.33 mm thick, to lessen the profile and hopefully patient awareness during use. The patch was designed to provide a burst release, with a duration of application of only 30 minutes, again to decrease the perceived inconvenience to the patient. Despite these efforts, we realize that the patch will still be less familiar than a pill or tablet, and thus potentially more prone to decreased patient compliance. While the duration of application is only 30 minutes, multiple applications per day will likely be needed, again potentially decreasing compliance. Patient compliance will be speculative until human trials are conducted. Patch adhesiveness may present yet another challenge. Our patch was tested on static buccal mucosa. Whether the adhesion is strong enough for mobile mucosa has not been tested. Also, it is unlikely that the patch would adhere well to natural mucosal folds, such as the floor of mouth or buccal vestibules.
**Stem Cell Retention**

Another challenge to using fenretinide as a sole chemopreventive agent is that while it has no known genotoxicity, it has not been shown to eliminate genetically altered stem cells. Thus, assuming excellent patient compliance, absorption, metabolism, and clinical and histologic regression of premalignant lesions, using fenretinide alone may not be sufficient in eliminating the risk of future OSCC. Again, concomitant use with another agent, such as BRB, may compliment a fenretinide patch. The anthocyanins in black raspberries have been shown to reduce LOH in treated tissue, making it more likely to eliminate altered stem cells. This action, when combined with fenretinide’s ability to promote maturation and/or apoptosis of dysplastic cells, may prove to be a potent therapeutic combination. Future studies will be needed to fully elucidate these interactions.

**Patch Delivery Safety**

Patch delivery, like any other known method of pharmacologic delivery, carries inherent risks. On a macroscopic level, a patient may accidentally ingest or aspirate a mucoadhesive patch. Our patch is small and thin enough to potentially pass through a human digestive tract, although one can never rule out a bowel obstruction, especially if a larger patch were to be used. The total dose of fenretinide contained within each patch was 500 μg, much less than the 1,900 mg used in a recent clinical trial using fenretinide tablets. Thus, in addition to achieving therapeutic concentrations of fenretinide within mucosa, the ingested patch would theoretically be significantly less
toxic than a typical oral dose. Our patch is designed for burst delivery of fenretinide, with a delivery time of 30 minutes. This duration eliminates the need for overnight application, which may increase aspiration risk, and also allows for minimal disruption of meals. However, based on our more recent patch formulation, this duration may be significantly reduced as needed.

Given the appropriate application of the patch (no ingestion or aspiration), there may be a risk of systemic toxicity given the high tissue concentrations achieved. Using a patch with a diameter of 11 mm, we measured no detectable amount of fenretinide in rabbit serum. A 70 kg human has a blood volume approximately 36.9 times that of a 2.71 kg rabbit (rabbit blood volume is 56 mL/kg, while human blood volume is 8% of body weight). Assuming a similar pattern of absorption in humans, an equivalent patch for a human would have a diameter of 66 mm. This is clearly a gross extrapolation, as human and rabbit mucosa may have different permeabilities to fenretinide or differ in transport mechanisms and metabolism. However, if this estimate is within reason, we could deliver fenretinide over a large mucosal surface without any detectable levels in human serum using a patch.

Finally, a fenretinide mucoadhesive patch may elicit local unwanted effects such as contact mucositis, hyperkeratinization, or even frank ulceration. We did not observe any of these effects during our study. Neither the positive control mucosa nor treated mucosa showed macroscopic or microscopic changes in morphology, as determined by examination daily after patch removal and analysis of hematoxylin and eosin stained biopsied tissue. It is our hypothesis that results with human mucosa will be similar in its reaction to the fenretinide patch.
References


49. Paulson JD, Oldham JW, Preston RF, Newman D: Lack of genotoxicity of the cancer chemopreventive agent N-(4-hydroxyphenyl)retinamide. Fundam Appl Toxicol 5:144, 1985


