Localized Transdermal Delivery of Chemopreventive Agents to the Breast

Abdulsalam Alqahtani
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LOCALIZED TRANSDERMAL DELIVERY OF CHEMOPREVENTIVE AGENTS TO THE BREAST

BY

ABDULSALAM ALQAHTANI

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Pharmaceutical Sciences

South Dakota State University

2019
LOCALIZED TRANSDERMAL DELIVERY OF CHEMOPREVENTIVE AGENTS TO THE BREAST

ABDULSALAM ALQAHTANI

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Pharmaceutical Sciences degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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Dissertation Advisor Date

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Head, Department of Pharmaceutical Sciences Date

Dean, Graduate School Date
This dissertation is dedicated to my family
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<tbody>
<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>AIs</td>
<td>Aromatase inhibitors</td>
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<td>ASA</td>
<td>Aspirin</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer 2</td>
</tr>
<tr>
<td>CDKs</td>
<td>Cyclin-dependent kinases</td>
</tr>
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<td>DCIS</td>
<td>Ductal carcinoma in-situ</td>
</tr>
<tr>
<td>EE%</td>
<td>Encapsulation efficiency in percent</td>
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<tr>
<td>ENX</td>
<td>Endoxifen</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERDs</td>
<td>Estrogen receptor down regulators</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Fenr</td>
<td>Fenretinide</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor type 2</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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</table>
IDC  Invasive ductal carcinoma
ILC  Invasive lobular carcinoma
LCIS Lobular carcinoma in-situ
LE% Loading efficiency in percent
MN Microneedles
mTOR Mammalian target of rapamycin
MTX Methotrexate
ND Not detectable
NSAID Non-steroidal anti-inflammatory drug
PARP Poly ADP ribose polymerase
PBS Phosphate buffered saline
PDI Polydispersity Index
PEG-DOX Polyethylenglycol-doxorubicin
PLD Pegylated liposomal doxorubicin
PR Progesterone receptor
R² Correlation coefficient
rpm Rotation per minute
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>SC</td>
<td>Stratum corneum</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SERMs</td>
<td>Selective estrogen receptor modulators</td>
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<td>TAM</td>
<td>Tamoxifen</td>
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<td>VEGF-A</td>
<td>Vascular endothelial growth factor A</td>
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ABSTRACT

LOCALIZED TRANSDERMAL DELIVERY OF CHEMOPREVENTIVE AGENTS TO THE BREAST

ABDULSALAM ALQAHTANI

2019

Breast cancer is the second leading cause of cancer death in women. About 1 in 8 women will develop breast cancer in their lifetime. The current systemic approaches are associated with significant side effects including some life-threatening toxicity, leading to poor patient compliance. To overcome this limitation, localized transdermal drug delivery system was investigated in the current study. In addition, majority of breast cancers originate from the ducts and lobules in the breast. Therefore, localized transdermal drug delivery system can maximize drug concentration in the breast and minimize systemic adverse effects, resulting in a potential approach for preventing and/or treating early stage breast cancer.

In the current work, three different chemopreventive agents with different mechanisms of action and physicochemical properties were used. This includes aspirin, endoxifen and fenretinide. Due to the systemic side effects and/or poor oral bioavailability of these chemopreventive agents, they were selected in the current work. To explore the feasibility of localized transdermal delivery of these agents to the breast through breast skin and mammary papilla (nipple), four different penetration enhancement approaches were evaluated. In the first approach, the influence of hydroalcoholic vehicles (0, 33, 50 and 66% ethanol, v/v) on drug permeation through
breast skin and mammary papilla was tested. In the second approach, the effect of different chemical penetration enhancers (sandalwood oil, limonene and eugenol) on drug permeation through breast skin and mammary papilla was studied. The third approach focused on the influence of different vesicular carrier systems (ethosomes and liposomes) on drug permeation through breast skin and mammary papilla. The fourth approach focused on the use of microneedles on drug permeation through breast skin.

The first goal was to investigate the feasibility of localized transdermal delivery of aspirin to the breast through breast skin and mammary papilla (nipple). Aspirin has been shown to have breast cancer prevention effects. Oral aspirin is associated with gastrointestinal (GI) side effects. Also, a large oral daily dose of aspirin is needed for its activity against breast cancer. To this end, the localized transdermal delivery of aspirin to the breast can enhance the drug concentration in the breast and minimize its GI side effects. The results demonstrated that aspirin permeated through the breast skin and through the mammary papilla. However, the permeation of aspirin through the breast skin was much higher than the permeation through mammary papilla. Among the hydroalcoholic vehicles tested, 33% ethanol showed the highest permeation through the breast skin, while 66% ethanol showed the highest permeation through mammary papilla. Among the chemical penetration enhancers, 5% sandalwood oil in 50% ethanol showed the highest aspirin permeation through the breast skin and mammary papilla. Compared to 50% ethanol, 5% sandalwood oil increased the aspirin permeation by 3.8-fold and 3.2-fold through the breast skin and mammary papilla, respectively. Ethosomes showed similar skin permeation to 33% ethanol, while liposomes showed less permeation in comparison to 33% ethanol. On the other hand, liposomes showed the highest permeation
through the mammary papilla in comparison to ethosomes and 33% ethanol. Microneedles significantly increased the aspirin permeation through breast skin in comparison to 33% hydroalcoholic vehicle.

The second goal was to investigate the feasibility of localized transdermal delivery of endoxifen to the breast through breast skin and mammary papilla. Endoxifen, an active metabolite of tamoxifen, has been shown to have a strong activity against breast cancer. Endoxifen has 100-fold greater binding affinity for estrogen receptors in the breast compared to tamoxifen. However, tamoxifen and its metabolites act as an antagonist estrogen receptor in breast tissue and an agonist estrogen receptor in other organs including liver, bone and uterus, leading to severe side effects. To this end, the localized transdermal delivery of endoxifen to the breast can enhance the drug concentration in the breast and minimize the systemic exposure of the drug. The results demonstrate that endoxifen permeated through breast skin but not through the mammary papilla. Among the different hydroalcoholic vehicles (0-66% ethanol, v/v) tested, 33% ethanol showed the highest endoxifen permeation through the breast skin. Among the different chemical penetration enhancers, 5% sandalwood oil co-treated with 50% ethanol showed the highest endoxifen permeation through the breast skin. Liposomes and ethosomes systems showed less permeation through the breast skin in comparison to 33% ethanol. Also, microneedles increased the endoxifen permeation through breast skin in comparison to 33% hydroalcoholic vehicle.

The final goal of the current work was to investigate the feasibility of localized transdermal delivery of fenretinide to the breast through breast skin and mammary papilla. Fenretinide has been shown to have a strong activity against breast cancer in pre-
clinical and clinical studies. However, fenretinide has poor oral bioavailability due to its poor water solubility and poor permeability, which limits its clinical therapeutic benefits. Taken together, the localized transdermal delivery of fenretinide to the breast can increase the drug concentration in the breast and overcome its oral delivery limitations. The findings demonstrated that fenretinide permeated through the breast skin but did not show any permeation through the mammary papilla. From the hydroalcoholic vehicles studies, an increase in the alcohol concentration in the vehicle increased the fenretinide permeation through the breast skin. Liposomes increased fenretinide permeation through the breast skin in comparison to ethosomes and 33% hydroalcoholic vehicle. Among the different chemical penetration enhancers tested, 5% sandalwood in 66% alcohol showed the highest fenretinide permeation through the breast skin. On the other hand, microneedles pre-treatment significantly increased the fenretinide permeation through the breast skin in comparison to 66% hydroalcoholic vehicle. Compared to 66% ethanol, microneedles enhanced the fenretinide permeation through the breast skin by 3.2-fold. In addition, the results from in vivo studies demonstrated that the localized transdermal delivery of fenretinide to the breast can achieve high fenretinide concentration in the breast with minimal fenretinide concentration in other organs. The in vivo results suggest that the localized transdermal delivery of fenretinide to the breast might lead to a safe strategy and an effective localized therapy approach for breast cancer.

Overall, the findings from the current work demonstrate the feasibility of localized transdermal delivery of three chemopreventive agents with different physicochemical properties (aspirin, endoxifen and fenretinide) to the breast using penetration enhancement methods. Among the four penetration enhancement methods,
microneedles-assisted system was found to be the best enhancement permeation approach for the three drugs. The results from this study can be utilized to develop localized therapeutic approaches for prevention and treatment of early stages of breast cancer.
CHAPTER ONE: GENERAL INTRODUCTION
1.1. Anatomy of human breast

The human breast is composed of lobules, ducts, nipple, areola, connective (fibrous) tissue and fat. The female breasts are formed on the anterior thoracic walls of the chest and extend from the second rib to the sixth rib. The skin, subcutaneous tissue and the breast tissue are the three major structures of the human breast. The skin of the human breast is thin and flexible, and it contains hair follicles, sebaceous glands and apocrine glands. The subcutaneous tissue of the human breast contains fat, connective tissue, nerves, blood vessels and lymphatics (Golan et al., 2005). The breast tissue in humans contains epithelial parenchymal and stromal elements (Javed and Lteif, 2013). The human breast is composed of 15 to 20 lobes which are divided further into lobules (milk glands that produce milk). The lobules drain into lactiferous ducts that open onto the nipple. The nipple is surrounded by the pigmented region, areola (Pandya and Moore, 2011). The nipple contains 4 to 18 major milk ducts, and only 5 to 9 ducts are on the surface of the nipple (Jesinger, 2014). The space that surrounds the lobes and the ducts are filled with adipose (fatty) tissue (Figure 1).
Figure 1. Anatomy of female human breast.

The shape and the size of the human breast vary from woman to woman. The skin of the human breast is connected to the breast tissue by ligaments called Cooper ligaments that support the structure of the skin. The skin of the breast is composed of epidermis and dermis, both have a thickness of 0.5-2 mm, and the epidermis is indistinguishable as a separate layer from dermis at imaging (Giess et al., 2011). The skin of the nipple-areola complex is slightly thicker than 2 mm. The nipple-areola complex contains the Montgomery glands, sebaceous glands that are transitional between mammary glands and sweet glands and can secret milk. The nipple-areola complex contains many nerve endings, abundant lymphatic system and smooth muscle (Nicholson et al., 2009) (Figure 2).
Figure 2. Anatomy of the nipple-areola complex.

Reproduced from Nicholson et al. (2009).
1.2. Breast cancer

Breast cancer is the second leading cause of cancer death in women. According to the American Cancer Society, one out of every eight women in the United States will develop breast cancer in her lifetime. In 2017, the estimated new cases of women that have breast cancer in the United States were 252,710 and 40,610 were estimated to die of breast cancer (Siegel et al., 2017). The predicted number of females that have breast cancer in the world will reach almost 3.2 million new cases per year by 2050 (Hortobagyi et al., 2005). Breast cancer is a serious health problem that affects women in all societies worldwide; therefore, there is a strong need to develop effective therapeutic and prevention strategies for breast cancer.

There are many factors that contribute to the development of breast cancer. The breast cancer risk factors include genetic, hormonal, environmental, family history and lifestyle. The two breast cancer genes, BRCA1 (breast cancer 1) and BRCA2 (breast cancer 2), are the most well-known genes that can get mutated. The mutations of these two genes are attributed to 5-10% of breast cancers (Walsh et al., 2006). Women with BRCA1 mutation have 55-65% lifetime risk of developing breast cancer, while women with BRCA2 mutation have 45% lifetime risk of developing breast cancer (Britt et al., 2014). There are also other unknown mutations that may cause breast cancer. Besides the genetic factors, environmental factors and lifestyle play a role in the development of breast cancer.

Based on the stage of the breast cancer, there are two types: non-invasive breast cancer (remain within the breast) and invasive breast cancer (spread into other parts of the body). Furthermore, based on the location of the tumor, there are two types: ductal
carcinoma (tumor begins in the milk-carrying ducts) and lobular carcinoma (tumor begins in the milk-producing lobules). Based on the stage of breast cancer and the location of the tumor, breast cancer can be classified as follows (Figure 3):

i. Ductal carcinoma in-situ (DCIS)
ii. Invasive ductal carcinoma (IDC)
iii. Lobular carcinoma in-situ (LCIS)
iv. Invasive lobular carcinoma (ILC)

Ductal carcinoma in-situ (DCIS) is non-invasive breast cancer. DCIS is the most common non-invasive breast cancer. According to the American Cancer Society, about 60,000 cases of DCIS are diagnosed in the United States every year, i.e. about 1 out of every 5 breast cancer cases. About 25% of breast cancers diagnosed in the United States are DCIS, and around one million women will be diagnosed with DCIS by 2020 (Virnig et al., 2009). DCIS is defined as the proliferation of malignant mammary ductal epithelial cells that are attached to the basement membrane of the breast ducts without invasion to surrounding breast tissue. DCIS has the risk of developing into invasive breast cancer. It has been found that the major molecular phenotypes identified with DCISs were also identified among invasive breast cancers (Tamimi et al., 2008). Thus, early diagnosis and treatment of DCIS is critical to prevent the occurrence of an invasive breast cancer (Fisher et al., 2001).

Invasive ductal carcinoma (IDC) is also a common type of breast cancer. According to the American Cancer Society, about 80,000 cases of IDC are diagnosed in
the United States every year. IDC is defined as the proliferation of malignant epithelial cells of the breast ducts and it can invade other tissues of the breast outside the duct as well as metastasizing to other parts of the body.

Lobular carcinoma in-situ (LCIS) is non-invasive breast cancer but it can develop to invasive breast cancer. LCIS is defined as an increase in the number of abnormal cells in the lobules, milk-producing glands at the end of breast ducts. LCIS was initially considered as a premalignant lesion, but now it is a marker of increased the risk for breast cancer (Afonso and Bouwman, 2008). It has been found that the incidence of LCIS in postmenopausal women is increasing (Li et al., 2005).

Invasive lobular carcinoma (ILC) is also a common type of breast cancer. About 14% of all invasive breast cancers are invasive lobular carcinoma (Martinez and Azzopardi, 1979). ILC is characterized by small uniform neoplastic cells that invade the stromal tissue and can surround mammary duct tissues and infiltrate the stromal and adipose tissues (Cristofanilli et al., 2005). ILC is difficult to palpate and visualize (mammographically and clinically) and it has a different metastatic spread (Cristofanilli et al., 2005). However, it has been found that patients with ILC have much better survival rate than patients with IDC (Toikkanen et al., 1997).
Figure 3. Different types of breast cancer.

Based on the molecular profile of the tumor, breast cancer can be classified to different types including luminal-like, basal-like and HER2 (human epidermal growth factor receptor type 2) enriched breast cancer.

Luminal-like breast cancer is named based on the expression profile of luminal breast epithelium and classified as luminal A, B and C breast cancers. Luminal A breast cancer is the most common among the luminal-like breast cancers. Luminal A breast cancer is characterized by overexpression of hormone receptors (estrogen receptor (ER)-positive and/or progesterone receptor (PR)-positive) and underexpression of HER2 receptors (HER2-negative). Around 40% of breast cancers are luminal A breast cancer (Hu et al., 2006; Kennecke et al., 2010; Voduc et al., 2010).

Basal-like breast cancer is also known as triple negative breast cancer. Triple negative breast cancer is characterized by the lack expression of estrogen receptors, progesterone receptors and HER2 receptors (ER-negative, PR-negative and HER2-negative). Around 15% of invasive breast cancers are triple negative breast cancer type (Carey et al., 2006; Bertucci et al., 2008).

HER2 (human epidermal growth factor receptor type 2) breast cancer is characterized by overexpression of HER2 receptors (HER2-positive) and underexpression of both ER and PR receptors (ER-negative and PR-negative). Around 20-30% of breast cancers are HER2 enriched breast cancer type (Perou et al., 2000; Pauletti et al., 1996; Pollack et al., 1999).
1.3 Treatment for breast cancer

Based on the clinical stage of breast cancer and on the type of the tumor, the treatment method is applied on breast cancer patients. The currently used treatment approaches for breast cancer are as follows:

i. Surgery

ii. Radiation therapy (Radiotherapy)

iii. Chemotherapy

iv. Targeted therapy

v. Hormonal therapy
1.3.1. Surgery

Surgery is the first line approach for advanced stage breast cancer. There are two main types of surgeries: lumpectomy and mastectomy. Lumpectomy surgery is done by just removing the tumor and a small amount of surrounding tissue while mastectomy surgery is done by removing the whole breast tissue. Surgery is often done for the invasive breast cancers.

1.3.2. Radiotherapy

Radiotherapy is usually done after the surgery to remove the remaining residual cancer cells in the breast. Radiation is also used to reduce the size of the tumor before the surgery. Radiotherapy utilizes a high energy beam to kill the cancer cells. Radiation therapy works by damaging the cell’s DNA, leading to cellular death of cancer cells. The radiation therapy could cause skin reaction such as itching, burning and redness.

1.3.3. Chemotherapy

Chemotherapy is a systemic therapy with one or more anticancer drugs to kill the cancer cells. Chemotherapy is given to treat both early and advanced stage breast cancers. Chemotherapy is used either before surgery or after surgery. If used before surgery, it is called neoadjuvant chemotherapy and if used after surgery, it is called adjuvant chemotherapy. Neoadjuvant chemotherapy is used to decrease the size of the tumor, while adjuvant chemotherapy is used to kill the growth of cancer cells. There are many chemotherapeutic agents used to treat breast cancer. These agents are shown in Table 1. These agents can be given as a one drug at a time or as a combination of two or more drugs at a time (Table 2).
Table 1. Chemotherapeutic agents used in the treatment of breast cancer

<table>
<thead>
<tr>
<th>Therapeutic class</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic inhibitor</td>
<td>- Albumin-bound paclitaxel</td>
</tr>
<tr>
<td></td>
<td>- Paclitaxel</td>
</tr>
<tr>
<td></td>
<td>- Docetaxel</td>
</tr>
<tr>
<td></td>
<td>- Vincristine</td>
</tr>
<tr>
<td></td>
<td>- Vinorelbine</td>
</tr>
<tr>
<td>Alkylating agent</td>
<td>- Carboplatin</td>
</tr>
<tr>
<td></td>
<td>- Cyclophosphamide</td>
</tr>
<tr>
<td></td>
<td>- Thiotepa</td>
</tr>
<tr>
<td>DNA intercalating agent</td>
<td>- Doxorubicin</td>
</tr>
<tr>
<td></td>
<td>- Daunorubicin</td>
</tr>
<tr>
<td></td>
<td>- Epirubicin</td>
</tr>
<tr>
<td></td>
<td>- Mitoxantrone</td>
</tr>
<tr>
<td>Antimetabolite</td>
<td>- 5-fluorouracil (5-FU)</td>
</tr>
<tr>
<td></td>
<td>- Gemcitabine</td>
</tr>
<tr>
<td></td>
<td>- Methotrexate</td>
</tr>
<tr>
<td></td>
<td>- Capecitabine</td>
</tr>
<tr>
<td>Microtubule inhibitor</td>
<td>- Ixabepilone</td>
</tr>
<tr>
<td></td>
<td>- Eribulin</td>
</tr>
<tr>
<td>Antineoplastic</td>
<td>- Mitomycin</td>
</tr>
</tbody>
</table>
Table 2. Combination chemotherapy used in the treatment of breast cancer

<table>
<thead>
<tr>
<th>Combination name</th>
<th>Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMF</td>
<td>Cyclophosphamide + methotrexate + 5-FU</td>
</tr>
<tr>
<td>FEC</td>
<td>5-FU + epirubicin + cyclophosphamide</td>
</tr>
<tr>
<td>CAF</td>
<td>Cyclophosphamide + doxorubicin (adriamycin) + 5-FU</td>
</tr>
<tr>
<td>TAC</td>
<td>Docetaxel (taxotere) + doxorubicin (adriamycin) + cyclophosphamide</td>
</tr>
<tr>
<td>GET</td>
<td>Gemcitabine + epirubicin + paclitaxel (taxol)</td>
</tr>
<tr>
<td>ACT</td>
<td>Doxorubicin (adriamycin) + cyclophosphamide + paclitaxel (taxol)</td>
</tr>
<tr>
<td>AT</td>
<td>Doxorubicin (adriamycin) + docetaxel (taxotere)</td>
</tr>
<tr>
<td>AC</td>
<td>Doxorubicin (adriamycin) + cyclophosphamide</td>
</tr>
</tbody>
</table>
Although chemotherapy is the major approach to treat breast cancer, there are significant limitations associated with using chemotherapeutic agents. These limitations include lack of selectivity, toxicity and multidrug resistance (Chidambaram et al., 2011). The systemic exposure of chemotherapeutic agents can lead to severe side effects including some life threatening adverse effects. These side effects include hair loss, fatigue, infection, diarrhea and constipation, nausea and vomiting, bleeding, anemia, weight changes, nerve and muscle issues, nail changes, brain damages, cardiac dysfunction, leukemia and kidney problems.

1.3.4. Targeted therapy

Targeted therapy is an effective approach that target specific cancer cells to treat breast cancer. Some of the targeted therapies use monoclonal antibodies and this type of targeted therapy is called immune targeted therapy, which targets the overexpressed receptors in the cancer cell. There are many targeted therapy agents used in the treatment of breast cancer and these agents are summarized in Table 3.
Table 3. Representative list of targeted therapy agents used in the treatment of breast cancer

<table>
<thead>
<tr>
<th>Molecular target class</th>
<th>Examples</th>
</tr>
</thead>
</table>
| HER2 positive receptor antagonist (monoclonal antibody) | - Trastuzumab  
- Pertuzumab  
- Ado-trastuzumab emtansine |
| Cyclin-dependent kinases (CDKs) inhibitor (CDK4 and CDK6 inhibitor) | - Palbociclib  
- Ribociclib  
- Abemaciclib |
| Tyrosine kinase inhibitor | - Labatinib  
- Neratinib |
| Mammalian target of rapamycin (mTOR) inhibitor | - Everolimus |
| Poly ADP ribose polymerase (PARP) inhibitor | - Olaparib |
| Vascular endothelial growth factor A (VEGF-A) inhibitor (monoclonal antibody) | - Bevacizumab |
With advances in molecular biology, a number of molecular targets have been identified for breast cancer and is a growing area of research. However, the targeted therapy has some challenges. One of the challenges is that targeted therapy is only effective in specific sub-set of patients. For instance, HER2 positive receptor antagonists are only effective in patients that have tumors with HER2 receptor overexpression, and PARP inhibitor is only effective in patients that have tumors with BRCA mutant (Tutt et al., 2010). Another challenge is the drug resistance that is found in most breast cancer patients in the advanced stages (Wong and Goodin, 2009). Also, targeted therapy agents are associated with systemic side effects. Besides the common side effects including mouth sores, rash, hair loss, fatigue, nausea, diarrhea and appetite loss, targeted therapy has severe adverse effects including infections, kidney failure, lung problems, heart problems, liver problems and blood clots.

1.3.5. **Hormonal therapy**

Hormonal therapy is another successful approach to prevent and/or treat breast cancer. Estrogen and progesterone are hormone receptors that play an important role in breast cancer. Thus, the hormonal therapy aims to regulate the level of estrogen and progesterone receptors in breast cancer cells. Hormonal therapy is used for hormone receptor positive breast cancers (estrogen receptor (ER)-positive and/or progesterone receptor (PR)-positive). The three main types of hormonal therapy are aromatase inhibitors (AIs), estrogen receptor down regulators (ERDs) and selective estrogen receptor modulators (SERMs).

Aromatase inhibitors (AIs) are used to stop the production of estrogen by blocking the enzyme aromatase which is the responsible for synthesizing estrogen in the
body. They mainly are used to treat breast cancer in postmenopausal women. The FDA-approved aromatase inhibitors are anastrozole, letrozole and exemestane.

Estrogen receptor down regulators (ERDs) are used to block the effects of estrogen in breast cells. Fulvestrant is an estrogen antagonist and has no estrogen agonist effects.

Selective estrogen receptor modulators (SERMs) are used to block the effects of estrogen by preventing estrogen from binding to the cell. Unlike ERDs, SERMs can serve as estrogen agonists beside its estrogen antagonist activity. SERMs block the action of estrogen in breast cells, but can activate the action of estrogen in other tissues including liver, bone and uterus. The FDA-approved SERMs include tamoxifen, raloxifene and toremifene. Tamoxifen (TAM) was approved by FDA in 1977 for the treatment of breast cancer (Osborne, 1998). TAM is a drug of choice for the treatment and prevention of estrogen receptor (ER)-positive breast cancer. It acts as an estrogen antagonist in breast tissue and used as a therapy for early stages of ER-positive breast cancer as well as a chemopreventive agent (Lewis and Jordan, 2005). TAM is considered as a prodrug and is metabolized to two active metabolites, 4-hydroxtamoxifen (4-OH-TAM) and 4-hydroxy-N-desmethyltamoxifen (endoxifen), by CYP2D6 and CYP3A4/5 (Jordan, 2007). Figure 4 illustrates the metabolic pathways for tamoxifen. It has been found that these two active metabolites (4-OH-TAM and endoxifen) have many times greater binding affinity for estrogen receptors than TAM (Lim et al., 2005, 2006). The change in the activity of cytochrome P450 isomers (CYP2D6 and CYP3A4/5) can affect the metabolic pathways of TAM, leading to variation in the plasma concentrations of TAM and its metabolites (4-
OH-TAM and endoxifen) in the body. As a result, the systemic exposure of TAM, 4-OH-TAM and endoxifen can vary widely from patient to patient (Desta et al., 2004).
Figure 4. Major metabolic pathways of tamoxifen.

Reproduced from Jordan (2007).
The most common short time side effects of SERMs include fatigue, hot flashes, headache, bone pain, nausea and vaginal discharge. There are also some possible serious side effects including the risk of developing uterine cancer, blood clots and strokes. In addition, cancer cells can develop resistance to SERMs (Clarke et al., 2003).

1.4. Chemoprevention of breast cancer

Chemoprevention is the use of drugs to prevent the development and progression of cancer. The best examples of chemopreventive agents that have been studied for the breast cancer are the aromatase inhibitors (example: exemestane and anastrozole) and SERMs (example: tamoxifen and raloxifene). The breast cancer chemopreventive agents approved by the FDA include tamoxifen and raloxifene. Tamoxifen has shown to significantly decrease the risk of breast cancer in premenopausal and postmenopausal women. For example, tamoxifen reduced the risk of breast cancer in women with LCIS by 46% and 56% in different studies (Fisher et al., 1998, 2005). Furthermore, raloxifene has shown a reduction in the risk of breast cancer in postmenopausal women. One study has shown that raloxifene reduced breast cancer risk in postmenopausal women and TAM showed similar amount of risk reduction of breast cancer in postmenopausal women (Vogel et al., 2006). In another study, raloxifene was not associated with an increased risk of uterine cancer and had lower risk of venous thromboembolic effects in comparison to tamoxifen (Vogel et al., 2010). In addition to SERMs, aromatase inhibitors (AIs) have also been reported to show chemoprevention activity against breast cancer. Exemestane, an AI, has shown to reduce breast cancer risk by 65% in postmenopausal women (Goss et al., 2011). Anastrozole, another AI, has been shown to reduce breast cancer risk in women at increased risk of breast cancer by 53% (Cuzick et al., 2014).
Also, investigations have focused on the use of other drugs including arzoxifene, NSAIDs and retinoids as chemopreventive agents (Rahme et al., 2005; Swede et al., 2005; Wu et al., 2002).

Although chemoprevention strategy should be considered for women with high risk of breast cancer, there are significant challenges associated with the chemoprevention strategy. These challenges include low adherence, low patient compliance and the side effects, especially the severe side effects that associated with SERMs. In addition, the major challenge of using chemopreventive agents is that these agents should be taken for a long period of time. For example, TAM is used for three months to five years.

1.5. Localized therapy

Localized delivery is an attractive approach to address the limitations of systemic therapy. In addition, given that majority of breast cancers originate from the duct and lobules in the breast, direct delivery of anti-cancer drugs to the breast can maximize the drug concentration within breast and reduce the systemic exposure. To this end, intraductal injection, transdermal drug delivery and transpapillary (through mammary papilla, nipple) drug delivery have been investigated to localize drug to the breast tissue.

1.5.1. Intraductal drug delivery

Intraductal drug delivery is a direct delivery of a drug through the ductal orifice via a catheter. This approach has been investigated in preclinical and clinical studies (Stearns et al., 2011; Love et al., 2012). As shown in the ductogram images of the breast (Figure 5) after intraductal injection, the dye uniformly distributes throughout the ductal
tree (Stearns et al., 2011). The intraductal delivery of anti-cancer agents has been investigated for the treatment and prevention of breast cancer (Table 4).
Figure 5. Ductogram images from two different women with breast cancer.

Reproduced from Stearns et al. (2011).
Table 4. Anti-cancer drugs tested for intraductal delivery

<table>
<thead>
<tr>
<th>Agent</th>
<th>Preclinical/clinical</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-hydroxytamoxifen (4-OHT)</td>
<td>Preclinical study</td>
<td>Murata et al., 2006</td>
</tr>
<tr>
<td>Pegylated liposomal doxorubicin (PLD)</td>
<td>Preclinical study</td>
<td>Murata et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Clinical studies</td>
<td>Stearns et al., 2011; Love et al., 2013; Mahoney et al., 2013</td>
</tr>
<tr>
<td>Polyethyleneglycol-doxorubicin (PEG-DOX)</td>
<td>Preclinical study</td>
<td>Gu et al., 2018</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Preclinical studies</td>
<td>Stearns et al., 2011; Okugawa et al., 2005</td>
</tr>
<tr>
<td>Methotrexate (MTX)</td>
<td>Preclinical study</td>
<td>Stearns et al., 2011</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>Preclinical study</td>
<td>Stearns et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Clinical study</td>
<td>Love et al., 2013</td>
</tr>
<tr>
<td>5-Fluorouracil (5-FU)</td>
<td>Preclinical study</td>
<td>Stearns et al., 2011</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Preclinical study</td>
<td>Chun et al., 2012</td>
</tr>
</tbody>
</table>
The intraductal administration of PLD and 4-OHT was effective in both the treatment and prevention of mammary tumors in animal (rat and mouse) models of breast cancer (Murata et al., 2006). The results from this study also demonstrated that the intraductal injection showed lower circulating level of the agents compared to the intravenous injection, with no evidence of systemic toxicity, resulting in direct access to the breast tumors with higher local drug concentration and lower systemic drug exposure (Murata et al., 2006). In another study, the effect of intraductal administration of different anticancer drugs including 5-FU, MTX, paclitaxel and carboplatin for the treatment of mammary tumors in a rat model has been investigated. In this study, the results showed that the intraductal administration of these agents showed lower tumors in mammary glands, with minimum side effects compared to the systemic administration or untreated animals (Stearns et al., 2011). Also, the intraductal administration of paclitaxel showed significant reduction in tumor burden and total number of mammary carcinomas, and there were no toxic side effects compared to the intraperitoneal injection group in the rat model (Okugawa et al., 2005). In another study, curcumin nanoparticles were administrated via intraductal injection in a rat model. The results from this study showed that the intraductal group showed a significant lower mammary tumor incidence compared to the control group or oral curcumin administrated group, suggesting the feasibility of combining nanoparticles encapsulated formulation of curcumin with the intraductal injection as a potential approach for breast cancer prevention (Chun et al., 2012). In a recent study, an intraductal administration of polyethylene glycol-doxorubicin (PEG-DOX) nanocarriers was investigated in a DCIS like rat model. In this study, the results showed that the intraductal injection of PEG-DOX was effective in reducing the
tumor and was less toxic compared to the free DOX group and the intravenous injection of free DOX or PEG-DOX groups (Gu et al., 2018). Moreover, it is important to understand the differences between the rats and human mammary gland anatomy. Rats have 6 pairs of mammary glands, and each mammary papilla (nipple) has one duct, while each human mammary papilla has 5 to 9 ducts (Love and Barsky, 2004).

In addition to the preclinical studies, the clinical studies of intraductal approach have been investigated. A phase 1 clinical trial (15-17 women scheduled for mastectomy) has been investigated to study the feasibility, safety and maximum tolerated dose of intraductal administration of pegylated liposomal doxorubicin (PLD). The results from this study showed that the intraducal injection showed lower systemic concentration of PLD compared to the intravenous injection, with less toxicity, suggesting that anti-breast cancer drugs can be delivered directly to the breast ducts in an outpatient setting (Stearns et al., 2011). Another phase 1 trial (30 women scheduled for mastectomy) demonstrated the feasibility, safety and pharmacokinetics properties of intraductal administration of two chemotherapy drugs (PLD and carboplatin) into multiple ducts within one breast in breast cancer patients. The results from this study showed that carboplatin was absorbed faster into the bloodstream than PLD and there was no systemic toxicity observed, demonstrating the influence of formulation on ductal retention of drugs (Love et al., 2013). Another clinical trial involved 13 women with Ductal Carcinoma In Situ (DCIS) treated before the surgery with intraductal PLD. The results demonstrated that the intraductal administration of PLD was feasible, safe and well tolerated, with no severe adverse effects, suggesting that the intraductal therapy is an attractive approach for the treatment and prevention of DCIS (Mahoney et al., 2013).
In spite of the promising preclinical and clinical studies of intraductal therapy, there are several challenges with intraductal administration. First challenge is that the intraductal administration is a difficult procedure that needs skilled expert to cannulate the duct for administration of the anti-breast cancer drugs. This procedure has to be done under anesthesia and a single duct has to be identified for drug administration. Secondly, the drug retention in the breast is limited and hence requires multiple injections, leading to a poor patient compliance. Overall, this approach is effective and safe, but its clinical application is limited by the need for multiple injections to sustain the drug level in the breast.

1.5.2. Localized transdermal drug delivery

Transdermal drug delivery is an effective method and offers many advantages including avoidance of first pass metabolism, providing sustained drug release for a long period of time and high patient compliance. However, the permeation of drugs is limited by the barrier posed by the skin (Figure 6).
Figure 6. Anatomy of human skin.

Reproduced from: https://www.azurlis.com/pages/skin-anatomy
Stratum corneum (SC), the outermost layer of the epidermis, provides the major barrier in the skin, limiting the penetration of drugs through skin (Blank, 1965; Barry, 1983; Scheuplein and Blank, 1971; Wiechers, 1989). As shown in Figure 7, the two major routes of drug penetration through stratum corneum are intercellular route (between the cells) and intracellular route (through the cell) (Barry, 2001). The physicochemical properties of the drug, such as aqueous solubility, lipophilicity (Log P), partition coefficient and molecular weight, play a crucial role in determining the feasibility of delivering drugs through the skin. The ideal physicochemical properties of a drug for skin permeation include molecular weight less than 500 Da, aqueous solubility more than 1 mg/ml and Log P in the range of 1-3 (Bos and Meinardi, 2000; Naik et al., 2000). As a result, the transdermal delivery is limited to drugs that have the above mentioned ideal physicochemical properties, and there is a need to utilize penetration enhancement methods to overcome this challenge. The major two methods for permeation enhancement are chemical methods and physical methods. The physical methods include electrical, mechanical, thermal and magnetics strategies. The physical approach examples include use of microneedles array, electric current, ultrasound, radiofrequency thermal ablation, lasers, pressure waves, thermophoresis, high velocity particles, ballistic liquid jet and magnetophoresis of diamagnetic solutes (Karande and Mitragotri, 2009). The physical enhancement approaches act mainly on the skin by disrupting the skin membrane to increase the transport of the drug through the skin. The second method for permeation enhancement is to use chemical permeation enhancers. The chemical permeation enhancer examples include water, alcohols, hydrocarbons, acids, amides, amines, esters, surfactants, terpenes and lipids.
Figure 7. Routes of drug penetration through stratum corneum.

Reproduced from Barry (2001).
Furthermore, mixtures of chemical enhancers can be used, and the examples of these mixtures include liposomes, ethosomes, niosomes, transfersomes and microemulsions. The chemical penetration enhancers can increase the permeation of the drug by acting directly on the skin or modifying the thermodynamic activity of the drug in the formulation.

Diffusion of the drug through breast skin or mammary papilla is considered to be a passive kinetic process where the drug diffuses from the area of high concentration to the area of low concentration (Lane, 2013). Steady state diffusion can be described by Fick’s law as shown below:

\[
J = \frac{DKC_v}{h}
\]

Where \( J \) = the flux,

\( D \) = the diffusion coefficient,

\( K \) = the partition coefficient,

\( C_v \) = the concentration gradient through membrane,

\( h \) = the thickness of the membrane.
To expand the number of drugs delivered through skin, there is a need to utilize penetration enhancement methods. Based on the previous equation, the increase in the drug flux can be achieved by changing $D$, $K$ or $C_p$, or by changing $h$ (Lane, 2013). The use of chemical enhancers including hydroalcoholic vehicles (alcohol and water) and terpenes can increase the drug permeation by altering the diffusion coefficient ($D$) and the partition coefficient ($K$). Moreover, the use of microneedles can increase the drug permeation through breast skin by altering the thickness of skin layers ($h$).

In addition to the localized transdermal drug delivery, transpapillary (through mammary papilla, nipple) drug delivery has been studied to localize drug within breast tissue. The localized transdermal delivery of anti-cancer drugs to the breast skin has been investigated. The direct transdermal delivery of tamoxifen’s metabolites (such as 4-OHT and endoxifen) to the breast has been investigated (Mansel et al., 2007; Lee et al., 2014; Yang et al., 2014; Lee et al., 2011). For instance, a phase II clinical trial demonstrated that the topical gel of 4-OHT was effective in the treatment of cyclical mastalgia with high safety and tolerability (Mansel et al., 2007). Also, in another phase II clinical trial, the same topical gel of 4-OHT has been investigated for the treatment of women diagnosed with ductal carcinoma in situ of the breast (Güngör, et al., 2013). In another clinical study on postmenopausal women with an invasive estrogen receptor positive breast cancer, direct topical delivery to the breast of 4-OHT gel (three doses: 0.5, 1 or 2 mg/day) was compared to oral tamoxifen (20 mg/day). The results showed that the topical gel of 4-OHT resulted in the same reductions in tumor proliferation as the oral tamoxifen and the 4-OHT systemic concentrations were significantly lower in the gel groups compared to oral tamoxifen group (Rouanet et al., 2005). Similar results were
found when 6 to 10 weeks of treatment with 4 mg/day of 4-OHT gel was compared to oral tamoxifen (20 mg/day) in pre- and postmenopausal women with DCIS (Lee et al., 2014).

Endoxifen, another metabolite of tamoxifen, has 100-fold greater binding affinity for estrogen receptors (ERs) than tamoxifen (Lim et al., 2005, 2006). Endoxifen has been shown to be an active agent for the treatment of breast cancer, and the oral administration of endoxifen hydrochloride has been investigated in clinical trials (Ahmad et al., 2010A, 2010B; Wu et al., 2009). Given the potential activity of oral endoxifen against breast cancer, the direct transdermal delivery of endoxifen to the breast has also been assessed (Lee et al., 2011; Mah et al., 2013). In an in vitro human skin permeation study, the permeation of endoxifen was investigated and oleic oil was tested as a permeation enhancer. The results showed that endoxifen showed higher permeation and retention in tissue and the addition of oleic acid enhanced the permeation of endoxifen through the human skin, suggesting that endoxifen can be an effective agent for local transdermal delivery to the breast (Lee et al., 2011). In another study, the permeation of endoxifen encapsulated in polymeric micelles was tested in hairless mouse skin and human skin. The results from that study demonstrated that the encapsulated endoxifen showed higher permeation through the skin, supporting that endoxifen can be localized and delivered transdermally to the breast (Yang et al., 2014).

In addition, the transdermal delivery of hydrophilic and hydrophobic molecules to the breast through the breast skin and mammary papilla has been demonstrated by our group and others (Dave et al., 2014, 2016, 2017; Alsharif et al., 2017; Lee et al., 2014; Lee et al., 2010; Pujol et al., 1995).
Taken together, it is reasonable to hypothesize that the direct transdermal delivery of chemopreventive agents to the breast through breast skin and/or through mammary papilla can be an effective approach for localized therapy of breast cancer.

1.6. Scope and goals of the present study

Breast cancer is the second leading cause of death due to cancer in women. More than 95% of breast cancers originate from the epithelium of mammary ducts and lobules. Currently used anti-cancer drugs are associated with severe side effects including some life-threatening adverse effects. To this end, localized delivery of anti-cancer agents can be very effective in maximize the drug concentration in breast and minimizing systemic drug exposure.

Also, the localized approach can be combined with other treatment approaches (radiation or surgery) to improve the clinical outcomes. Intraductal injection has been investigated for the prevention and the treatment of breast cancer. However, this approach is an invasive process and requires professional expertise. To this end, transdermal delivery of chemopreventive agents to the breast is an attractive alternative non-invasive approach for the prevention and treatment of breast cancer.

Given the barrier nature of skin, it is imperative to explore the use of penetration enhancement methods to expand the number of drugs delivered through this route. Furthermore, it is also important to compare the relative permeability of drugs through the breast skin and mammary papilla to develop a combinatorial approach to achieve effective drug concentrations in the breast.
Taken together, the main objective of this dissertation is to explore the feasibility of transdermal delivery of promising chemopreventive agents belonging to different therapeutic classes and physicochemical properties through breast skin and/or through mammary papilla. The focus of this study is to investigate the influence of different permeation enhancement methods including hydroalcoholic vehicles, chemical penetration enhancers, vesicular carriers and microneedles for localized transdermal drug delivery to the breast.

The specific goals of the study are as follows:

1- Investigate the feasibility of transdermal delivery of aspirin to the breast through the breast skin and through the mammary papilla using different penetration enhancement methods.

2- Investigate the feasibility of transdermal delivery of endoxifen to the breast through the breast skin and through the mammary papilla using different penetration enhancement methods.

3- Investigate the feasibility of transdermal delivery of fenretinide to the breast through the breast skin and through the mammary papilla using different penetration enhancement methods.

The outcome from this study will help guide the development of effective transdermal delivery approaches for the prevention and treatment of breast cancer.
CHAPTER TWO: TRANSDERMAL DELIVERY OF ASPIRIN TO THE BREAST
2.1. Introduction

Aspirin, a non-steroidal anti-inflammatory drug (NSAID), is the most commonly used analgesic, antipyretic and anti-inflammatory drug. Aspirin is also used in the prevention of heart diseases. In particular, aspirin has shown anti-cancer activity in human breast cancer cells (Dachineni et al., 2016). It has also shown to have chemopreventive activity against breast cancer (Rothwell et al., 2011; Holmes et al., 2010). The regular use of aspirin has shown to decrease the risk of breast cancer deaths (Holmes et al., 2010). Low dose of aspirin for women diagnosed with breast cancer was associated with increased patient survival (Fraser et al., 2014). However, the systemic delivery of aspirin for long term treatment is limited by the cardiovascular side effects and severe gastrointestinal side effects. Therefore, the transdermal delivery of aspirin is an attractive alternative approach.

Transdermal drug delivery is an attractive approach to overcome some of the limitations of oral drug delivery. Transdermal drug delivery can also avoid the first pass metabolism and increase patient compliance. The transdermal delivery of aspirin has been evaluated earlier (Levang et al., 1999; Ammar et al., 2006; Zuo et al., 2014). However, the focus of this study is localized transdermal delivery of aspirin to the breast through the breast skin and mammary papilla. Aspirin (Figure 8) has a Log P of 1.23, which is suitable for transdermal delivery approach (Lien and Gaot, 1995). The goal for localized transdermal delivery of aspirin to the breast is to maximize drug concentration in the breast and reduce systemic drug levels.
Figure 8. Chemical structure of aspirin.
The specific aims of this study are as follows:

i. Investigate the influence of hydroalcoholic vehicles on aspirin permeation through breast skin and mammary papilla.

ii. Investigate the effect of chemical penetration enhancers on aspirin permeation through breast skin and mammary papilla.

iii. Investigate the influence of vesicular systems on aspirin permeation through breast skin and mammary papilla.

iv. Investigate the effect of microneedles on aspirin permeation through breast skin.
2.2. Materials and Methods

2.2.1. Materials

Aspirin was purchased from Sigma-Aldrich, St. Louis, MO, USA. Phosphoric acid and HPLC grade acetonitrile were obtained from Fisher Scientific, Hampton, NH, USA. Sandalwood oil was obtained from Organic Infusions, Camarillo, CA, USA. Eugenol and (R)-(+)−Limonene were purchased from Sigma-Aldrich, St. Louis, MO, USA. Phospholipid (phospholipon® 90G) was obtained from Lipoid LLC, Newark, NJ, USA. Adminpatch stainless steel microneedle arrays (AdminPatch® 0900 microneedle array) were purchased from AdminMed nanoBioSciences LLC, Sunnyvale, CA, USA.

2.2.2. Preparation of the breast skin and mammary papilla

Female porcine breast tissues were obtained from a local slaughterhouse. The underlying fat tissue was removed using a scalpel and the hair on the skin surface was removed using a hair clipper. The breast skin surrounding the nipple was collected and dermatomed to 760 μm thickness using an Electric Dermatome Model B (Padgett Instruments®, Integra Lifescinces Corporation, Plainsboro, NJ, USA). The breast skin and mammary papilla were examined for any visible damage. The keratin plug on the surface of the nipple was removed using 70% alcohol. The tissues were stored at -20 °C and used within three months.

2.2.3. Preparation of hydroalcoholic vehicles and chemical penetration enhancers

In this study, different hydroalcoholic vehicles including 33, 50 and 66% (v/v) were tested. For the sub-saturated concentration studies, 10 mg/ml was used for all
hydroalcoholic vehicles. For the saturated concentration studies, 30, 80 and 170 mg/ml were used for 33, 50 and 66% hydroalcoholic vehicles, respectively.

For the chemical penetration enhancers studies, sandalwood oil, eugenol and limonene were used and aspirin was co-treated with 5% (v/v) of the terpenes in 50% (v/v) hydroalcoholic vehicle. Aspirin concentration of 10 mg/ml was used for all chemical penetration enhancers studies.

2.2.4. Preparation and characterization of vesicular carriers

For the preparation of the vesicular carrier systems, aspirin liposomes and ethosomes were prepared using thin film method (Fang et al., 2008). Briefly, phospholipon® 90G (20 mg/ml) and aspirin (10 mg/ml) were dissolved in chloroform in a dry round bottom flask. Then, the flask was placed in a rotary vacuum evaporator at 55 °C (above the phospholipon® 90G transition temperature) and kept under vacuum overnight to remove the residual chloroform. The resulting dry lipid film was hydrated with distilled water. After hydration, the liposomes were vortexed and then sonicated using bath sonicator at 55 °C for 10-15 minutes. The final preparation was extruded six times through 100 nm polycarbonate membrane at 55 °C using Avanti mini extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA). In case of aspirin ethosomes, phospholipon® 90G (20 mg/ml) was dissolved in chloroform in a dry round bottom flask. The flask then was placed in a rotary vacuum evaporator at 55 °C and kept under vacuum overnight to remove the residual of chloroform. The resulting thin film was hydrated with 33% (v/v) hydroalcoholic solution containing aspirin (10 mg/ml). Then, ethosomes were vortexed followed by sonication using bath sonicator at 55 °C for 10-15 minutes. The final preparation was extruded six times through 100 nm polycarbonate membrane at 55
⁰C using Avanti mini extruder. At the end of the preparation of the liposomes and ethosomes, the particle size, polydispersity index (PDI) and zeta potential were determined by dynamic light scattering method using Malvern Zetasizer Nano ZS (Malvern Instruments, Inc., Westborough, MA, USA). To measure the encapsulation efficiency (EE%) and the loading efficiency (LE%), the obtained carriers were frozen and lyophilized in a freeze drying system using lyophilizer (SP Scientific, Stone Ridge, NY, USA) for 24 hours. After weighing the frozen carriers and after appropriate dilutions, EE% and LE% were measured using HPLC. The EE% and LE% were calculated using the following equations:

\[
EE\% = \frac{\text{Weight of aspirin in carriers}}{\text{Weight of aspirin initially added}} \times 100
\]

\[
LE\% = \frac{\text{Weight of aspirin in carriers}}{\text{Weight of carriers}} \times 100
\]

2.2.5. Microneedles assisted transdermal delivery

Adminpatch stainless steel microneedle arrays were applied onto breast skin to study the effect of microneedles on aspirin permeation through breast skin. This microneedle array has 85 microneedles located within 1 cm² circular area, and each microneedle is 800 μm long. The diameter of the entire device is 20 mm, and it is made of medical grade SS316L stainless steel (NanoBioSciences LLC, Sunnyvale, CA, USA). The microneedle arrays used in this study is shown in Figure 9.
Figure 9. Stainless steel microneedle arrays.

Reproduced from: http://adminmed.com/array0900
2.2.6. Solubility studies

The solubility studies of aspirin were carried out in different hydroalcoholic vehicles including 33, 50 and 66% alcohol. Briefly, an excess amount of aspirin was added to 1ml of the hydroalcoholic vehicle and kept in a shaker at 100 rpm at 37 °C for 24 hours. The samples were kept at 37 °C with no shaking for further 24 hours in order to allow the phase separation to occur. At the end of the total 48 hours, the samples were filtered through a 0.2 μm membrane filter (Millex® GN Syringe Filter, MilliporeSigma, Burlington, MA, USA). After appropriate dilutions, the drug concentration was determined using HPLC.

2.2.7. Partition coefficient studies

The epidermal partition coefficient (epidermis/vehicle partition coefficient) studies of aspirin were carried out in different hydroalcoholic vehicles including 33, 50 and 66% alcohol. The epidermal membranes were separated from the breast skin using the heat-separation method (Kligman and Christophers, 1963). Briefly, full fresh thickness porcine breast skin was placed in a water bath at 60 °C for 2 min. Then, the epidermis was carefully separated from the dermis using forceps and washed with phosphate buffered saline (PBS, pH 7.4). The epidermis sheet was dried using Kim wipe and then stored in a desiccator at -20 °C until use. Prior to the partition coefficient studies, the epidermal sheet was hydrated by floating on a solution of PBS for 6 hours. Then, the hydrated epidermal sheet was placed in a glass vial containing 1mg of the drug per 1ml of the vehicle (33, 50 and 66% alcohol) and equilibrated in a shaker water bath at 37 °C for 24 hours. At the end of 24 hours, the epidermal sheet was gently blotted dry using Kim wipe to remove the excess drug solutions from the epidermis, digested and
kept in a shaker water bath at 37 °C for overnight. The concentration of drug in the epidermal sheet was determined using HPLC. The concentration of drug remaining in the vehicle after partitioning was also determined using HPLC. The epidermal partition coefficient \((K)\) value was calculated using the following equation:

\[
K = \frac{\text{Drug concentration in epidermis}}{\text{Drug concentration in vehicle}}
\]

2.2.8. In vitro permeation studies

The penetration studies for both breast skin and mammary papilla were conducted in a vertical in-vitro Franz diffusion cell (PermeGear, Inc., Hellertown, PA, USA). The breast skin or mammary papilla was sandwiched between receptor and donor compartments with the stratum corneum facing towards the donor compartment (Figure 10). The available surface area for permeation was 0.64 cm\(^2\). The receptor compartment (5 ml) was filled with phosphate buffered saline (PBS, pH 7.4). The receptor compartment was stirred using a magnetic bar at 100 rpm and maintained at 37 °C. The donor compartment was filled with 500 μl of the formulation that contained aspirin (saturated and sub-saturated). In the case of microneedles studies, the permeation area (0.64 cm\(^2\)) of the breast skin was pretreated with microneedle arrays before applying the aspirin formulation. The donor compartment and the sampling port were occluded with Parafilm in order to prevent the evaporation of the solution. The Franz diffusion cells were covered with aluminum foil. At specified intervals (6, 12, 24, 30, 36 and 48 hours), 200 μl samples were withdrawn from the receptor compartment and same amount of the fresh receptor medium system was added in order to maintain a constant volume. All the
experiments were run for 48 hours. At the end of the study, the breast skin and mammary papilla were digested to determine the drug concentration in the tissue. The drug concentration in all experiments was determined using HPLC.
Figure 10. In-vitro Franz diffusion cells system.
2.2.9. Analytical method

Aspirin concentration was determined by HPLC (Waters Corporation, Milford, MA, USA). The chromatographic resolution of aspirin was done using a C\textsubscript{18} reverse phase column (5 μm, 4.6 × 150 mm, Waters Corporation, Milford, MA, USA) attached with a guard column (3.9 × 20 mm, Waters). The mobile phase was a mixture of water: acetonitrile: phosphoric acid (75: 25: 0.1, v/v). The mobile phase was filtered through a 0.2 μm membrane filter (Whatman® membrane filters, GE Healthcare Life Sciences, Pittsburgh, PA, USA). The flow rate was 1 ml/min and the detection wavelength was 234 nm. The injection volume was 50 μl and the standard drug solutions were prepared in 50% (v/v) ethanol. The concentration range used for the calibration curve was 0.1-10 μg/ml (R\textsuperscript{2} = 0.9998).

2.2.10. Data analysis

The cumulative amount of aspirin permeated through breast skin (through 0.64 cm\textsuperscript{2}) was plotted as a function of time. The flux was determined from steady-state portion of the curve (the slope). The lag time was obtained by extrapolating the liner portion to x-axis. The correlation coefficient of linear portion of the curve was > 0.99. Statistical analysis was carried out using Minitab 18 Statistical Software. One-way ANOVA was used to determine the difference between the treatment groups. The results were expressed as mean ± standard deviation (SD). The results were considered to be significant at p-value < 0.05.
2.3. Results

2.3.1. Influence of hydroalcoholic vehicles on aspirin permeation

2.3.1.1. Breast skin

The drug permeation was tested using sub-saturated and saturated aspirin concentration. In the permeation studies of aspirin through breast skin using different hydroalcoholic vehicles (sub-saturated concentration), 33% alcohol vehicle showed the highest aspirin permeation through the breast skin (Figure 11 A). Among the hydroalcoholic vehicles tested, 33% hydroalcoholic vehicle showed the highest flux (7.14 μg/cm²/h) and 66% hydroalcoholic vehicle showed the lowest flux (Table 5). Also, the longest lag time (4.31 hours) was observed with 66% hydroalcoholic vehicle (Table 5). The highest skin retention of aspirin was observed with 33% alcohol (Figure 11 B). In addition, the saturated concentrations of aspirin permeation studies were tested. The 33% hydroalcoholic vehicle showed the highest aspirin permeation through the breast skin in comparison to 50 and 66% hydroalcoholic vehicles (Figure 12 A). The highest flux of aspirin (12.55 μg/cm²/h) was observed with 33% hydroalcoholic vehicle (Table 6). The highest skin retention of aspirin was observed with 66% alcohol (Figure 12 B).

The solubility studies (Figure 13; Table 7) showed that increasing concentration of alcohol in the vehicle increased the solubility of aspirin. Compared to 33% alcohol, 50% alcohol increased the solubility of aspirin by 2.5-fold, and 66% alcohol increased the solubility of aspirin by 5.5-fold. Also, 66% alcohol increased the aspirin solubility by 2-fold in comparison to 50% alcohol. The results indicate that the solubility of aspirin is influenced by the alcohol concentration in the vehicle.
The epidermal partition coefficient studies (Figure 13; Table 7) showed that increasing the concentration of alcohol in the vehicle resulted in decrease the epidermal partition coefficient of aspirin. Compared to 33% alcohol, 50% alcohol decreased the partition coefficient of aspirin by 1.5-fold, and 66% alcohol decreased the partition coefficient of aspirin by 2.4-fold. The partition coefficient studies results indicate that the epidermal partition coefficient of aspirin is influenced by the alcohol concentration in the vehicle.

Taken together, the results show that aspirin solubility and epidermal/vehicle partition coefficient were inversely correlated, i.e. an increase in aspirin solubility decreased the epidermal/vehicle partition coefficient and vice-versa.
Figure 11. Permeation of aspirin through breast skin using different hydroalcoholic vehicles (sub-saturated concentration).

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin permeated through breast skin. ‘a’ indicates a significant difference in comparison to 66% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. (B) Retained amount of aspirin in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 66% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol.
Table 5. In vitro permeation parameters of aspirin permeated through breast skin using different hydroalcoholic vehicles (sub-saturated concentration)

<table>
<thead>
<tr>
<th>Vehicle (% of alcohol)</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours(μg/cm²)</th>
<th>Retained amount at 48 hours(μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>2.27±0.56a</td>
<td>7.14±0.91a,b</td>
<td>323.85±29.95</td>
<td>106.14±20.18</td>
<td>0.33</td>
</tr>
<tr>
<td>50</td>
<td>3.61±0.84</td>
<td>4.70±0.33a</td>
<td>230.33±15.49</td>
<td>86.32±15.58</td>
<td>0.37</td>
</tr>
<tr>
<td>66</td>
<td>4.31±0.66</td>
<td>2.83±0.75</td>
<td>140.40±38.83</td>
<td>67.91±7.33</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 10 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 66% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. The values are significant at p-value < 0.05.
Figure 12. Permeation of aspirin through breast skin using different hydroalcoholic vehicles (saturated concentration).

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin (asa) permeated through breast skin. ‘a’ indicates a significant difference in comparison to 66% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. (B) Retained amount of aspirin in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol.
Table 6. In vitro permeation parameters of aspirin permeated through breast skin using different hydroalcoholic vehicles (saturated concentration)

<table>
<thead>
<tr>
<th>Vehicle (% of alcohol)</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>3.62±0.31a</td>
<td>12.55±0.19ab</td>
<td>602.66±12.51</td>
<td>335.11±36.57</td>
<td>0.56</td>
</tr>
<tr>
<td>50</td>
<td>3.68±0.26a</td>
<td>7.02±0.17a</td>
<td>338.67±9.81</td>
<td>424.54±36.77</td>
<td>1.25</td>
</tr>
<tr>
<td>66</td>
<td>2.79±0.20a</td>
<td>4.93±0.05</td>
<td>255.77±2.10</td>
<td>558.21±34.22</td>
<td>2.18</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 30, 80 and 170 mg/ml were used for 33, 50 and 66% hydroalcoholic vehicles, respectively. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 66% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. The values are significant at p-value < 0.05.
Figure 13. Correlation between saturated solubility and epidermal partition coefficient of aspirin as a function of different alcohol concentrations.

The data is presented as mean ± SD (n=3).
Table 7. Saturated solubility and epidermal partition coefficient of aspirin in presence of different chemical penetration enhancers

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Solubility (mg/ml)</th>
<th>Epidermal partition coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% alcohol</td>
<td>27.65±0.05</td>
<td>0.173±0.003</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>68.72±0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.113±0.005&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>66% alcohol</td>
<td>153.11±0.58&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.073±0.003&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% sandalwood oil + 50% alcohol</td>
<td>64.56±0.62&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>0.123±0.003&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

5% sandalwood oil was co-treated with 50% alcohol. Values are presented as mean ± SD (n=3). ‘a’ indicates a significant difference in comparison to 33% alcohol, ‘b’ indicates a significant difference in comparison to 50% alcohol and ‘c’ indicates a significant difference in comparison to 66% alcohol. The values are significant at p-value < 0.05.
2.3.1.2. Mammary papilla

In permeation studies of aspirin (sub-saturated concentration) through mammary papilla using different hydroalcoholic vehicles, 66% alcohol vehicle showed the highest aspirin permeation (Figure 14 A). Among all the hydroalcoholic vehicles tested, 66% hydroalcoholic vehicle showed the highest flux (0.69 μg/cm²/h) (Table 8). The longest lag time (6.51 hours) was observed with 50% hydroalcoholic vehicle (Table 8). The highest tissue retention of aspirin was observed with 66% alcohol (Figure 14 B). In addition, the saturated concentration of aspirin permeation studies was tested. The 66% hydroalcoholic vehicle showed the highest aspirin permeation through the mammary papilla in comparison to 33 and 50% hydroalcoholic vehicles (Figure 15 A). Similar to the results from sub-saturated concentration, highest flux of aspirin (2.20 μg/cm²/h) was observed with 66% hydroalcoholic vehicle (Table 9). Similarly, highest tissue retention of aspirin was also observed with 66% alcohol (Figure 15 B).
Figure 14. Permeation of aspirin through mammary papilla using different hydroalcoholic vehicles (sub-saturated concentration).

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin permeated through mammary papilla. ‘a’ indicates a significant difference in comparison to 50% alcohol and ‘b’ indicates a significant difference in comparison to 33% alcohol. (B) Retained amount of aspirin in nipple (μg/g). ‘a’ indicates a significant difference in comparison to 50% alcohol and ‘b’ indicates a significant difference in comparison to 33% alcohol.
Table 8. In vitro permeation parameters of aspirin permeated through mammary papilla using different hydroalcoholic vehicles (sub-saturated concentration)

<table>
<thead>
<tr>
<th>Vehicle (% of alcohol)</th>
<th>Lag time (h)</th>
<th>Flux (µg/cm²/h)</th>
<th>Cumulative amount at 48 hours (µg/cm²)</th>
<th>Retained amount at 48 hours (µg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>5.66±0.14</td>
<td>0.49±0.01</td>
<td>23.16±0.65</td>
<td>790.37±39.60</td>
<td>34.13</td>
</tr>
<tr>
<td>50</td>
<td>6.51±0.53</td>
<td>0.37±0.02</td>
<td>20.12±0.69</td>
<td>661.16±20.12</td>
<td>32.86</td>
</tr>
<tr>
<td>66</td>
<td>6.48±0.14</td>
<td>0.69±0.01</td>
<td>35.28±0.23</td>
<td>899.37±22.57</td>
<td>25.49</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the mammary papilla at 48 hours divided by the cumulative amount at 48 hours. 10 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. The values are significant at p-value < 0.05.
Figure 15. Permeation of aspirin through mammary papilla using different hydroalcoholic vehicles (saturated concentration).

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin (asa) permeated through mammary papilla. ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. (B) Retained amount of aspirin in nipple (μg/g). ‘a’ indicates a significant difference in comparison to 33% alcohol.
Table 9. In vitro permeation parameters of aspirin permeated through mammary papilla using different hydroalcoholic vehicles (saturated concentration)

<table>
<thead>
<tr>
<th>Vehicle (% of alcohol)</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>3.33±0.38</td>
<td>1.75±0.03</td>
<td>79.50±1.99</td>
<td>925.14±42.58</td>
<td>11.64</td>
</tr>
<tr>
<td>50</td>
<td>2.02±0.41</td>
<td>1.81±0.02</td>
<td>83.22±2.25</td>
<td>811.18±17.05</td>
<td>9.75</td>
</tr>
<tr>
<td>66</td>
<td>1.59±0.40</td>
<td>2.20±0.06</td>
<td>104.30±2.64</td>
<td>1078.16±37.89</td>
<td>10.34</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the mammary papilla at 48 hours divided by the cumulative amount at 48 hours. 30, 80 and 170 mg/ml were used for 33, 50 and 66% hydroalcoholic vehicles, respectively. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. The values are significant at p-value < 0.05.
2.3.2 Influence of chemical penetration enhancers on aspirin permeation

2.3.2.1. Breast skin

To further enhance the skin permeation of aspirin, terpenes (sandalwood oil, limonene and eugenol) were tested. Sub-saturated concentration (10 mg/ml) was used in this study. The results showed that all terpenes in 50% alcohol significantly enhanced the cumulative amount of aspirin permeated through the breast skin (Figure 16 A). Sandalwood oil showed the greatest influence on aspirin permeation through skin followed by eugenol and limonene. Compared to 50% hydroalcoholic vehicle, sandalwood oil, eugenol and limonene increased aspirin flux by 3.8-fold, 2.7-fold and 1.6-fold, respectively (Table 10). Furthermore, sandalwood oil and limonene significantly reduced the lag time, while there was no significant difference in lag time between eugenol and 50% hydroalcoholic vehicle (Table 10). The highest skin retention of aspirin was observed with eugenol, while sandalwood oil showed the lowest skin retention (Figure 16 B).
Figure 16. Permeation of aspirin through breast skin using different chemical penetration enhancers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin permeated through breast skin. ‘a’ indicates a significant difference in comparison to 50% alcohol, ‘b’ indicates a significant difference in comparison to 5% limonene and ‘c’ indicates a significant difference in comparison to 5% eugenol. (B) Retained amount of aspirin in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 50% alcohol, ‘b’ indicates a significant difference in comparison to 5% sandalwood oil and ‘c’ indicates a significant difference in comparison to 5% limonene.
Table 10. In vitro permeation parameters of aspirin permeated through breast skin using different chemical penetration enhancers

<table>
<thead>
<tr>
<th>Penetration enhancer</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% alcohol</td>
<td>3.61±0.84</td>
<td>4.70±0.33</td>
<td>230.33±15.49</td>
<td>86.32±15.58</td>
<td>0.37</td>
</tr>
<tr>
<td>Sandalwood oil</td>
<td>1.32±0.39a,c</td>
<td>17.94±0.28a,b,c</td>
<td>881.67±19.33</td>
<td>63.30±6.71</td>
<td>0.07</td>
</tr>
<tr>
<td>Limonene</td>
<td>1.62±0.17a</td>
<td>7.29±0.20a</td>
<td>345.70±13.63</td>
<td>95.61±4.54</td>
<td>0.28</td>
</tr>
<tr>
<td>Eugenol</td>
<td>2.95±0.25b</td>
<td>12.56±0.07a,b</td>
<td>603.42±7.11</td>
<td>128.62±4.92</td>
<td>0.21</td>
</tr>
</tbody>
</table>

5% of terpenes were used. Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 10 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 50% alcohol, ‘b’ indicates a significant difference in comparison to limonene and ‘c’ indicates a significant difference in comparison to eugenol. The values are significant at p-value < 0.05.
2.3.2.2. Mammary papilla

All the three terpenes in 50% alcohol increased the permeation of aspirin in comparison to 50% alcohol alone (Figure 17 A). Similar to breast skin, sandalwood oil had the greatest influence on aspirin permeation through mammary papilla followed by eugenol and limonene. Compared to 50% hydroalcoholic vehicle, sandalwood oil, eugenol and limonene increased aspirin flux by 4-fold, 3.6-fold and 2-fold, respectively (Table 11). Moreover, sandalwood oil, eugenol and limonene significantly reduced the lag time in comparison to 50% hydroalcoholic vehicle (Table 11). The highest mammary papilla retention of aspirin was observed with sandalwood oil, while limonene showed the lowest retention amount of aspirin in mammary papilla (Figure 17 B).
Figure 17. Permeation of aspirin through mammary papilla using different chemical penetration enhancers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin (asa) permeated through mammary papilla. ‘a’ indicates a significant difference in comparison to 50% alcohol and ‘b’ indicates a significant difference in comparison to 5% limonene. (B) Retained amount of aspirin in nipple (μg/g). ‘a’ indicates a significant difference in comparison to 5% eugenol, ‘b’ indicates a significant difference in comparison to 5% limonene and ‘c’ indicates a significant difference in comparison to 50% alcohol.
Table 11. In vitro permeation parameters of aspirin permeated through mammary papilla using different chemical penetration enhancers

<table>
<thead>
<tr>
<th>Penetration enhancer</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% alcohol</td>
<td>6.51±0.53</td>
<td>0.37±0.02</td>
<td>20.12±0.69</td>
<td>661.16±20.12</td>
<td>32.86</td>
</tr>
<tr>
<td>Sandalwood oil</td>
<td>1.09±0.42&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.46±0.12&lt;sup&gt;a,h&lt;/sup&gt;</td>
<td>64.85±3.53</td>
<td>765.79±21.62</td>
<td>11.81</td>
</tr>
<tr>
<td>Limonene</td>
<td>3.55±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.84±1.52</td>
<td>458.58±38.71</td>
<td>11.81</td>
</tr>
<tr>
<td>Eugenol</td>
<td>3.52±0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.35±0.02&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>65.77±0.80</td>
<td>598.62±29.33</td>
<td>9.10</td>
</tr>
</tbody>
</table>

5% of terpenes were co-treated with 50% alcohol. Tissue affinity ratio was calculated as the retained amount in the mammary papilla at 48 hours divided by the cumulative amount at 48 hours. 10 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 50% alcohol, ‘b’ indicates a significant difference in comparison to limonene and ‘c’ indicates a significant difference in comparison to eugenol. The values are significant at p-value < 0.05.
2.3.3. Influence of vesicular carriers on aspirin permeation

2.3.3.1. Breast skin

The characteristic of aspirin loaded ethosomes and liposomes are shown in Table 12. The aspirin-ethosomes and aspirin-liposomes showed 66% and 84% of encapsulation efficiency, respectively, and the particle size of liposomes (294 nm) was higher than ethosomes (194 nm) (Table 12). The ethosomes system showed higher aspirin permeation through the breast skin in comparison to liposomes, while there was no significant difference in aspirin permeation through breast skin between ethosomes and 33% hydroalcoholic vehicle (Figure 18 A). Compared to 33% hydroalcoholic vehicle, liposomes reduced the flux of aspirin by 6-fold and ethosomes showed no significant difference in the flux of aspirin (Table 13). Also, there was no significant difference in lag time among all three formulations (Table 13). The liposomes showed the highest skin retention of aspirin and ethosomes showed no significant difference in the retention amount of aspirin in breast skin in comparison to 33% hydroalcoholic vehicle (Figure 18 B).
Table 12. Characteristic of aspirin ethosomes and liposomes

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>EE (%)</th>
<th>LE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank ethosomes</td>
<td>56.71±0.90</td>
<td>0.21±0.01</td>
<td>-17.16±1.70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aspirin-ethosomes</td>
<td>194.53±25.6</td>
<td>0.40±0.04</td>
<td>13.75±0.21</td>
<td>66.35±0.37</td>
<td>41.16±0.23</td>
</tr>
<tr>
<td>Blank liposomes</td>
<td>89.64±0.90</td>
<td>0.29±0.01</td>
<td>-15.26±2.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aspirin-liposomes</td>
<td>294.43±2.15</td>
<td>0.35±0.16</td>
<td>12.90±0.10</td>
<td>84.24±0.99</td>
<td>38.93±0.46</td>
</tr>
</tbody>
</table>

PDI: Polydispersity Index; EE%: Encapsulation efficiency in percent; LE%: Loading efficiency in percent. Values are presented as mean ± SD (n=3).
Figure 18. Permeation of aspirin through breast skin using vesicular carriers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin (ASA) permeated through breast skin. ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes. (B) Retained amount of aspirin in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes.
Table 13. In vitro permeation parameters of aspirin permeated through breast skin using vesicular carriers

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Lag time (h)</th>
<th>Flux (µg/cm²/h)</th>
<th>Cumulative amount at 48 hours (µg/cm²)</th>
<th>Retained amount at 48 hours (µg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% alcohol</td>
<td>2.27±0.56</td>
<td>7.14±0.91</td>
<td>323.85±29.95</td>
<td>106.14±20.18</td>
<td>0.33</td>
</tr>
<tr>
<td>Ethosomes</td>
<td>3.61±0.68</td>
<td>6.53±0.63</td>
<td>308.86±31.84</td>
<td>154.85±18.52</td>
<td>0.50</td>
</tr>
<tr>
<td>Liposomes</td>
<td>2.52±0.90</td>
<td>1.14±0.13&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>55.09±8.02</td>
<td>261.11±30.90</td>
<td>4.74</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 10 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes. The values are significant at p-value < 0.05.
2.3.3.2. Mammary papilla

The liposomes system showed highest aspirin permeation in comparison to the ethosomes and 33% alcohol vehicle (Figure 19 A). Unlike in breast skin, liposomes increased the flux of aspirin by 1.3-fold and ethosomes decreased the flux of aspirin by 1.5-fold (Table 14). Also, liposomes system increased the lag time by 1.3-fold in comparison to 33% hydroalcoholic vehicle while there was no significant difference in lag time between ethosomes and liposomes (Table 14). The ethosomes system showed higher retention amount of aspirin in mammary papilla in comparison to liposomes system and 33% hydroalcoholic vehicle system, while there was no significant difference between liposomes and 33% hydroalcoholic vehicle (Figure 19 B).
Figure 19. Permeation of aspirin through mammary papilla using vesicular carriers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin (asa) permeated through mammary papilla. ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes. (B) Retained amount of aspirin in nipple (μg/g). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes.
Table 14. In vitro permeation parameters of aspirin permeated through mammary papilla using vesicular carriers

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% alcohol</td>
<td>5.66±0.14</td>
<td>0.49±0.01</td>
<td>23.16±0.65</td>
<td>790.37±39.60</td>
<td>34.13</td>
</tr>
<tr>
<td>Ethosomes</td>
<td>6.74±0.91</td>
<td>0.31±0.05</td>
<td>14.58±2.40</td>
<td>1065.18±24.32</td>
<td>73.06</td>
</tr>
<tr>
<td>Liposomes</td>
<td>7.52±0.32</td>
<td>0.63±0.07</td>
<td>29.12±2.51</td>
<td>911.98±20.45</td>
<td>31.32</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the mammary papilla at 48 hours divided by the cumulative amount at 48 hours. 10 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes. The values are significant at p-value < 0.05.
2.3.4. Influence of microneedles on aspirin permeation through breast skin

In case of microneedles, it was only tested in the breast skin since mammary papilla has natural ductal openings for drug transport. The 33% alcohol showed the highest permeation through breast skin and hence was tested in combination with microneedles. Microneedles with 33% alcohol significantly increased the permeation of aspirin from that of 33% alcohol vehicle alone (Figure 20 A). Compared to 33% hydroalcoholic vehicle, microneedles increased the cumulative amount of aspirin permeated through breast skin by 3-fold and decreased the lag time by 3.8-fold (Table 15). However, there was no significant difference in the skin retention amount after pretreatment with microneedles (Figure 20 B).
Figure 20. Permeation of aspirin through breast skin using microneedles.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of aspirin (ASA) permeated through breast skin. ‘a’ indicates a significant difference in comparison to 33% alcohol. (B) Retained amount of aspirin in breast skin (μg/g). MN: microneedles.
Table 15. In vitro permeation parameters of aspirin permeated through breast skin after pretreatment with microneedles

<table>
<thead>
<tr>
<th>Vehicle/MN</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% alcohol</td>
<td>2.27±0.56</td>
<td>7.14±0.91</td>
<td>323.85±29.95</td>
<td>106.14±20.18</td>
<td>0.33</td>
</tr>
<tr>
<td>MN</td>
<td>0.59±0.35ᵃ</td>
<td>20.13±0.73ᵃ</td>
<td>972.22±28.64</td>
<td>109.85±15.78</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 10 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol. The values are significant at p-value < 0.05. MN: microneedles.
Taken together, the results from this study suggest that aspirin can be delivered transdermally to the breast skin and to the mammary papilla. Overall, the findings from this study demonstrate the feasibility of delivering aspirin through breast skin using a variety of skin permeation enhancement approaches.
2.4. Discussion

The main objective of this study was to study the feasibility of transdermal delivery of aspirin to the breast using different penetration enhancement strategies.

Diffusion of the drug through breast skin or mammary papilla is considered to be a passive kinetic process where the drug diffuses from the area of high concentration to the area of low concentration (Lane, 2013). The steady state diffusion of the drug can be described by Fick’s law. Based on Fick’s equation, the increase of the drug flux can be mainly achieved by changing the drug diffusion through the skin, drug solubility in the solution, drug partitioning from the solution to the epidermis or the thickness of the skin. Therefore, the use of chemical penetration enhancers, such as alcohol and terpenes, can increase the drug permeation by altering the diffusion coefficient, drug solubility or the partition coefficient, while the use of microneedles can increase the drug permeation through breast skin by altering the skin layers. In the current study, the effects of various hydroalcoholic vehicles (33, 50 and 66%), terpenes, vesicular carriers and microneedles on aspirin permeation through the breast skin and mammary papilla were investigated with the aim of demonstrating the feasibility of transdermal delivery of aspirin to the breast.

The effect of ethanol content in the vehicle on solubility and partition coefficient of aspirin was investigated in order to study the influence of ethanol on aspirin through breast skin and nipple. Epidermal partition coefficient studies and solubility studies revealed an inverse correlation relationship between solubility and partition coefficient of aspirin in different hydroalcoholic vehicles. The solubility of aspirin increased with increasing the ethanol concentration in the vehicle; in contrast, the partition coefficient of
aspirin decreased with increasing the ethanol concentration in the vehicle. The same findings, the inverse correlation between solubility and partition coefficient, have been found in previous reports (Pershing et al., 1990; Ghanem et al., 1987; Megrab et al., 1995; Alsharif et al., 2017).

In breast skin permeation studies, 33% alcohol showed the highest permeation and flux of aspirin compared to 50 and 66% alcohol. These findings can also be explained by the epidermal partition coefficient results; in other words, increasing the concentration of ethanol in the vehicle decreased the partitioning of aspirin from the vehicle into the stratum corneum, resulting in decreasing the penetration of aspirin through breast skin. Furthermore, the decrease in the flux of aspirin with increasing the alcohol concentration in the vehicle was due to the lower uptake of the drug into the stratum corneum (Kurihara-Bergstrom et al., 1990).

Another effective approach to enhance the permeation of aspirin through breast skin is to utilize natural penetration enhancers that have potential chemopreventive activities against breast cancer. Sandalwood oil is an essential oil that obtained from the steam distillation barks of sandalwood tree and it has been shown to have anti-cancer effects in breast cancer (Santha et al., 2013; Bommareddy et al., 2015). In addition, due to the presence of terpenes, sandalwood oil can serve as a skin penetration enhancer. Thus, sandalwood oil has been co-treated with 50% alcohol to enhance the permeation of aspirin through breast skin in this study. Limonene is a major constituent in the oil of several citrus fruits including orange, lemon and grapefruits. Limonene has been shown to be effective against breast cancer (Elegbede et al., 1984; Miller et al., 2013). In addition to its chemoprevention activity against breast cancer, limonene is well known to
have skin penetration enhancement effects on both hydrophilic and hydrophobic drugs (Williams and Barry, 1991; Amnuaikit et al., 2005). For these reasons, limonene was used in this study. In addition to sandalwood oil and limonene, eugenol has been used as a potent penetration enhancer (Zhao and Singh, 1998, 2000). Eugenol has also been shown to have anti-cancer activity against breast cancer cells in both in-vitro and in-vivo studies (Al-Sharif et al., 2013). Due to its chemoprevention breast cancer effects and skin penetration enhancement effects, eugenol was used in this study. To this end, three terpenes (sandalwood oil, limonene and eugenol) have been chosen for this study using only 5% of the terpenes co-treated with 50% alcohol. The results demonstrated that sandalwood oil showed the highest cumulative amount of aspirin permeated through breast skin after 48 hours compared to limonene, eugenol and 50% hydroalcoholic vehicle. These findings could be mainly attributed to the fact that terpenes can disrupt the intercellular bilayer lipids, increase drug solubility and/or increase drug partitioning into the stratum corneum (Williams and Barry, 1991; Cornwell et al., 1996). As shown in Table 7, 5% sandalwood oil plus 50% ethanol increased the epidermal partition coefficient of aspirin and decreased the solubility of aspirin in comparison to 50% ethanol alone, indicating that the increase of aspirin permeation through the breast skin was due to the increase in the partitioning of aspirin into the stratum corneum. These findings are consistent with previous reports in which limonene and eugenol have shown to enhance the skin permeation of butylparaben and tamoxifen (Koyama et al., 1994; Zhao and Singh, 1998, 2000). Moreover, all terpenes significantly decreased the lag time compared to 50% alcohol vehicle alone. This finding is unlike other study where terpenes
increased the permeation and the lag time of diclofenac sodium in comparison to the control that has no terpenes (Arellano et al., 1996).

In another set of experiments, aspirin-ethosomes showed higher permeation through breast skin compared to aspirin-liposomes and there was no significant difference between ethosomes system and 33% hydroalcoholic vehicle alone. These findings can be explained by the fact that ethosomes system increased the aspirin permeation by a synergistic mechanism between vesicles, ethanol and skin lipids; in other words, ethanol disturbs the stratum corneum lipid bilayer and increases its lipid fluidity, and the flexibility of ethosomes (less rigid than liposomes) enables it to penetrate through the stratum corneum lipid bilayer (Touitou et al., 2000). These findings are consistent with a previous study where ethosomes showed higher permeation of diclofenac sodium through skin and a significant higher flux in comparison to liposomes (Ghanbarzadeh and Arami, 2013).

Microneedles-assisted drug delivery can enhance drug permeation through skin by disrupting the barrier of SC by creating microchannels (small pores) in the skin, allowing the drug to transport easily through skin (Prausnitz, 2004). Unlike the use of hypodermic injections, the application of microneedles can provide convenient and painless delivery of a variety of drugs through the skin (Wermeling et al., 2008). In this present study, the microneedle arrays application on the skin has been investigated with the aim of testing the feasibility of increasing the permeation of aspirin through breast skin. It was found that the pretreated skin with microneedles significantly enhanced the cumulative amount of aspirin compared to 33% alcohol. In comparison to 33% alcohol, the application of microneedles increased the flux of aspirin and decreased the lag time.
These findings could be explained by the fact that the micropores created by the microneedles enhanced the diffusion rate of aspirin through the stratum corneum.

In mammary papilla permeation studies, the cumulative amount and the flux of aspirin increased with increasing the alcohol concentration in the vehicle as a result of the increase in the solubility of aspirin in the vehicle and into the stratum corneum lipids. These findings are in agreement with previous reports (Watkinson et al., 2009). These results also may reflect the effects of ethanol on the skin structure by altering the lipid bilayer in the skin, increasing the lipid fluidity and lipid extraction (Berner et al., 1989; Kim et al., 1996; Panchagnula et al., 2001). Furthermore, all terpenes co-treated with 50% alcohol increased the permeation of aspirin through the mammary papilla in comparison to 50% alcohol, which is similar to breast skin results. Eugenol and sandalwood oil showed the highest cumulative amount of aspirin permeated through the mammary papilla after 48 hours compared to limonene and 50% hydroalcoholic vehicle. Also, all terpenes significantly decreased the lag time compared to 50% alcohol vehicle alone. In addition, aspirin-liposomes showed higher permeation through the mammary papilla compared to aspirin-ethosomes or 33% alcohol alone. These findings can be explained by the fact that liposomes typically work by disrupting the lipid bilayer fluidity of the stratum corneum, resulting in changing the stratum corneum structure and increasing the partitioning of the drug (Fresta and Puglisi, 1996; Maghraby et al., 2006). These findings are consistent with previous reports (Puglia et al., 2004; Bhatia et al., 2004; E. Ramón et al., 2005).

From both breast skin and mammary papilla permeation results, aspirin showed great permeation through breast skin. Thus, it is reasonable to apply aspirin to the entire
breast area (breast skin and nipple) in order to increase the concentration of aspirin within breast tissue, resulting in producing a useful breast cancer prevention outcome. The results from the present study showed that the highest cumulative amount of aspirin permeated through breast skin at 24 hours is 95.34 μg/ml (Table 16), which is less than the IC\textsubscript{50} value (900 μg/ml) of aspirin in human breast cancer cells (Nath et al., 2009). Given that the average of the entire surface area of the human breast is 200 cm\textsuperscript{2} and mass of 750 g of tissue, simple extrapolations of the in vitro permeation of aspirin was applied (Güngör, et al., 2013; Alsharif et al., 2017; Table 16). From Table 16, the highest delivery rate of aspirin permeated through breast skin at 24 hours is 5.30 μg per gram of breast tissue per hour. This delivery rate is not comparable to the IC\textsubscript{50} value of aspirin. Hence, further studies are required to enhance the transdermal delivery of aspirin to the breast. It is important to mention that the local clearance of the drug from the breast tissue may also affect the total drug concentration in the breast.
Table 16. Transdermal delivery rate of aspirin through breast skin and mammary papilla

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cumulative at 24 hours (Q&lt;sub&gt;24&lt;/sub&gt;) (μg/cm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>flux(μg/cm&lt;sup&gt;2&lt;/sup&gt;/h) (Q&lt;sub&gt;24&lt;/sub&gt;/24)</th>
<th>Area (cm&lt;sup&gt;2&lt;/sup&gt;) required to deliver 1 mg in 24 hours (1000/Q&lt;sub&gt;24&lt;/sub&gt;)</th>
<th>Delivery rate (μg/h per g of tissue)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN+33% alcohol (skin)</td>
<td>476.68±30.06</td>
<td>19.86±1.25</td>
<td>2.10</td>
<td>5.30</td>
</tr>
<tr>
<td>50% alcohol+5% sandalwood oil (skin + nipple)</td>
<td>437.29±8.30</td>
<td>18.22±0.35</td>
<td>2.29</td>
<td>4.86</td>
</tr>
<tr>
<td>50% alcohol+5% eugenol (skin + nipple)</td>
<td>238.59±9.74</td>
<td>9.94±0.41</td>
<td>4.19</td>
<td>2.65</td>
</tr>
<tr>
<td>33% alcohol (skin + nipple)</td>
<td>205.79±11.04</td>
<td>8.57±0.46</td>
<td>4.86</td>
<td>2.29</td>
</tr>
</tbody>
</table>

*The delivery rate values are calculated as the flux multiplied by 200 cm<sup>2</sup> (average area of human breast) divided by 750 g of tissue. MN: microneedles.
Taken together, the in vitro permeation studies of aspirin demonstrate the feasibility of enhancing transdermal delivery of aspirin to the breast. Although these formulations and microneedles application showed to have great influence on the transport of aspirin through breast skin and through mammary papilla, further studies are required to test other chemical and physical permeation enhancement methods. In addition, in vivo studies are required to explore the efficacy studies (chemopreventive effects studies) of aspirin and to understand the pharmacokinetics of aspirin.

2.5. Conclusions

The results from the study demonstrate the feasibility of enhancing the transdermal delivery of aspirin to the breast. Among the hydroalcoholic vehicles, 33% alcohol showed the highest cumulative amount permeated through breast skin and 66% alcohol showed the highest cumulative amount of aspirin permeated through mammary papilla. Ethosomes system showed higher permeation of aspirin through skin and liposomes showed higher permeation through mammary papilla. Among the three terpenes, 5% sandalwood oil showed the highest flux. In addition, microneedles significantly enhanced the skin permeation of aspirin. Overall, the findings from this study can be used design penetration enhancement strategies for localized transdermal delivery of compounds with similar physicochemical properties to aspirin.
CHAPTER THREE: TRANSDERMAL DELIVERY OF ENDOXIFEN TO THE BREAST
3.1. Introduction

Majority of breast cancers are estrogen receptors positive (ER+). Oral tamoxifen is a drug of choice for the treatment of ER+ breast cancer. Tamoxifen acts as an estrogen receptor antagonist in breast tissue. However, it has estrogenic agonist activity in the uterus and therefore carries the risk of uterine cancer. In addition, tamoxifen use is associated with other systemic side effects including hot flashes, retinopathy, venous thrombosis and cataract (Morello et al., 2002; Cohen, 2004; De Lima et al., 2003).

Tamoxifen is considered to be a produrg and undergoes extensive metabolism in the liver by CYP2D6 and CYP3A4 enzymes to produce its active metabolites. One of the main active metabolites of tamoxifen is endoxifen which has 100-fold greater binding affinity for estrogen receptors in the breast compared to tamoxifen (Lim et al., 2005, 2006). Endoxifen has been shown to be an active anti-estrogen agent for the treatment of breast cancer, and the oral administration of endoxifen hydrochloride has been investigated in clinical trials (Ahmad et al., 2010A, 2010B; Wu et al., 2009). As opposed to systemic delivery, the localized transdermal delivery can maximize endoxifen concentration in the breast and minimize its systemic drug exposure.

The direct transdermal delivery of tamoxifen’s metabolites has been studied (Mansel et al., 2007; Lee et al., 2014; Yang et al., 2014; Lee et al., 2011). In particular, the direct transdermal delivery of endoxifen to the breast has been evaluated (Lee et al., 2011; Mah, et al., 2013). In another study, preclinical studies on localized transdermal delivery of different agents, including endoxifen, to the breast have been evaluated (Lee et al., 2015). The results from this study show that the mammary tissue concentration of endoxifen was significantly higher in mammary glands of the gel treated animals than
systemically treated animals, while the plasma concentrations of endoxifen were similar in gel and systemically treated animals. These results suggest that endoxifen can be transdermally delivered to the breast to provide breast cancer prevention outcome. However, endoxifen is a lipophilic agent (Figure 21) with a high Log P value (5.45) that limits its transport to the deeper breast tissue (Güngör, et al., 2013). Thus, there is a need to develop penetration enhancement strategies for localized transdermal delivery of endoxifen.
Figure 21. Chemical structure of endoxifen.
The main goal of this chapter is to investigate the feasibility of localized transdermal delivery of endoxifen to the breast through breast skin and/or through the mammary papilla. The specific aims of this chapter are as follows:

i. Investigate the influence of hydroalcoholic vehicles on endoxifen permeation through the breast skin and mammary papilla.

ii. Investigate the effect of chemical penetration enhancers on endoxifen permeation through breast skin and mammary papilla.

iii. Investigate the influence of vesicular carriers on endoxifen permeation through breast skin and mammary papilla.

iv. Investigate the effect of microneedles on endoxifen permeation through the breast skin.
3.2. Materials and methods

3.2.1. Materials

Endoxifen was purchased from Cayman Chemical Company, Ann Arbor, MI, USA. Tween® 80 and acetic acid were purchased from Acros Organics, Bridgewater, NJ, USA. HPLC grade acetonitrile and methanol were obtained from Fisher Scientific, Hampton, NH, USA. All other materials and chemicals used in this study were similar to the ones described in chapter 2 (section 2.2.1.).

3.2.2. Preparation of the breast skin and mammary papilla

The preparation of tissues used in this study was similar to the methods described in chapter 2 (section 2.2.2.).

3.2.3. Preparation of hydroalcoholic vehicles and penetration enhancers

The preparation of hydroalcoholic vehicles and chemical penetration enhancers used in this study was similar to the methods described in chapter 2 (section 2.2.3.). The concentration of endoxifen used in this study was 2 mg/ml.

3.2.4. Preparation and characterization of vesicular carriers

The preparation of endoxifen-liposomes and endoxifen-ethosomes was similar to the methods described in chapter 2 (section 2.2.4.). The concentration of endoxifen used in this study was 2 mg/ml.

3.2.5. Microneedles assisted transdermal delivery

The microneedles used in this study were similar to the ones described in chapter 2 (section 2.2.5.).
3.2.6. **Solubility studies**

The solubility studies of endoxifen were carried out using the same method as described in chapter 2 (section 2.2.6.).

3.2.7. **Partition coefficient studies**

The epidermal partition coefficient (epidermis/vehicle partition coefficient) studies of endoxifen were carried out using the same method as described in chapter 2 (section 2.2.7.).

3.2.8. **In vitro permeation studies**

The penetration studies of endoxifen for both breast skin and mammary papilla were conducted using the same method as described in chapter 2 (section 2.2.8.). The receptor compartment (5 ml) was composed of 70% phosphate buffered saline (PBS, pH 7.4), 20% alcohol, and 10% Tween® 80 to maintain sink conditions. The donor compartment was filled with 500 μl of the endoxifen formulation, and the endoxifen concentration (2 mg/ml) was same in all permeation experiments.

3.2.9. **Analytical method**

Drug concentrations were determined by HPLC (Waters Corporation, Milford, MA, USA). The chromatographic resolution of endoxifen was achieved on a C_{18} reverse phase column (5 μm, 4.6 × 150 mm, Waters Corporation, Milford, MA, USA) with a guard column (3.9 × 20 mm, Waters). The mobile phase was a mixture of acetonitrile: methanol: water: acetic acid: triethanolamine (35: 35: 30: 0.2: 0.1, v/v). The mobile phase was filtered through a 0.2 μm membrane filter (Whatman® membrane filters, GE Healthcare Life Sciences, Pittsburgh, PA, USA). The flow rate was 0.70 ml/min and the
detection wavelength was 244 nm. The injection volume was 50 μl and the standard concentrations were prepared in 50% (v/v) ethanol solvent. The concentration range used for the calibration curve was 0.2-10 μg/ml ($R^2 = 0.9981$) and was used to determine the endoxifen concentration in the in-vitro permeation studies.

3.2.10. Data analysis

The permeation parameters of endoxifen were calculated as described in chapter 2 (section 2.2.10.). Statistical analysis was carried out using Minitab 18 Statistical Software using one-way ANOVA Tukey posthoc test. The results were expressed as mean ± standard deviation (SD). A 0.05 level of probability (p < 0.05) was considered to be significant.

3.3. Results

3.3.1. Influence of hydroalcoholic vehicles on endoxifen permeation

The permeation studies of endoxifen were studied using porcine breast skin and mammary papilla. The 33 % hydroalcoholic vehicle showed the highest endoxifen permeation through the breast skin and there was no measurable permeation with 66% hydroalcoholic vehicle (Figure 22 A). The 33% hydroalcoholic vehicle showed 1.3-fold and 15.5-fold increase in endoxifen flux in comparison to 0% and 50% alcohol, respectively (Table 17). The skin retention of endoxifen decreased with increase in alcohol concentration in the vehicle (Figure 22 B).

In permeation studies of endoxifen through mammary papilla, endoxifen showed highest tissue retention with 0% alcohol, but there was no permeation into the receptor medium (Figure 23).
The solubility studies (Figure 24; Table 18) showed that increasing the concentration of alcohol in the vehicle increased the solubility of endoxifen. Compared to 33% alcohol, 50% alcohol increased the solubility of endoxifen by 2.3-fold, and 66% alcohol increased the solubility of endoxifen by 3.5-fold.

On the other hand, the epidermal partition coefficient studies (Figure 24; Table 18) showed that increasing the concentration of alcohol in the vehicle decreased the epidermal partition coefficient of endoxifen. Compared to 33% alcohol, 50% alcohol decreased the partition coefficient of endoxifen by 2.3-fold, and 66% alcohol decreased the partition coefficient of endoxifen by 4.6-fold. Also, 66% alcohol reduced the partition coefficient of endoxifen by 2-fold in comparison to 50% alcohol. The partition coefficient studies indicate that the epidermal partition coefficient of endoxifen is influenced by the concentration of alcohol in the vehicle.
Figure 22. Permeation of endoxifen through breast skin using different hydroalcoholic vehicles.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of endoxifen permeated through breast skin. ‘a’ indicates a significant difference in comparison to 50% alcohol and ‘b’ indicates a significant difference in comparison to 0% alcohol. (B) Retained amount of endoxifen in breast skin.
(µg/g). ‘a’ indicates a significant difference in comparison to 66% alcohol, ‘b’ indicates a significant difference in comparison to 50% alcohol and ‘c’ indicates a significant difference in comparison to 33% alcohol.
Table 17. In vitro permeation parameters of endoxifen permeated through breast skin with different hydroalcoholic vehicles

<table>
<thead>
<tr>
<th>Vehicle (% of alcohol)</th>
<th>Lag time (h)</th>
<th>Flux ($\mu g/cm^2$/h)</th>
<th>Cumulative amount at 48 hours ($\mu g/cm^2$)</th>
<th>Retained amount at 48 hours ($\mu g/cm^2$)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.61±1.49</td>
<td>4.65±0.17</td>
<td>216.07±8.22</td>
<td>232.35±7.00</td>
<td>1.08</td>
</tr>
<tr>
<td>33</td>
<td>1.92±0.13</td>
<td>6.05±0.50$^a$</td>
<td>285.52±33.02</td>
<td>177.81±17.12</td>
<td>0.62</td>
</tr>
<tr>
<td>50</td>
<td>5.64±0.57$^{a,b}$</td>
<td>0.39±0.03$^{a,b}$</td>
<td>18.30±1.00</td>
<td>86.29±21.27</td>
<td>4.72</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 0% alcohol and ‘b’ indicates a significant difference in comparison to 33% alcohol. The values are significant at p-value < 0.05.
Figure 23. Retained amount of endoxifen in mammary papilla (μg/g) using different hydroalcoholic vehicles.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. ‘a’ indicates a significant difference in comparison to 66% alcohol, ‘b’ indicates a significant difference in comparison to 50% alcohol and ‘c’ indicates a significant difference in comparison to 33% alcohol.
Figure 24. Correlation between saturated solubility and epidermal partition coefficient of endoxifen as a function of different alcohol concentrations.

The data is presented as mean ± SD (n=3).
Table 18. Saturated solubility and epidermal partition coefficient of endoxifen from different hydroalcoholic vehicles

<table>
<thead>
<tr>
<th>Vehicle (% of alcohol)</th>
<th>Solubility (mg/ml)</th>
<th>Epidermal partition coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>7.72±0.73</td>
<td>0.415±0.004</td>
</tr>
<tr>
<td>50</td>
<td>17.59±1.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.184±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>66</td>
<td>26.76±1.88&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.091±0.007&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD (n=3). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. The values are significant at p-value < 0.05.
3.3.2. Influence of chemical penetration enhancers on endoxifen permeation

To further enhance the endoxifen permeation through breast skin, 5% of terpenes in 50% alcohol were used. The results showed that all terpenes in 50% alcohol significantly enhanced the cumulative amount of endoxifen permeated through breast skin compared to 50% hydroalcoholic vehicle (Figure 25 A). The greatest influence on endoxifen permeation through skin was shown by sandalwood oil followed by limonene and eugenol. Compared to 50% hydroalcoholic vehicle, sandalwood oil, limonene and eugenol increased the endoxifen flux by 7.3-fold, 5.7-fold and 3.3-fold, respectively (Table 19). Also, all the three terpenes significantly decreased the lag time in comparison to 50% hydroalcoholic vehicle (Table 19). Compared to 50% hydroalcoholic vehicle, the three terpenes increased the skin retention of endoxifen and the highest skin retention was observed with eugenol (Figure 25 B).

In permeation studies of endoxifen through mammary papilla, there was no permeation into the receptor medium. All the three terpenes increased the tissue retention in comparison to 50% hydroalcoholic vehicle. Similar to breast skin, the highest retention in the mammary papilla was observed with eugenol (Figure 26).
Figure 25. Permeation of endoxifen through breast skin using different chemical penetration enhancers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of endoxifen (ENX) permeated through breast skin. ‘a’ indicates a significant difference in comparison to 50% alcohol, ‘b’ indicates a significant
difference in comparison to 5% eugenol and ‘c’ indicates a significant difference in comparison to 5% limonene. (B) Retained amount of endoxifen in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 50% alcohol, ‘b’ indicates a significant difference in comparison to 5% sandalwood oil and ‘c’ indicates a significant difference in comparison to 5% limonene.
Table 19. In vitro permeation parameters of endoxifen permeated through breast skin using different chemical penetration enhancers

<table>
<thead>
<tr>
<th>Penetration enhancer</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours(μg/cm²)</th>
<th>Retained amount at 48 hours(μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% alcohol</td>
<td>5.64±0.57</td>
<td>0.39±0.03</td>
<td>18.30±1.00</td>
<td>86.29±21.27</td>
<td>4.72</td>
</tr>
<tr>
<td>Sandalwood oil</td>
<td>2.24±0.65ᵃᵇᶜ</td>
<td>2.86±0.14ᵃᵇᶜ</td>
<td>133.69±6.23</td>
<td>206.73±23.71</td>
<td>1.55</td>
</tr>
<tr>
<td>Limonene</td>
<td>3.47±0.31ᵃ</td>
<td>2.22±0.08ᵃᵇ</td>
<td>106.15±3.39</td>
<td>231.78±13.69</td>
<td>2.18</td>
</tr>
<tr>
<td>Eugenol</td>
<td>3.32±0.19ᵃ</td>
<td>1.28±0.01ᵃ</td>
<td>61.23±0.69</td>
<td>307.31±26.42</td>
<td>5.02</td>
</tr>
</tbody>
</table>

5% of terpenes were co-treated with 50% alcohol. Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 50% alcohol, ‘b’ indicates a significant difference in comparison to eugenol and ‘c’ indicates a significant difference in comparison to limonene. The values are significant at p-value < 0.05.
Figure 26. Retained amount of endoxifen in mammary papilla tissue (μg/g) using different chemical penetration enhancers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. ‘a’ indicates a significant difference in comparison to 50% alcohol, ‘b’ indicates a significant difference in comparison to 5% sandalwood oil and ‘c’ indicates a significant difference in comparison to 5% limonene.
3.3.3. Influence of vesicular carriers on endoxifen permeation

The characteristic of ethosomes and liposomes are shown in Table 20. The particle size of ethosomes and liposomes was 54 nm and 90 nm, respectively (Table 20). The encapsulation efficiency (EE) for endoxifen was above 90% in ethosomes and liposomes, and the loading efficiency (LE) for endoxifen was below 10% in ethosomes and liposomes (Table 20).

In permeation of endoxifen through the breast skin studies, the vesicular systems (ethosomes and liposomes) showed no enhancement of endoxifen permeation through skin in comparison to 33% hydroalcoholic vehicle, and there was no significant difference in endoxifen permeation through breast skin between ethosomes and liposomes (Figure 27 A). Compared to 33% hydroalcoholic vehicle, ethosomes reduced the flux of endoxifen by 11-fold and liposomes also decreased the flux of endoxifen by 16.8-fold (Table 21). The vesicular systems showed less skin retention of endoxifen in comparison to 33% alcohol, and there was no significant difference in skin retention between ethosomes and liposomes (Figure 27 B).

In permeation of endoxifen through the mammary papilla studies, liposomes increased the tissue retention in comparison to ethosomes and 33% hydroalcoholic vehicle, and there was no permeation into the receptor medium (Figure 28).
### Table 20. Characteristic of endoxifen ethosomes and liposomes

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>EE (%)</th>
<th>LE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank ethosomes</td>
<td>56.71±0.90</td>
<td>0.21±0.01</td>
<td>-17.16±1.70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Endoxifen-ethosomes</td>
<td>54.39±1.02</td>
<td>0.24±0.02</td>
<td>61.57±1.80</td>
<td>94.72±3.01</td>
<td>10.38±0.33</td>
</tr>
<tr>
<td>Blank liposomes</td>
<td>89.64±0.90</td>
<td>0.29±0.01</td>
<td>-15.26±2.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Endoxifen-liposomes</td>
<td>90.54±0.72</td>
<td>0.25±0.01</td>
<td>49.57±0.57</td>
<td>90.23±1.62</td>
<td>5.84±0.11</td>
</tr>
</tbody>
</table>

PDI: Polydispersity Index; EE%: Encapsulation efficiency in percent; LE%: Loading efficiency in percent. Values are presented as mean ± SD (n=3).
Figure 27. Permeation of endoxifen through breast skin using vesicular carriers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of endoxifen (ENX) permeated through breast skin. ‘a’ indicates a significant difference in comparison to liposomes and ‘b’ indicates a significant difference in comparison to ethosomes. (B) Retained amount of endoxifen in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to liposomes and ‘b’ indicates a significant difference in comparison to ethosomes.
Table 21. In vitro permeation parameters of endoxifen permeated through breast skin using vesicular carriers

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% alcohol</td>
<td>1.92±0.13</td>
<td>6.05±0.50</td>
<td>285.52±33.02</td>
<td>177.81±17.12</td>
<td>0.62</td>
</tr>
<tr>
<td>Ethosomes</td>
<td>7.03±0.75</td>
<td>0.54±0.05</td>
<td>25.73±1.85</td>
<td>59.66±3.63</td>
<td>2.32</td>
</tr>
<tr>
<td>Liposomes</td>
<td>3.41±1.52</td>
<td>0.36±0.04</td>
<td>16.95±2.49</td>
<td>30.51±2.50</td>
<td>1.80</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to liposomes and ‘b’ indicates a significant difference in comparison to ethosomes. The values are significant at p-value < 0.05.
Figure 28. Retained amount of endoxifen in the mammary papilla tissue (μg/g) using vesicular carriers

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes.
3.3.4. **Influence of microneedles on endoxifen permeation through breast skin**

Microneedles were only tested in breast skin. Because 33% alcohol showed the highest permeation through breast skin, it was used in combination with microneedles. Microneedles pretreatment followed by 33% alcohol significantly increased the cumulative amount of endoxifen permeated through the breast skin in comparison to 33% alcohol alone (Figure 29 A). However, the microneedles did not significantly increase the flux of endoxifen, but decreased the lag time by 8-fold in comparison to 33% alcohol vehicle (Table 22). Moreover, microneedles significantly decreased the skin retention of endoxifen in comparison to 33% alcohol (Figure 29 B).
Figure 29. Permeation of endoxifen through breast skin using microneedles.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of endoxifen (enx) permeated through breast skin. ‘a’ indicates a significant difference in comparison to 33% alcohol. (B) Retained amount of endoxifen in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 33% alcohol. MN: microneedles.
Table 22. In vitro permeation parameters of endoxifen permeated through breast skin after pretreatment with microneedles

<table>
<thead>
<tr>
<th>Vehicle/MN</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% alcohol</td>
<td>1.92±0.13</td>
<td>6.05±0.50</td>
<td>285.52±33.02</td>
<td>177.81±17.12</td>
<td>0.62</td>
</tr>
<tr>
<td>MN</td>
<td>0.24±0.18,a</td>
<td>6.61±0.08</td>
<td>325.88±4.85</td>
<td>156.09±14.02</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol. The values are significant at p-value < 0.05. MN: microneedles.
3.4. Discussion

The main objective of this study was to study the feasibility of transdermal delivery of endoxifen to the breast.

As described in chapter 2, the drug flux can be increased by changing the drug diffusion through the skin, drug solubility in the solution, drug partitioning from the solution to the epidermis or the thickness of the skin (Fick’s law). Chemical penetration enhancers can increase endoxifen permeation by altering the diffusion coefficient, drug solubility or the partition coefficient, while the use of microneedles can increase endoxifen permeation through breast skin by creating pores in skin. In this study, the effect of various hydroalcoholic vehicles, terpenes, vesicular carriers and microneedles on the permeation of endoxifen through the breast skin and mammary papilla were investigated.

The effect of ethanol content in the vehicle on solubility and partition coefficient of endoxifen was investigated to study the influence of ethanol on endoxifen permeation through breast skin and nipple. Epidermal partition coefficient studies and solubility studies revealed an inverse correlation relationship between solubility and partition coefficient of endoxifen in different hydroalcoholic vehicles. The solubility of endoxifen increased with increasing the ethanol concentration in the vehicle; in contrast, the partition coefficient of endoxifen decreased with increasing the ethanol concentration in the vehicle. The same findings have been found in previous reports (Pershing et al., 1990; Ghanem et al., 1987; Megrab et al., 1995; Alsharif et al., 2017). Also, the same findings were found with aspirin as described in chapter 2.
In the breast skin permeation studies, 33% alcohol showed the highest flux of endoxifen among the other hydroalcoholic vehicles, and there was no permeation with 66% alcohol. In consistent to these results, estradiol showed lower penetration through human skin and lower flux with higher concentration of alcohol (higher than 60%) in the vehicle (Megrab et al., 1995). These findings can also be explained by the partition coefficient results; in other words, increasing the concentration of ethanol in the vehicle decreased the partitioning of endoxifen from the vehicle into the stratum corneum, resulting in decreasing the penetration of endoxifen through breast skin. Also, the same findings were found with aspirin as described in chapter 2.

Another effective approach to enhance the permeation of endoxifen through breast skin is to use penetration enhancers that have potential chemopreventive activity against breast cancer. Terpenes have been widely investigated in transdermal drug delivery as chemical penetration enhancers. Terpenes, such as limonene and eugenol, have shown to enhance permeation of different drugs through the skin by mainly improving their partitioning into the stratum corneum (Williams and Barry, 1991; Yamane et al., 1995; Cornwell et al., 1996). In addition, sandalwood oil can serve as a skin penetration enhancer. Due to the chemoprevention breast cancer effects and skin penetration enhancement effects, the three terpenes (sandalwood oil, eugenol and limonene) have been chosen for this study using only 5% of the terpenes co-treated with 50% alcohol. Sandalwood oil showed the highest cumulative amount of endoxifen permeated through breast skin compared to limonene, eugenol and 50% hydroalcoholic vehicle. These findings are consistent with previous reports where limonene and eugenol have shown to enhance the skin permeation of lipophilic compounds including
butylparaben and tamoxifen (Koyama et al., 1994; Zhao and Singh, 1998, 2000). The same results were also found with aspirin as described in chapter 2. Moreover, the three terpenes significantly decreased the lag time in comparison to 50% alcohol vehicle alone.

In addition, the microneedle arrays application on the skin was investigated with the aim of testing the feasibility of increasing the permeation of endoxifen through breast skin. It was found that the pretreated skin with microneedles significantly enhanced the cumulative amount of endoxifen compared to 33% alcohol. In comparison to 33% alcohol, the application of microneedles slightly increased the flux of endoxifen and significantly decreased the lag time. These findings could be explained by the fact that the micropores created by the microneedles enhanced the diffusion rate of endoxifen and helped the drug to reach the steady state rapidly, resulting in a higher flux and a shorter lag time. Similar results, in previous reports, have been found with different molecules that have moderate (log P = 2.7-3.8) lipophilicity (Kaur et al., 2014; Nguyen and Banga, 2015). Unlike aspirin (chapter 2), microneedles did not significantly increase the flux of endoxifen in comparison to 33% alcohol, and this is mainly due to the high lipophilicity of endoxifen compared to aspirin.

In mammary papilla permeation studies, endoxifen did not show any measurable permeation through nipple and this could be due to the high lipophilicity of endoxifen, resulting in slowing down its transport rate through the mammary papilla. These observations are consistent with a recent mechanistic study illustrated that the hydrophilic dye (sulforhodamine B, Log P = -2) was more permeable than the hydrophobic dye (Nile red, Log P = 5) through mammary papilla (Kurtz and Lawson, 2018). In that study, the cumulative amount of both dyes permeated through skin was much higher than the
mammary papilla, which is also in agreement with our results in which endoxifen penetrated through breast skin but not through mammary papilla. More importantly, the average of human nipple has 5-9 ductal orifices and porcine nipple has only 2 ductal orifices (Love and Barsky, 2004; Martineau et al., 2012). Therefore, it is reasonable to predict that localized transdermal delivery of endoxifen to human breast can result in higher endoxifen concentration in human mammary papilla in comparison to porcine mammary papilla.

From both breast skin and nipple permeation results, endoxifen showed higher permeation through the breast skin. However, it is reasonable to apply endoxifen to the entire breast area (breast skin and nipple) in order to increase the concentration of endoxifen within breast tissue. The results from the present study showed that the highest cumulative amount of endoxifen permeated through breast skin at 24 hours is 30.61 μg/ml (Table 23), which is 122-fold higher than the IC₅₀ value (0.25 μg/ml) of endoxifen in human breast cancer cells (Zhang et al., 2015; Alsharif et al., 2017). Similar to aspirin in chapter 2, simple extrapolation of the in vitro permeation of endoxifen was used to check the in-vivo feasibility (Table 23). From Table 23, the highest delivery rate of endoxifen permeated through breast skin at 24 hours was 1.70 μg per gram of breast tissue per hour, which is 6.8-fold higher than the IC₅₀ value of endoxifen in human breast cancer cells. Since the delivery is comparable to the IC₅₀ values of endoxifen, it is reasonable to predict that optimizing more transdermal delivery formulations of endoxifen would increase the delivery rate of the drug per hour, leading to a higher therapeutic level of the drug in breast. However, the local clearance of the drug from the breast tissue may affect the total drug concentration in the breast.
Table 23. Calculation of transdermal delivery rate of endoxifen

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cumulative at 24 hours ($Q_{24}$) ($\mu g/cm^2$)</th>
<th>Calculated flux ($\mu g/cm^2/h$) ($Q_{24}/24$)</th>
<th>Area (cm$^2$) required to deliver 1 mg in 24 hours ($1000/Q_{24}$)</th>
<th>Delivery rate ($\mu g/h$ per g of tissue)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN</td>
<td>153.05±5.24</td>
<td>6.38±0.22</td>
<td>6.53</td>
<td>1.70</td>
</tr>
<tr>
<td>33% alcohol</td>
<td>112.23±11.51</td>
<td>4.68±0.48</td>
<td>8.91</td>
<td>1.25</td>
</tr>
<tr>
<td>Sandalwood oil</td>
<td>73.97±7.98</td>
<td>3.08±0.33</td>
<td>13.52</td>
<td>0.82</td>
</tr>
<tr>
<td>Limonene</td>
<td>35.02±1.07</td>
<td>1.46±0.04</td>
<td>28.56</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*The delivery rate values are calculated as the calculated flux ($Q_{24}/24$) multiplied by 200 cm$^2$ (average area of human breast) divided by 750 g of tissue. MN: microneedles.
Taken together, the in vitro permeation studies of endoxifen demonstrate the feasibility of transdermal delivery of endoxifen to the breast. Furthermore, results show the influence of the hydroalcoholic vehicles, terpenes, vesicular carriers and microneedles on the transdermal delivery of endoxifen to the breast. In vivo studies are required to investigate the pharmacokinetics and efficacy of transdermal endoxifen delivery to the breast.

3.5. Conclusions

The results from the study demonstrate the feasibility of endoxifen transdermal delivery to the breast. Among the hydroalcoholic vehicles, 33% alcohol showed the highest cumulative amount of endoxifen permeated through breast skin. All three chemical penetration enhancers (sandalwood oil, limonene and eugenol) increased the permeation of endoxifen through breast skin compared to 50% alcohol, and sandalwood oil showed the highest flux among other penetration enhancers. Ethosomes and liposomes did not enhance the transdermal delivery of endoxifen. Similarly, microneedles pretreatment only slightly enhanced the skin permeation of endoxifen. On the other hand, there was hardly any permeation through the mammary papilla. Overall, the findings from this study show that the chemical enhancers can be used to enhance the transdermal permeation of endoxifen.
CHAPTER FOUR: TRANSDERMAL DELIVERY OF FENRETINIDE TO THE BREAST
4.1. Introduction

Fenretinide, $N$-4-hydroxyphenyl-retinamide (Figure 30), is a synthetic derivative of retinoic acid. Fenretinide has been investigated in preclinical studies and clinical trials for the prevention of breast cancer (Moon et al., 1979; Veronesi et al., 1999). It has been shown to have a strong activity against breast cancer in animal models (Moon et al., 1979). Since fenretinide showed high activity in inhibiting mammary carcinogenesis in preclinical models, oral administration of fenretinide has been investigated in clinical trials for breast cancer prevention (Veronesi et al., 1999, 2006; Formelli et al., 2003; Cobleigh et al., 1993).

However, fenretinide has poor oral bioavailability due to its low water solubility ($\log P = 8.03$) and low permeability (Desai et al., 2011). As a result, the delivery of fenretinide is a major limitation in realizing its clinical benefits. To overcome this limitation, localized transdermal delivery of fenretinide to the breast is a potential alternative approach. Furthermore, clinical studies have shown that on systemic administration, fenretinide shows a strong accumulation in human breast tissue (Mehta et al., 1991; Formelli et al., 1993). Taken together, the transdermal application of fenretinide can overcome the delivery challenges to maximize the drug concentration in the breast.

However, the poor physicochemical properties of fenretinide may limit its transdermal delivery. Therefore, our goal in this study is to investigate the feasibility of transdermal delivery of fenretinide using various penetration enhancement strategies similar to aspirin and endoxifen.
Figure 30. Chemical structure of fenretinide.
To this end, the specific aims of this study are as follows:

i. Investigate the influence of hydroalcoholic vehicles on the permeation of fenretinide to the breast through breast skin and mammary papilla.

ii. Investigate the influence of chemical penetration enhancers on the permeation of fenretinide to the breast through breast skin and mammary papilla.

iii. Investigate the influence of vesicular carrier systems on the permeation fenretinide to the breast through breast skin and mammary papilla.

iv. Investigate the effect of microneedles pretreatment on the permeation of fenretinide through the breast skin.

v. Study the in-vivo transdermal delivery of fenretinide to the breast in rats.
4.2. Materials and methods

4.2.1. Materials

Fenretinide was purchased from LC Laboratories, Woburn, MA, USA. Glacial acetic acid was obtained from Fisher Scientific, Hampton, NH, USA. All other materials and chemicals used in this study were similar to the ones described in chapter 2 (section 2.2.1.).

4.2.2. Preparation of the breast skin and mammary papilla

The breast skin and mammary papilla were prepared as described in chapter 2 (section 2.2.2.).

4.2.3. Preparation of hydroalcoholic vehicles and chemical penetration enhancers

The hydroalcoholic vehicles used in this study were prepared as described in chapter 2 (section 2.2.3.). Sub-saturated concentration (0.20 mg/ml) and saturated concentration (4 mg/ml) of fenretinide were used in the study.

Sandalwood oil, limonene and eugenol were used and fenretinide was co-treated with 5% (v/v) of the terpenes in 66% (v/v) hydroalcoholic vehicle. The concentration used in this study was 4 mg/ml.

4.2.4. Preparation and characterization of vesicular carriers

The fenretinide liposomes and ethosomes were prepared using the same method as described in chapter 2 (section 2.2.4.). The concentration of fenretinide used in this study was 4 mg/ml.
4.2.5. Microneedles assisted transdermal delivery

The method was similar to that described in chapter 2 (section 2.2.5.).

4.2.6. Solubility studies

The solubility studies of fenretinide were carried out using the same method as described in chapter 2 (section 2.2.6.).

4.2.7. Partition coefficient studies

The epidermal partition coefficient (epidermis/vehicle partition coefficient) studies of fenretinide were carried out using the same method as described in chapter 2 (section 2.2.7.).

4.2.8. In vitro permeation studies

The penetration studies of fenretinide for both breast skin and mammary papilla were conducted as described in chapter 2 (section 2.2.8.). The receptor compartment (5 ml) was composed of 70% phosphate buffered saline (PBS, pH 7.4), 20% alcohol, and 10% Tween® 80 to maintain sink conditions. The donor compartment was filled with 500 μl of the formulation that contained fenretinide. Saturated (4 mg/ml) or sub-saturated (0.20 mg/ml) concentration of fenretinide was used for permeation experiments.

4.2.9. In vivo permeation studies

The animal experiments were carried out after approval from the Institutional Animal Care and Use Committee (IACUC) at South Dakota State University. Female Sprague Dawley rats (7-9 weeks old of age, 200-250 grams of weight) were randomly assigned to one of the treatment groups (Charles River Laboratories, Wilmington, MA, USA). The treatment groups included 66% hydroalcoholic vehicle, 5% sandalwood oil
co-treatment with 66% alcohol and pretreatment with microneedles followed by fenretinide delivery in 66% alcohol. Briefly, the hair around the breast skin and mammary papilla was shaved one day before the study using a hair clipper. The formulations were applied on the breast skin and mammary papilla (on two breast glands) under isoflurane anesthesia for 4 hours (500 µl of the formulation containing 4 mg of fenretinide was applied on each mammary gland). After 8 hours of starting the treatment, the rats were euthanized by CO₂ asphyxiation. Blood samples were collected by cardiac puncture in heparinized blood collection tubes. The plasma was collected by centrifuging at 4000 rpm for 15 minutes. In addition, the mammary glands, liver, kidneys, spleen, heart and lungs were collected. The tissues were weighed and homogenized in phosphate buffered saline (PBS, pH 7.4) to determine the drug concentration.

4.2.10. Analytical method

Drug concentrations were determined by HPLC (Waters Corporation, Milford, MA, USA). The chromatographic resolution of fenretinide was achieved on a C₁₈ reverse phase column (5 µm, 4.6 x 150 mm, Waters Corporation, Milford, MA, USA) attached with a guard column (3.9 x 20 mm, Waters). The mobile phase was a mixture of acetonitrile: water: glacial acetic acid (80: 18: 2, v/v). The mobile phase was filtered through a 0.2 µm membrane filter (Whatman® membrane filters, GE Healthcare Life Sciences, Pittsburgh, PA, USA). The flow rate was 0.80 ml/min and the detection wavelength was 340 nm. The injection volume was 50 µl and the standard concentrations were prepared in 90% (v/v) ethanol solvent. The concentration range used for the calibration curve was 0.1-10 µg/ml ($R^2 = 0.9995$).
For in-vivo studies, fenretinide was extracted from the plasma and tissues by adding an equal volume of ethyl acetate and n-hexane mixture (50:50, v/v) followed by centrifugation at 10000 rpm for 10 minutes. The supernatant was collected, and the extraction process was repeated three times to ensure complete fenretinide extraction. The organic solvent mixture was evaporated by dry nitrogen, and the residue was then reconstituted in 90% ethanol and used for HPLC analysis. The fenretinide concentration in the plasma was determined by HPLC using a calibration plot prepared by spiking known amount of fenretinide (0.25-5 μg/ml) in rat plasma ($R^2 = 0.9983$). The extraction efficiency of fenretinide from the rat plasma was greater than 97.76%. Fenretinide was extracted from the tissue using the same extraction method used for the plasma samples. The fenretinide concentration in the tissue was determined by HPLC using a calibration plot prepared by spiking known amount of fenretinide (0.25-5 μg/ml) in corresponding rat tissue ($R^2 = 0.9967$), and the extraction efficiency was greater than 94.41%.

4.2.11. Data analysis

The permeation parameters of fenretinide were calculated as described in chapter 2 (section 2.2.10.). Statistical analysis was carried out using Minitab 18 Statistical Software using one-way ANOVA Tukey posthoc test. The results were expressed as mean ± standard deviation (SD). A 0.05 level of probability (p < 0.05) was considered to be significant.
4.3. Results

4.3.1. Influence of hydroalcoholic vehicles on fenretinide permeation

The permeation studies of fenretinide were studied using porcine breast skin and mammary papilla. To study the effect of fenretinide concentration in the hydroalcoholic vehicles on fenretinide permeation through the breast skin, sub-saturated and saturated concentrations were used in this study. The results from the saturated concentration (4 mg/ml) studies showed that an increase in alcohol concentration in the vehicle increased the fenretinide permeation through the breast skin and there was no measurable permeation with 0% alcohol (Figure 31 A). An increase in alcohol concentration from 33% to 66% alcohol increased the cumulative permeated amount and the flux of fenretinide by 4-fold and 2-fold, respectively (Table 24). The highest skin retention of fenretinide was observed with 33% alcohol (Figure 31 B). In addition, the sub-saturated concentration (0.20 mg/ml) studies were carried out. The results showed that an increase in alcohol concentration in the vehicle increased the fenretinide permeation through the breast skin and there was no measurable permeation with 0% alcohol, which are the same results from saturation fenretinide permeation studies (Figure 33; Table 25). From both saturated and sub-saturated concentrations studies, the saturated concentration in 66% alcohol increased the flux of fenretinide by 4.8-fold in comparison to the sub-saturated concentration in 66% alcohol (Table 24; Table 25).

In permeation studies of fenretinide through mammary papilla, an increase in alcohol concentration in the vehicle increased the tissue retention, but there was no permeation into the receptor medium (Figure 32).
The solubility studies (Figure 34; Table 26) showed that increasing the concentration of alcohol in the vehicle increased the solubility of fenretinide. Compared to 33% alcohol, 50% alcohol increased the solubility of fenretinide by 6-fold, and 66% alcohol increased the solubility of fenretinide by 10.5-fold. Also, 66% alcohol increased the fenretinide solubility by 2-fold in comparison to 50% alcohol.

The epidermal partition coefficient studies (Figure 34; Table 26) showed that increasing the concentration of alcohol in the vehicle resulted in decreasing the epidermal partition coefficient of fenretinide. Compared to 33% alcohol, 50% alcohol decreased the partition coefficient of fenretinide by 1.4-fold, and 66% alcohol decreased the partition coefficient of fenretinide by 6-fold. Also, 66% alcohol reduced the partition coefficient of fenretinide by 4-fold in comparison to 50% alcohol.
Figure 31. Permeation of fenretinide through breast skin using different hydroalcoholic vehicles (saturated concentration).

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of fenretinide permeated through breast skin. ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. (B) Retained amount of fenretinide in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 0% alcohol, ‘b’ indicates a significant difference in comparison to 33% alcohol and ‘c’ indicates a significant difference in comparison to 50% alcohol.
Table 24. In vitro permeation parameters of fenretinide permeated through breast skin using different hydroalcoholic vehicles (saturated concentration)

<table>
<thead>
<tr>
<th>Vehicle (% of alcohol)</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>5.19±1.03</td>
<td>0.58±0.03</td>
<td>27.12±1.13</td>
<td>1269.1±354.43</td>
<td>46.80</td>
</tr>
<tr>
<td>50</td>
<td>1.00±0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.36±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.19±1.87</td>
<td>1244.18±154.99</td>
<td>19.38</td>
</tr>
<tr>
<td>66</td>
<td>0.98±0.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.11±0.05&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>98.15±1.66</td>
<td>903.37±91.76</td>
<td>9.20</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 4 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. The values are significant at p-value < 0.05.
Figure 32. Retained amount of fenretinide in nipple (μg/g) using different hydroalcoholic vehicles (saturated concentration).

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. ‘a’ indicates a significant difference in comparison to 0% alcohol, ‘b’ indicates a significant difference in comparison to 33% alcohol and ‘c’ indicates a significant difference in comparison to 50% alcohol.
Figure 33. Permeation of fenretinide through breast skin using different hydroalcoholic vehicles (sub-saturated concentration).

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of fenretinide (fenr) permeated through breast skin. ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. (B) Retained amount of fenretinide in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 0% alcohol, ‘b’ indicates a significant difference in comparison to 33% alcohol and ‘c’ indicates a significant difference in comparison to 50% alcohol.
Table 25. In vitro permeation parameters of fenretinide permeated through breast skin using different hydroalcoholic vehicles (sub-saturated concentration)

<table>
<thead>
<tr>
<th>Vehicle (% of alcohol)</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>5.33±0.32</td>
<td>0.15±0.005</td>
<td>7.10±0.23</td>
<td>110.90±14.45</td>
<td>15.62</td>
</tr>
<tr>
<td>50</td>
<td>5.32±0.19</td>
<td>0.25±0.01³</td>
<td>11.65±0.45</td>
<td>154.69±11.44</td>
<td>13.28</td>
</tr>
<tr>
<td>66</td>
<td>4.27±0.35⁴,⁵</td>
<td>0.44±0.01⁴,⁵</td>
<td>20.0±0.44</td>
<td>198.79±10.93</td>
<td>9.94</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 0.20 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. The values are significant at p-value < 0.05.
Figure 34. Correlation between solubility and epidermal partition coefficient of fenretinide as a function of different alcohol concentrations.

The data is presented as mean ± SD (n=3).
Table 26. Saturated solubility and epidermal partition coefficient of fenretinide in presence of different chemical penetration enhancers

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Solubility (mg/ml)</th>
<th>Epidermal partition coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% alcohol</td>
<td>0.33±0.01</td>
<td>0.333±0.008</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>1.92±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.238±0.012&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>66% alcohol</td>
<td>3.47±0.13&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.055±0.005&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% sandalwood oil + 66% alcohol</td>
<td>3.36±0.02&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.071±0.004&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD (n=3). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to 50% alcohol. The values are significant at p-value < 0.05.
4.3.2. Influence of chemical penetration enhancers on fenretinide permeation

In chemical penetration studies, all terpenes in 66% alcohol significantly enhanced the cumulative amount of fenretinide permeated through breast skin (Figure 35A). The greatest influence on fenretinide permeation through skin was seen with sandalwood oil followed by limonene and eugenol. Compared to 66% hydroalcoholic vehicle, sandalwood oil increased the fenretinide flux by 2.2-fold, while there was very minimal increase in permeation with eugenol and limonene (Table 27). The highest skin retention of fenretinide was observed with eugenol, while sandalwood oil showed the lowest skin retention (Figure 35 B).

In permeation studies of fenretinide through mammary papilla, there was no permeation into the receptor medium and terpenes did not significantly alter the tissue retention compared to 66% alcohol (Figure 36).
Figure 35. Permeation of fenretinide through breast skin using different chemical penetration enhancers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of fenretinide (fenr) permeated through breast skin. ‘a’ indicates a significant difference in comparison to 66% alcohol, ‘b’ indicates a significant difference in comparison to 5% eugenol and ‘c’ indicates a significant difference in comparison to 5% limonene. (B) Retained amount of fenretinide in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 5% sandalwood oil, ‘b’ indicates a significant difference in comparison to 5% limonene and ‘c’ indicates a significant difference in comparison to 66% alcohol.
Table 27. In vitro permeation parameters of fenretinide permeated through breast skin using different chemical penetration enhancers

<table>
<thead>
<tr>
<th>Penetration enhancer</th>
<th>Lag time (h)</th>
<th>Flux (µg/cm²/h)</th>
<th>Cumulative amount at 48 hours (µg/cm²)</th>
<th>Retained amount at 48 hours (µg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>66% alcohol</td>
<td>0.98±0.81</td>
<td>2.11±0.05</td>
<td>98.15±1.66</td>
<td>903.37±91.76</td>
<td>9.20</td>
</tr>
<tr>
<td>Sandalwood oil</td>
<td>4.86±0.04</td>
<td>4.13±0.04</td>
<td>186.19±1.05</td>
<td>646.32±14.77</td>
<td>3.47</td>
</tr>
<tr>
<td>Limonene</td>
<td>2.38±0.18</td>
<td>2.78±0.03</td>
<td>130.53±1.97</td>
<td>697.71±32.52</td>
<td>5.35</td>
</tr>
<tr>
<td>Eugenol</td>
<td>4.56±0.24</td>
<td>2.29±0.05</td>
<td>107.60±0.93</td>
<td>725.28±28.72</td>
<td>6.74</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 4 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 66% alcohol, ‘b’ indicates a significant difference in comparison to eugenol and ‘c’ indicates a significant difference in comparison to limonene. The values are significant at p-value < 0.05.
Figure 36. Retained amount of fenretinide in nipple (μg/g) using different chemical penetration enhancers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. ‘a’ indicates a significant difference in comparison to 5% sandalwood oil, ‘b’ indicates a significant difference in comparison to 5% limonene and ‘c’ indicates a significant difference in comparison to 5% eugenol.
4.3.3. Influence of vesicular carriers on fenretinide permeation

The characteristic of ethosomes and liposomes are shown in Table 28. The particle size of ethosomes (61 nm) was lower than liposomes (101 nm), and the encapsulation efficacy of ethosomes (92%) was higher than liposomes (68%). Both liposomes and ethosomes showed higher permeation through the breast skin than 33% alcohol. However, the liposomes showed higher permeation than ethosomes (Figure 37 A). Compared to 33% hydroalcoholic vehicle, liposomes enhanced the flux 5-fold, and ethosomes increased the cumulative permeated amount by 2-fold (Table 29). The carrier systems showed lower skin retention of fenretinide than 33% alcohol (Figure 37 B).

In permeation studies of fenretinide through mammary papilla, the liposomes and ethosomes did not show any measurable permeation across the mammary papilla. However, the liposomes showed significantly higher tissue retention in comparison to ethosomes and 33% alcohol (Figure 38).
Table 28. Characteristic of fenretinide ethosomes and liposomes

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>EE (%)</th>
<th>LE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank ethosomes</td>
<td>56.71±0.90</td>
<td>0.21±0.01</td>
<td>-17.16±1.70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fenretinide-ethosomes</td>
<td>61.59±0.33</td>
<td>0.23±0.01</td>
<td>-13.33±1.35</td>
<td>92.24±3.78</td>
<td>6.12±0.25</td>
</tr>
<tr>
<td>Blank liposomes</td>
<td>89.64±0.90</td>
<td>0.29±0.01</td>
<td>-15.26±2.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fenretinide-liposomes</td>
<td>101.1±0.79</td>
<td>0.37±0.01</td>
<td>-13±1.53</td>
<td>68.42±1.07</td>
<td>5.3±0.08</td>
</tr>
</tbody>
</table>

PDI: Polydispersity Index; EE%: Encapsulation efficiency in percent; LE%: Loading efficiency in percent. Values are presented as mean ± SD (n=3).
Figure 37. Permeation of fenretinide through breast skin using vesicular carriers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of fenretinide (fenr) permeated through breast skin. ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes (Etho). (B) Retained amount of fenretinide in breast skin (μg/g). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes.
Table 29: In vitro permeation parameters of fenretinide permeated through breast skin using vesicular carriers

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours (μg/cm²)</th>
<th>Retained amount at 48 hours (μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>33% alcohol</td>
<td>5.19±1.03</td>
<td>0.58±0.03</td>
<td>27.12±1.13</td>
<td>1269.1±354.43</td>
<td>46.80</td>
</tr>
<tr>
<td>Ethosomes</td>
<td>4.78±0.35</td>
<td>0.98±0.12</td>
<td>46.03±5.41</td>
<td>134.73±41.38</td>
<td>2.93</td>
</tr>
<tr>
<td>Liposomes</td>
<td>1.86±0.41</td>
<td>3.92±0.06</td>
<td>177.98±2.03</td>
<td>224.96±27.64</td>
<td>1.26</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 4 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes. The values are significant at p-value < 0.05.
Figure 38. Retained amount of fenretinide in nipple (μg/g) using vesicular carriers.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. ‘a’ indicates a significant difference in comparison to 33% alcohol and ‘b’ indicates a significant difference in comparison to ethosomes.
4.3.4. Influence of microneedles on fenretinide permeation through breast skin

Pretreatment of skin with microneedles significantly increased the permeation of fenretinide from 66% alcohol (Figure 39 A). The microneedles significantly increased the cumulative amount of fenretinide permeated through breast skin and the flux by 2.3-fold and 2.4-fold, respectively (Table 30). In addition, microneedles significantly decreased the skin retention of fenretinide (Figure 39 B).
Figure 39. Permeation of fenretinide through breast skin using microneedles.

The data is presented as mean ± SD (n=3-4) and the values are significant at p-value < 0.05. (A) Cumulative amount of fenretinide permeated through breast skin. ‘a’ indicates a significant difference in comparison to 66% alcohol. (B) Retained amount of fenretinide in breast skin tissue (μg/g). ‘a’ indicates a significant difference in comparison to 66% alcohol. MN: microneedles.
Table 30. In vitro permeation parameters of fenretinide permeated through breast skin after pretreatment with microneedles

<table>
<thead>
<tr>
<th>Vehicle/MN</th>
<th>Lag time (h)</th>
<th>Flux (μg/cm²/h)</th>
<th>Cumulative amount at 48 hours(μg/cm²)</th>
<th>Retained amount at 48 hours(μg/cm²)</th>
<th>Tissue affinity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>66% alcohol</td>
<td>0.98±0.81</td>
<td>2.11±0.05</td>
<td>98.15±1.66</td>
<td>903.37±91.76</td>
<td>9.20</td>
</tr>
<tr>
<td>MN</td>
<td>0.62±0.44</td>
<td>4.44±0.10ᵃ</td>
<td>224.70±15.82</td>
<td>417.37±40.05</td>
<td>1.86</td>
</tr>
</tbody>
</table>

Tissue affinity ratio was calculated as the retained amount in the skin at 48 hours divided by the cumulative amount at 48 hours. 4 mg/ml was used. Values are presented as mean ± SD (n=3-4). ‘a’ indicates a significant difference in comparison to 66% alcohol. The values are significant at p-value < 0.05. MN: microneedles.
4.3.5. In vivo permeation studies

In vivo permeation studies, the main goal was to validate the in vitro findings. Three formulations including 66% hydroalcoholic vehicle, 5% sandalwood in 66% alcohol and 66% alcohol with microneedles pretreatment were evaluated in rats. Based on the results from the in-vitro studies, 8 hours was chosen as the time point for measuring the tissue and plasma concentration. The trend was similar to in-vitro studies. Microneedles showed the highest permeation followed by sandalwood oil and then 66% hydroalcoholic vehicle, and microneedles also showed the highest plasma concentration and drug concentration in the mammary glands (Figure 40; Table 31). Microneedles significantly increased the mammary glands concentration of fenretinide by 1.8-fold in comparison to 66% hydroalcoholic vehicle, and sandalwood oil only slightly increased the mammary glands concentration of fenretinide compared to 66% hydroalcoholic vehicle (Figure 40). Similarly, microneedles significantly increased the plasma concentration of fenretinide (98.50±7 ng/ml) by 1.8-fold in comparison to 66% hydroalcoholic vehicle, while there was no significant difference in plasma concentration of fenretinide between 66% hydroalcoholic vehicle and 5% sandalwood oil treatment group (Table 31). In contrast, there were no detectable drug levels in the organs, except in the liver for microneedles treated group (Table 31).
Figure 40. Mammary glands concentration of fenretinide after localized transdermal delivery to the breast in rats for 8 hours (μg/g tissue).

The data is presented as mean ± SD (n=3) and the values are significant at p-value < 0.05. ‘a’ indicates a significant difference in comparison to 66% alcohol and ‘b’ indicates a significant difference in comparison to 5% sandalwood oil. MN: microneedles. 8 mg/ml was used.
Table 31. Plasma concentration of fenretinide and organ distribution of fenretinide after localized transdermal delivery in rats

<table>
<thead>
<tr>
<th>Plasma/tissue</th>
<th>MN (8 mg/ml)</th>
<th>Sandalwood oil</th>
<th>66% alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma level (ng/ml)</td>
<td>98.50±7.46</td>
<td>62.57±2.97</td>
<td>55.06±4.34</td>
</tr>
<tr>
<td>Liver (µg/g tissue)</td>
<td>0.52±0.06</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Kidneys</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spleen</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Heart</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lungs</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not detectable; MN: microneedles. 8 mg/ml was used. The data is presented as mean ± SD (n=3). ‘a’ indicates a significant difference in comparison to 66% alcohol and ‘b’ indicates a significant difference in comparison to 5% sandalwood oil. The values are significant at p-value < 0.05.
4.4. Discussion

The main objective of this study was to study the feasibility of transdermal delivery of fenretinide to the breast through breast skin and mammary papilla. Fenretinide is highly lipophilic drug with a log P (8.03) which can limit its delivery through skin. Thus, different skin permeation enhancement approaches were used in the current study.

As discussed in the earlier chapters, the transdermal permeation is governed by Fick’s law. The hydroalcoholic vehicles and terpenes can increase fenretinide permeation by altering the diffusion coefficient, drug solubility and/or the partition coefficient, while the use of microneedles increased fenretinide permeation through breast skin by altering the skin barrier.

The effect of ethanol content in the vehicle on solubility and partition coefficient of fenretinide was investigated to study the influence of ethanol on the permeation of fenretinide through breast skin and mammary papilla. Similar to aspirin and endoxifen, epidermal partition coefficient studies and solubility studies showed an inverse correlation relationship between solubility and partition coefficient of fenretinide in different hydroalcoholic vehicles. The solubility of fenretinide increased with increasing the ethanol concentration in the vehicle; in contrast, the partition coefficient of fenretinide decreased with increasing the ethanol concentration in the vehicle. The same findings were found with aspirin and endoxifen in chapter 2 and 3.

In the breast skin permeation studies, the cumulative amount and the flux of fenretinide increased with increasing the alcohol concentration in the vehicle as a result
of the increase in the solubility of fenretinide in the vehicle and into the stratum corneum lipids, and these findings are in agreement with previous reports (Watkinson et al., 2009). These findings also may reflect the effects of ethanol on the skin structure by altering the lipid bilayer in the skin, increasing the lipid fluidity and lipid extraction (Berner et al., 1989; Kim et al., 1996; Panchagnula et al., 2001). In addition, the permeation studies through breast skin using sub-saturated fenretinide concentration (0.20 mg/ml) with same hydroalcoholic vehicles (33, 50 and 66% alcohol) were carried out. The results showed the same results (an increase in alcohol concentration in the vehicle increased the flux of fenretinide) that were found with saturated fenretinide concentration (4 mg/ml) permeation studies. Unlike aspirin and endoxifen results, the results from the hydroalcoholic vehicles showed that the fenretinide solubility in the vehicle play a critical role in fenretinide permeation through the breast skin.

As shown in chapter 2 and 3, the penetration enhancers, which have potential chemopreventive activities against breast cancer, can be used to increase the drug permeation though the breast skin and/or mammary papilla. Therefore, sandalwood oil, limonene and eugenol have been co-treated with 66% alcohol to enhance the permeation of fenretinide through breast skin in this study. Sandalwood oil showed the highest cumulative amount of fenretinide permeated through breast skin compared to limonene, eugenol and 66% hydroalcoholic vehicle. These findings could be mainly attributed to the fact that terpenes can disrupt the intercellular bilayer lipids, increase the fenretinide solubility and/or increase the partitioning of fenretinide into the stratum corneum (Williams and Barry, 1991; Cornwell et al., 1996). As shown in Table 26, sandalwood oil in 66% alcohol did not significantly change the solubility and partition coefficient of
fenretinide in comparison to 66% alcohol alone. These results indicate that sandalwood oil mainly acts on skin by disrupting the bilayer lipids, resulting in an increase in fenretinide permeation through the breast skin. Although terpenes significantly increased the permeation of fenretinide through the breast skin, they increased the lag time compared to 66% alcohol vehicle.

To study the effect of vesicular carriers on fenretinide permeation through breast skin and mammary papilla, ethosomes and liposomes systems were used in the current work. The results showed that fenretinide-liposomes showed higher permeation through skin compared to fenretinide-ethosomes or 33% alcohol alone. These findings can be explained by the fact that liposomes work by disrupting the lipid bilayer fluidity of the stratum corneum, resulting in changing the stratum corneum structure and increasing the partitioning of the drug (Fresta and Puglisi, 1996; Maghraby et al., 2006). The liposomes findings are consistent with previous reports (Puglia et al., 2004; Bhatia et al., 2004; E. Ramón et al., 2005). Furthermore, a mechanistic study demonstrated that the lipophilic fluorescent probe models incorporated into liposomes penetrated across the SC and reached the deepest layers of the epidermis, suggesting that lipophilic molecules formulated in liposomes can effectively penetrate through the SC and into deeper layers of the skin (Carrer et al., 2008).

In addition, microneedles significantly enhanced the cumulative amount of fenretinide compared to 66% alcohol. In comparison to 66% alcohol, the application of microneedles increased the flux of fenretinide and decreased the lag time. These findings could be explained by the fact that the micropores created by the microneedles enhanced the diffusion rate of fenretinide and helped the drug to reach the steady state rapidly,
resulting in a higher flux and a shorter lag time. Similar results have been found with different drug molecules in previous reports (Kaur et al., 2014; Nguyen and Banga, 2015). The same findings were found with aspirin and endoxifen in chapter 2 and 3.

In mammary papilla permeation studies, fenretinide did not show any measurable permeation through nipple and this could be due to the higher lipophilicity of fenretinide, resulting in slowing down its transport rate through the mammary papilla. Further studies should focus on the mechanistic pathways of fenretinide through the mammary papilla by using a dye that has a relative high Log P. A recent mechanistic study has evaluated the transport of Nile red dye with a Log P of 5 through mammary papilla and the results have shown that the Nile red was more permeable through skin than mammary papilla (Kurtz and Lawson, 2018).

From both breast skin and nipple permeation results, fenretinide showed better permeation through breast skin. Thus, it is reasonable to apply fenretinide to the entire breast area (breast skin and nipple) in order to increase the concentration of fenretinide within breast tissue. The results from the present study showed that the highest cumulative amount of fenretinide permeated through breast skin at 24 hours is 21.52 μg/ml (Table 32), which is 12-fold higher than the IC$_{50}$ value (1.76 μg/ml) of fenretinide in human breast cancer cells (Lim et al., 2002). From Table 32, the highest delivery rate of fenretinide permeated through breast skin at 24 hours was 1.20 μg per gram of breast tissue per hour, which is close to the IC$_{50}$ value of fenretinide in human breast cancer cells. Further optimization of fenretinide formulations can increase the delivery rate of the drug per hour, leading to a higher therapeutic level of the drug. However, the local
clearance of the drug from the breast tissue may affect the total drug concentration in the breast.

From the in vivo studies, the results demonstrate that the localized transdermal delivery of fenretinide can achieve a higher concentration of fenretinide in the mammary glands and a lower concentration of fenretinide in plasma and other organs, suggesting that the localized transdermal delivery of fenretinide to the breast might lead to a safe strategy and an effective localized therapy approach for breast cancer. Further in vivo time points should be studied to characterize the transdermal fenretinide pharmacokinetics. In addition, in-vivo studies should be done to demonstrate the efficacy of transdermally delivered fenretinide.
Table 32. Transdermal delivery rates of fenretinide

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cumulative at 24 hours ($Q_{24}$) ($\mu g/cm^2$)</th>
<th>Calculated flux ($\mu g/cm^2/h$) ($Q_{24}/24$)</th>
<th>Area ($cm^2$) required to deliver 1 mg in 24 hours ($1000/Q_{24}$)</th>
<th>Delivery rate ($\mu g/h$ per g of tissue)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN</td>
<td>107.61±2.06</td>
<td>4.48±0.09</td>
<td>9.29</td>
<td>1.20</td>
</tr>
<tr>
<td>Sandalwood oil</td>
<td>68.32±0.86</td>
<td>2.85±0.04</td>
<td>14.64</td>
<td>0.76</td>
</tr>
<tr>
<td>Liposomes</td>
<td>75.29±4.82</td>
<td>3.14±0.20</td>
<td>13.28</td>
<td>0.84</td>
</tr>
<tr>
<td>66% alcohol</td>
<td>48.42±1.80</td>
<td>2.02±0.07</td>
<td>20.65</td>
<td>0.54</td>
</tr>
</tbody>
</table>

*The delivery rate values are calculated as the calculated flux ($Q_{24}/24$) multiplied by 200 cm$^2$ (average area of human breast) divided by 750 g of tissue. MN: microneedles.
4.5. Conclusions

The results from the study demonstrate the feasibility of transdermal delivery to the breast. Among the hydroalcoholic vehicles, 66% alcohol showed the highest cumulative amount permeated through breast skin. Among the three chemical penetration enhancers, 5% sandalwood oil showed the highest flux. In comparison to ethosomes, liposomes showed higher flux and permeation. On the other hand, among all the approaches tested, microneedles showed the highest permeation. Furthermore, the results from in vivo studies demonstrated that the localized transdermal delivery of fenretinide to the breast can achieve high fenretinide concentration in the breast with minimal fenretinide concentration in other organs. Overall, the findings from this study show that the high lipophilic drug, fenretinide, can be transdermally delivered to the breast.
5. Summary

The findings from the current work demonstrate the feasibility of localized transdermal delivery of chemopreventive agents, including endoxifen, aspirin and fenretinide, to the breast through breast skin and/or through mammary papilla. Endoxifen (Log P = 5.45) and fenretinide (Log P = 8.03) did not show penetration through mammary papilla, while aspirin (Log P = 1.23) showed permeation through the mammary papilla. The three agents (aspirin, endoxifen and fenretinide) showed penetration through breast skin.

The alcohol concentration in the vehicle influenced the permeation of all three drugs. Higher (66%) alcohol concentration showed higher fenretinide permeation through breast skin; in contrast, the lowest alcohol concentration (33% alcohol) showed the highest permeation of endoxifen and aspirin. In addition, an increase in the alcohol concentration in the vehicle increased the solubility of the three drugs and decreased the epidermal partition coefficient of the three drugs. Also, an increase in the saturation of the drug in the vehicle increased the drug permeation through breast skin in comparison to sub-saturated concentration.

Microneedles showed the best enhancement permeation approach for all three drugs compared to other approaches. Sandalwood oil showed the best chemical penetration enhancer for all three drugs in comparison to other chemical penetration enhancers. Also, liposomes system showed a significant enhancement effect on fenretinide permeation through breast skin, but not for the other two drugs. On the other hand, ethosomes system showed same enhancement effect on aspirin permeation through
the breast skin in comparison to 33% alcohol, and less permeation for endoxifen and fenretinide.

In addition to the in-vitro studies, in-vivo studies showed that microneedles increased the fenretinide concentration in mammary glands compared to 66% alcohol and sandalwood oil. Also, the in-vivo studies demonstrated that localized transdermal delivery of fenretinide to the breast can be an effective approach to maximize the drug concentration in the breast and minimize the drug concentration in other organs.

Overall, the results from the current work demonstrate the feasibility of localized transdermal delivery of promising chemopreventive agents belonging to different therapeutic classes and physicochemical properties through breast skin and/or through mammary papilla. Furthermore, different permeation enhancement methods including hydroalcoholic vehicle systems, chemical penetration enhancers and microneedles showed to be effective approaches for localizing the chemopreventive agents to the breast. The overall summary of the current work is shown in Table 33.
Table 33. Comparison of different permeation enhancement approaches for localized transdermal delivery

<table>
<thead>
<tr>
<th>Permeation enhancement strategy</th>
<th>Aspirin</th>
<th>Endoxifen</th>
<th>Fenretinide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>X</td>
<td>XX</td>
<td>X</td>
</tr>
<tr>
<td>Chemical penetration enhancer</td>
<td>XX</td>
<td>X</td>
<td>XX</td>
</tr>
<tr>
<td>Liposomes</td>
<td>X</td>
<td>X</td>
<td>XX</td>
</tr>
<tr>
<td>Ethosomes</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Microneedles</td>
<td>XXX</td>
<td>XXX</td>
<td>XXX</td>
</tr>
</tbody>
</table>

(X) Fair; (XX) good; (XXX) excellent.
6. Future directions

The findings from this work demonstrate the feasibility of localized transdermal delivery of chemopreventive agents with different physicochemical properties using different penetration enhancement approaches. The results from this study can be used to design future studies as described below.

- Expand the localized transdermal delivery to other anti-cancer agents with similar physicochemical properties.
- Perform mechanistic studies using different dyes that have similar Log Ps to aspirin, endoxifen and fenretinide to understand the drug transport pathways through the skin and mammary papilla with various penetration enhancement methods.
- Characterize the preclinical pharmacokinetics of localized transdermal delivery of aspirin, endoxifen and fenretinide.
- Evaluate the in-vivo efficacy of localized transdermal delivery of aspirin, endoxifen and fenretinide to the breast in preclinical models of breast cancer.
- Study the influence of other chemical penetration enhancers including esters, fatty acids, surfactant among others for transdermal delivery of aspirin, endoxifen and fenretinide to the breast.
- Explore other carriers including microemulsions, nanoemulsions, micelles and solid lipid nanoparticles for localized transdermal delivery of aspirin, endoxifen and fenretinide to the breast.
• Investigate the influence of other physical penetration enhancement methods including application of heat and sonophoresis on the transdermal delivery of aspirin, endoxifen and fenretinide to the breast.
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