

Enhancing the Buccal Permeability Potential of Model ARV Drugs: Permeability and Histo-morphological Evaluations

By

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Date submitted: December 2013

ABSTRACT

Buccal delivery of antiretroviral (ARV) drugs may overcome disadvantages such as low bioavailability due to extensive first pass effects and gastrointestinal degradation associated with the oral route. However, the small mucosal surface area and low membrane permeability are challenges to buccal drug permeation. Identification of new permeation enhancers as well as new permeation enhancing strategies have been shown to overcome these limitations, thereby delivering adequate amounts of drug through the buccal mucosa. Polymeric excipients with previously reported mucoadhesive and controlled release properties could also possess additional buccal permeation enhancing effects and may therefore serve as multifunctional excipients in a buccal drug delivery system. Therefore the aims of this study were: 1) to identify and compare the buccal permeability potential of tenofovir (TNF) and didanosine (ddl). 2) to identify the buccal permeation effects of potential multifunctional excipients i.e. carboxymethylcellulose (CMC), sodium alginate (SA), polyacrylic acid (PAA) and polyethylene glycol (PEG) for TNF and ddl, and 3) to identify the buccal permeation potential of saquinavir (SQV) and assess the effect of high-energy ball milling on its permeability.

All *in vitro* permeation studies across porcine buccal mucosa were performed using vertical Franz diffusion cells with TNF, ddl and SQV being quantified using UV spectrophotometry at 262 nm, 250 nm and 239 nm respectively. The histomorphological evaluations were undertaken by light microscopy (LM) and Transmission Electron Microscopy (TEM). Ball milling of SQV samples for 1, 3, 15 and 30 hours was performed in a high-energy planetary mill. The integrity of the buccal mucosa was assessed by transepithelial electrical resistance (TEER) measurements using a Millicell ERS meter connected to a pair of chopstick electrodes.

Both TNF and ddl were able to permeate the buccal mucosa in a concentration-dependent manner. A higher permeability was observed for ddl (Flux = $181.62 \pm 23.62 \mu\text{g}/\text{cm}^2\text{h}$) as compared to TNF (Flux = $102.10 \pm 19.80 \mu\text{g}/\text{cm}^2\text{h}$). The permeation of these drugs in the absence of enhancers was attributed to passive diffusion via the paracellular route with transcellular route being an additional possibility for ddl. The addition of PAA, SA, CMC and PEG increased the permeability of TNF whilst only PEG was able to increase the permeability of ddl. The effect of these polymeric excipients appeared to be dependent on their ionic charges as well as that of the respective drugs. Permeability enhancement ratios for ddl and TNF were 1.63 and 1.74 respectively with PEG (0.5 %w/v) and CMC (0.5 %w/v) respectively. A maximum enhancement ratio of 2.13 for TNF was achieved with 4 %w/v PEG. Furthermore PEG was identified as the optimal permeation enhancer for TNF and ddl. Histological investigations revealed no significant loss in cellular integrity for mucosa treated with either TNF or ddl alone or when coupled with PEG as an enhancer. The differences in histomorphological changes in response to TNF and ddl alone could support the greater permeation observed with ddl. The histological findings proved useful in assessing the effects of drug and enhancer on mucosal integrity and provided insight into permeation pathways across the mucosa. Selective polymeric excipients therefore provide an effective means to increase the penetration of ddl and TNF. Their previously reported mucoadhesive and controlled release properties coupled with their permeation enhancing effects shown in this study highlight their potential use as multifunctional excipients for the design of buccal drug delivery systems.

SQV, a candidate for buccal drug delivery, is limited by its poor solubility. Therefore, Aim 3 identified the effects of high energy ball milling on the buccal permeability of SQV and compared its enhancing effect to the conventional use of common chemical enhancers together with unmilled SQV i.e. ethylenediaminetetraacetic acid (EDTA), sodium lauryl sulphate (SLS), PEG

and beta cyclodextrin (β -cyclodextrin). Unmilled SQV was able to permeate through the buccal mucosa with a flux of $3.99 \pm 0.11 \mu\text{g}/\text{cm}^2\text{h}$. Ball milling of SQV at all the time periods led to an increase in its permeability with optimal enhancement obtained at 15 hours with an enhancement ratio of 2.61. The enhanced permeability of the milled SQV samples was attributed to a contribution of various factors such as solubility, particle size, surface area, crystallinity, morphology and the formation of solid dispersions. The chemical permeation enhancers were also able to increase the permeability of unmilled SQV across the buccal mucosa, with SLS achieving the greatest enhancement ratio of 1.75. However, ball milling of SQV without any chemical permeation enhancers displayed to a greater enhancement ratio (2.61) as compared to the best permeation enhancer SLS at 0.5 %w/v (1.75). Histological investigations revealed no significant loss in cellular integrity for mucosa treated with either unmilled or milled SQV samples. The presence of larger intercellular spaces in the treated tissue suggests that SQV also uses the paracellular route of transport in combination with the transcellular route across the mucosa. High energy ball milling of SQV is therefore an effective approach for increasing buccal permeability when formulating SQV for a buccal delivery system, as compared to incorporating common chemical enhancers studied at 0.5% w/v for this purpose.

The findings in this study will therefore contribute to formulation optimization strategies for the development of novel buccal delivery systems for ARV drugs, thereby optimising treatment of patients with HIV and AIDS.

Keywords: Buccal permeability, didanosine, histology, permeation enhancer, polymers, tenofovir, saquinavir, ball milling

SUPERVISOR CONSENT

I, Prof. Thirumala Govender as supervisor of the M. Med Science (Pharmaceutics) study titled “Enhancing the buccal permeability potential of model ARV drugs: Permeability and histomorphological evaluations” hereby consent to the submission of this dissertation.

Signed:

Date:

I, Mrs Elizabeth Ojewole as co-supervisor of the M. Med Science (Pharmaceutics) study titled “Enhancing the buccal permeability potential of model ARV drugs: Permeability and histomorphological evaluations” hereby consent to the submission of this dissertation.

Signed:

Date:

DECLARATION ONE – PLAGIARISM

I, Mr Sanjeev K. Rambharose, declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original work.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
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5. Where I have reproduced a publication of which I am an author, co-author or editor, I have indicated in detail which part of the publication was actually written by myself alone and have fully referenced such publications.
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RESEARCH OUTPUTS FROM THE STUDY

PUBLICATIONS

The following original research articles were published in international ISI peer reviewed journals from data generated during this study:

1. Rambharose S, Ojewole E, Mackraj I, Govender T. Comparative buccal permeability enhancement of didanosine and tenofovir by potential multifunctional polymeric excipients and their effects on porcine buccal histology. **Pharmaceutical Development and Technology** 2013; doi:10.3109/10837450.2012.752505
2. Rambharose S, Ojewole E, Branham M, Kalhapure R, Govender T. High-energy ball milling of Saquinavir increases permeability across the buccal mucosa. **Drug Development and Industrial Pharmacy** , 2013, In Press (Ref: LDDI-2013-0486.R1).

CONFERENCE PRESENTATIONS

Research findings from this study were presented at various conferences as follows:

1. Sanjeev Rambharose, Elizabeth B. Ojewole, Irene Mackraj and Thirumala Govender. Identifying the buccal permeability potential of Tenofovir, 2nd conference on "Innovation in drug delivery : From Preformulation to Development through Innovative Evaluation Process", Aix-en-Provence (France), 3-6 October 2010.
2. Sanjeev Rambharose, Elizabeth B. Ojewole, Irene Mackraj and Thirumala Govender. Investigating the Potential of Multifunctional Excipients for Enhancing the Permeability of Tenofovir (TNF), 71st congress of FIP, Hyderabad –India, 3- 8 September 2011.
3. Sanjeev Rambharose, Elizabeth Ojewole, and Thirumala Govender. Nanosizing by high-energy ball milling increases buccal permeability of Saquinavir, 9th Central European Symposium on Pharmaceutical Technology with focus on Nanopharmaceuticals and Nanomedicine, LIBERTAS RIXOS Hotel in Dubrovnik, Croatia, 20-22 September 2012.

4. Sanjeev Rambharose, Elizabeth B. Ojewole, Irene Mackraj and Thirumala Govender. Identifying the buccal permeability potential of Tenofovir, 31st Annual Conference of the Academy of Pharmaceutical Sciences of South Africa, Limpopo, 14-17 September 2010.

5. Sanjeev Rambharose, Elizabeth B. Ojewole, Irene Mackraj and Thirumala Govender. Investigating the Potential of Polyethylene Glycol (PEG) for Enhancing the Buccal Permeability of Tenofovir (TNF) and Didanosine (DDI), 6th International Conference on Pharmaceutical and Pharmacological Sciences, Coastlands Hotel, Umhlanga, Durban, SA, 25-27 September 2011.

DECLARATION TWO – PUBLICATIONS

Details of contributions to publications that form part and/or include research presented in this thesis (*include published and accepted articles giving details of the contributions of each author to the publication.*)

Article 1: Rambharose S, Ojewole E, Mackraj I, Govender T. Comparative buccal permeability enhancement of didanosine and tenofovir by potential multifunctional polymeric excipients and their effects on porcine buccal histology. **Pharmaceutical Development and Technology** 2013; doi:10.3109/10837450.2012.752505

Mr S. Rambharose performed all experimental work and specifically undertook the harvesting and excision of buccal mucosa from pigs, and also performed all *in vitro* permeation studies, drug quantification and LM/TEM experiments. He contributed to modification of methods, data analysis and interpretation and writing of the paper. Professor I. Mackraj assisted with interpretation of some histomicrographs. The remaining authors served as supervisor and co-supervisor.

Article 2: Rambharose S, Ojewole E, Branham M, Kalhapure R, Govender T. High-energy ball milling of Saquinavir increases permeability across the buccal mucosa. **Drug Development and Industrial Pharmacy**, 2013, In Press (Ref: LDDI-2013-0486.R1).

Mr S. Rambharose performed all experimental work and specifically undertook the ball milling of saquinavir samples, harvesting and excision of buccal mucosa from pigs, and also performed all *in vitro* permeation studies, drug quantification, LM/TEM and TEER experiments. He contributed to modification of methods, data analysis and interpretation and writing of the paper. Dr M. Branham is a post doctorate fellow that provided guidance on the ball milling technique and Dr R. Kalhapure is a post doctorate fellow that assisted with interpretation of solid state characteristics of SQV. The remaining authors served as supervisor and co-supervisor.

Signed:

Date:

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LIST OF ABBREVIATIONS

β-cyclodextrin	Beta cyclodextrin
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance
ARV	Antiretroviral
ATR-IR	Attenuate total reflectance-infrared spectroscopy
AZT	Zidovudine
CMC	Carboxymethyl cellulose
ddl	Didanosine
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
EDTA	Ethylenediamineteraacetic acid
ER	Enhancement ratio
GIT	Gastrointestinal tract
H&E	Hematoxylin and Eosin
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
LM	Light microscopy
MW	Molecular weight
NDDS	Novel drug delivery system
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitor
NS	Normal saline
PAA	Polyacrylic acid
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RNA	Ribonucleic acid
SA	Sodium alginate
SD	Standard deviation
SLS	Sodium lauryl sulphate
SQV	Saquinavir
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
TGA	Thermogravimetric Analysis
TNF	Tenofovir
UNAIDS	Joint United Nations Programme on HIV/AIDS
UV	Ultraviolet
WHO	World Health Organization
XRD	X-ray diffractometry

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CHAPTER 1 - INTRODUCTION

1.1 INTRODUCTION

This chapter describes the background and rationale for this study, outlining the challenges encountered with current antiretroviral therapy and the approaches to overcome these limitations. The significance and the novelty of the study are also outlined. This chapter is concluded with the overview of this dissertation.

1.2 BACKGROUND TO THIS STUDY

Oral antiretroviral (ARV) drug therapy has significantly improved the treatment of human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS), which are considered among the most serious public health diseases (1,2) in South Africa. With current statistics indicating that more than 2.3 million new infections worldwide were recorded in 2012, it is evident that the prevalence of the disease is still high. This is also the case in sub-Saharan Africa, where there are currently more than 25 million people living with HIV (3). With no cure for HIV or an effective vaccine, ARVs are the only treatment available to patients living with HIV. The benefits of current treatment are a reduced risk of progression to AIDS and a decreased risk of early death. Treatment reduces the risk of acquiring opportunistic infections such as tuberculosis and also improves mental and physical health.

Additional benefits include reduced mother-to-child transmission and also a decreased risk of transmission of the disease to sexual partners (4). The effectiveness of treatment depends on patient compliance, the complexity of treatment regimens (due to pills burden and dosing frequency) and adverse effects, which may reduce patient adherence (4). Some ARV drugs have low bioavailabilities due to extensive gastrointestinal degradation and first pass

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metabolism, and have short half-lives, requiring frequent drug administration. Their frequent administration leads to severe dose-dependent side-effects, which further exacerbate the decrease in patient compliance (5). All these limitations i.e. decreased patient compliance, low bioavailability and short half-lives of many of these ARV drugs, negatively impact on the effective treatment of HIV and AIDS. Successful treatment largely depends on the ARV plasma concentration being relatively high in order to completely suppress viral replication (6).

In order to address these limitations of ARV treatment, formulation scientists have been exploring the use of novel drug delivery systems and alternate routes of drug administration (5). Alternate routes of drug administration that obviate the gastrointestinal tract as well as hepatic first pass metabolism and deliver drug directly into the systemic circulation will be beneficial to many ARVs that are susceptible to the above mentioned limitations. Therefore, there has been much interest in the use of the mucosal lining of body cavities as alternative routes of drug administration (7). Potential sites for this type of drug delivery are: the oral cavity, vaginal, rectal, and nasal as well as the ocular route. The oral cavity comprises of three different categories of drug delivery i.e. sublingual, buccal and local drug delivery (8). These oral mucosal sites differ significantly from each other in terms of structure, the ability to retain a delivery system as well as permeability.

Compared to the skin for transdermal delivery, the permeability of the buccal mucosa is higher, cell recovery is more rapid, and it has easier access than nasal, rectal and vaginal mucosa (9). The buccal mucosa is also more resistant to irritation, and has greater patient acceptability than other non-oral routes. The buccal mucosa is more suited for sustained/controlled release dosage forms than the rest of the oral mucosa. This route is also advantageous for paediatrics and patients with swallowing problems. The buccal mucosa therefore presents as an attractive

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site for ARV drug delivery (10, 11). While ARV drugs have been more widely studied for their transdermal route of administration (12-15), research on their buccal permeability properties appear to be limited. The literature indicates that only Didanosine and Zalcitabine have been studied for their buccal permeation properties (16, 17), necessitating further studies on optimizing their delivery via this route.

The advantages of the buccal route has led to the design and evaluation of various buccal delivery systems, such as wafers, films, patches, tablets, pastes and gels (12). However, one of the main disadvantages of the buccal route is the low mucosal permeability, necessitating the use of various classes of permeation enhancers to facilitate drug permeation (18). The permeability of drugs from these delivery systems can therefore be improved with the incorporation of permeation enhancers into the formulations. Both physical as well as chemical methods have been investigated to enhance the permeation of drugs across the buccal mucosa. Chemical permeation enhancers are most widely studied for the buccal route (18). There are several classes of chemical permeation enhancers, including surfactants, fatty acids, bile salts, inclusion complexes, chelators, and polymers (19). These enhancers alter mucosal permeability by various mechanisms that decrease the barrier properties of either the mucosal surface, or the transport pathways across the mucosa.

In order to facilitate prolonged retention of the formulation on the mucosa allowing for sustained delivery and permeation, buccal delivery systems also need to display mucoadhesivity, i.e. they need to adhere to the mucus layer covering the buccal mucosa. To meet the challenges of buccal delivery, the key properties needed for a buccal drug delivery system are drug permeation enhancement, mucoadhesivity and controlled drug release. The identification of formulation excipients to provide these properties is therefore critical. Various polymers, such as

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cellulose derivatives, and those based on polymethacrylic acid, polysaccharides and alginic acid, have successfully shown mucoadhesive and sustained drug release properties (20).

The identification of excipients with potential multifunctional properties, such as mucoadhesivity, drug permeation enhancement and controlled drug release, will significantly contribute to the optimization of the design of buccal delivery systems. The multifunctional properties required for buccal delivery systems have led investigators to study, different classes of excipients separately, as permeation enhancers and polymers that have either or both mucoadhesive and controlled release properties. There is a need for the use of multifunctional pharmaceutical excipients in the design of buccal delivery systems, i.e. excipients that could simultaneously provide mucoadhesivity, sustained drug release and permeation enhancement. The use of multifunctional excipients for buccal drug delivery will decrease the number and quantity of excipients required in product formulation. The advantages will include decreased manufacturing costs, adverse effects, allergic reactions and excipient incompatibilities.

In addition, for drugs that have poorly aqueous solubility, approaches that increase this may also prove advantageous for their buccal delivery. Common approaches used to enhance low aqueous solubility of drugs include methods that reduce particle size (including microsizing and nanosizing), increase their solubilisation in co-solvents, improve their complexation with cyclodextrins, or for the delivery of lipophilic drugs, use lipid-based vehicles as well as solid dispersion technology (21,22). The success of increasing the solubility of a poorly soluble drug is dependent on the physicochemical properties of that drug, although many of these techniques have been shown to be effective at enhancing oral bioavailability for specific compounds. Poorly soluble drugs, including some poorly soluble ARVs, have been presented in the literature using a number of these strategies to enhance their aqueous solubility.

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The formation of amorphous solids or solid dispersions also presents a promising approach to overcome limited solubility of poorly soluble drug (23). Although milling is an approach widely used to reduce particle size, it is not commonly used for making amorphous dispersions, despite being shown to be suitable for this purpose (23). Recent studies have shown that ball milling is a viable process for producing amorphous and solid dispersions (24, 25). The advantage of high energy ball milling is generating an amorphous system while simultaneously stimulating the formation of reduced particle size (26). Using such a technique for a drug with low aqueous solubility to improve its solubility could prove to be very beneficial, with regards to its buccal permeability potential. This technique could therefore obviate the use of chemical permeation enhancers in formulations that incorporate a poorly soluble drug, thus limiting the risks associated with the use of chemical enhancers. If successful, this strategy would widen the pool of approaches used to enhance permeation across the buccal mucosa.

1.2 AIMS AND OBJECTIVES OF THIS STUDY

The aims of this study were therefore to:

1. Identify and compare the buccal permeability potential of tenofovir (TNF) and didanosine (ddl).
2. Identify potential multifunctional excipients as buccal permeation enhancers for TNF and ddl.
3. Identify the buccal permeation potential of saquinavir (SQV), and assess the effect of high-energy ball milling on the buccal permeability of SQV.

In order to achieve Aims 1 and 2 the objectives were to:

- Determine and compare the *in vitro* buccal permeability parameters for TNF and ddl.

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- Assess the histomorphological effects of TNF and ddl on the buccal mucosa.
- Determine the effects of poly (acrylic acid) (PAA), sodium alginate (SA), poly(ethylene glycol) (PEG) and carboxymethylcellulose (CMC) as possible multifunctional excipients on the permeability parameters of TNF and ddl.
- Determine the concentration effects of the best identified multifunctional excipient on the permeability parameters of TNF and ddl, as well as to assess the histomorphological effects of this excipient on the buccal mucosa.

In order to achieve Aim 3 the objectives were to:

- Identify the *in vitro* buccal permeability parameters of unmilled SQV and ball milled SQV.
- Compare the permeation enhancement effect of ball milled SQV to common chemical enhancers.
- Assess the histomorphological effects of unmilled SQV and milled SQV samples on the buccal mucosa.

1.3 SIGNIFICANCE OF THIS STUDY

The findings of this study will be significant as there are currently very limited reports on the buccal mucosa as an alternate route of drug administration for ARVs. The successful delivery of the model ARV drugs (TNF, ddl and SQV) via this route would overcome several disadvantages associated with their current therapy. The successful identification of polymers that have mucoadhesive, controlled release as well as buccal permeation enhancement properties would decrease the amount of excipients being incorporated into a dosage form as one multifunctional excipient would bring about these desired affects. The advantages of such a formulation would be decreased drug-polymer interaction, as the numbers of polymers are

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limited in the formulation. There would also be a decrease in adverse or allergic reactions to the patient, and also a decrease in excipient incompatibilities. This type of formulation would also decrease manufacturing costs.

The identification of high-energy ball milling as an alternate method to chemical permeation enhancers for buccal permeation enhancement will widen the pool of available strategies used for permeation enhancement. Methods that preclude the use of chemical enhancers for this purpose will decrease the reliance of incorporating chemical enhancers into buccal drug delivery formulations. The advantages of using high-energy ball milling as a strategy would include decreased chemical interactions or incompatibilities experienced between excipients and drugs in drug formulations, as high-energy ball milling is a physical method of promoting drugs permeability across the buccal mucosa. Therefore, this study will contribute to formulation optimization strategies for buccal delivery systems of ARV drugs.

1.4 NOVELTY OF THIS STUDY

While other routes for the delivery of antiretroviral drugs have been extensively studied and reported, there is a scarcity of data on their buccal delivery potential, which can overcome several disadvantages associated with current therapy. For the first time, this study identifies the buccal delivery potential of tenofovir (TNF). It also compares the permeability potential of tenofovir, and didanosine, two different classes of ARV drugs. Histomorphological evaluations are also used to predict their suitability for retention on the mucosae and to predict possible permeation mechanisms. The identification of excipients possessing multifunctional properties such as mucoadhesivity, controlled release and buccal permeation would be useful for the formulation of buccal drug delivery systems. To date, the only polymeric excipient reported so

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far with these three properties is chitosan. This study shows for the first time that PEG, PAA, SA and CMC as polymeric excipients, also have buccal permeation enhancing properties and can therefore be considered as multifunctional excipients for buccal delivery systems. The study also shows that neither of the two ARV's alone, or in combination with PEG has any adverse effects on the cellular integrity of the buccal mucosa.

Ball milling is widely used as an approach to reduce particle size, as well as for making amorphous dispersions that serve to increase the solubility of poorly soluble drugs. We show for the first time that ball milling of SQV can be used as a physical method for enhancing its permeation across the buccal mucosa. This study therefore displays that ball milling can be used as an attractive alternative to chemical enhancers for buccal permeation. For the first time, this study also reports on the buccal delivery potential of Saquinavir (SQV).

1.5 OVERVIEW OF THIS DISSERTATION

Chapter 1 provides an introduction and outlines the background to the study, describing the challenges faced with current antiretroviral drug therapy and drug delivery via the buccal route. This chapter also explores the rationale for the study and highlights the aim and objectives of the study.

Chapter 2 is a literature review, focusing on HIV/AIDS with the current treatments, its limitations and strategies employed to overcome these limitations. This chapter also describes the buccal route of drug delivery focusing on the structure, advantages and disadvantages of this route, barriers of the buccal mucosa to permeation, routes available for permeation across the mucosa and methods used to assess mucosal permeation. It explains the need for buccal permeation enhancers, as well as the approaches and classes of both physical and chemical enhancers.

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The mechanisms of chemical permeation enhancers are described in this chapter. This chapter describes the evaluation of buccal mucosal integrity testing during permeation experiments.

Chapter 3 is an original research article published in an ISI international journal: Journal of Pharmaceutical Development and Technology, titled: “Comparative Buccal Permeability Enhancement of Didanosine and Tenofovir by Potential Multifunctional Polymeric Excipients and their Effects on Porcine Buccal Histology.” This chapter is presented in the required format of the journal and is the final revised accepted version.

Chapter 4 is an original research article accepted for publication in an ISI international journal: Drug Development and Industrial Pharmacy, titled: “High-energy ball milling of Saquinavir increases permeability across the buccal mucosa.” Manuscript ID: LDDI-2013-0486.R1. This chapter is presented in the required format of the journal and is the final revised accepted version.

Chapter 5 is comprised of the conclusions and future recommendations of the study.

Chapter 1 - Introduction

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CHAPTER 2 – LITERATURE REVIEW

INTRODUCTION

This chapter provides an overview of literature relevant to the background of this study. This includes the current status of HIV infections, current therapy for HIV and AIDS and the limitations to the current therapy. The review describes the approaches to overcome these limitations, and the buccal route as an alternate route of drug administration. The advantages and disadvantages associated with the buccal route are outlined, together with the methods employed to study permeation, and the current strategies to enhance drug permeation, across the buccal mucosa.

2.1 HIV AND AIDS

Human immunodeficiency virus (HIV) is an infectious agent that causes Acquired Immune Deficiency Syndrome (AIDS). HIV and AIDS are serious public health diseases and one of the main causes of death in sub-Saharan Africa (1, 2). There are currently 35.3 million people living with HIV globally, of whom 71% live in sub-Saharan Africa. Although new HIV infections have been reduced by 33% globally since 2001, there were 2.3 million new infections in 2012 (3). Despite the decrease in the number of new infections, recent statistics indicate that much still remains to be done to stem the pandemic.

HIV is a retrovirus that primarily infects components of the human immune system such as, macrophages, dendritic cells and CD4⁺ T cells, and directly and indirectly destroys the latter. Once inside the cell the viral RNA genome is converted into double-stranded DNA by a virally encoded reverse transcriptase which is transported with the viral genome in the virus particle. The virus and the infected host cell can avoid detection by the immune system if the virus

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becomes latent after integration with the host cell. Alternatively, the replication cycle can be initiated if the virus is transcribed, producing new RNA genomes and viral proteins (4,5).

Two types of HIV have been characterized: HIV-1 and HIV-2. HIV-1 is more virulent and more infective, and is the cause of the majority of HIV infections globally (5). Ultimately, HIV causes AIDS by depleting CD4⁺ T cells that weakens the immune system and allows opportunistic infections. The primary causes of death from HIV and AIDS are opportunistic infections, which is frequently the result of the progressive failure of the immune system (5). In 2012, Tuberculosis co-infection was at the forefront of the sicknesses that caused death in those with HIV and AIDS. Tuberculosis is present in a third of all HIV infected people, and caused 25% of HIV related deaths (6). HIV can also invade the central nervous system; causing severe neurological damage and loss that often leads to HIV associated dementia. Without treatment, HIV infection is nearly uniformly fatal (5).

2.1.1 Current therapy for HIV and AIDS

There is currently no cure or effective HIV vaccine, although ARV drug therapy has significantly improved the treatment of HIV and AIDS (1,2). Presently, ARV drugs are classified under the following four categories including, protease inhibitors (PI), nucleoside reverse transcriptase inhibitors (NRTI), nucleotide reverse transcriptase inhibitors (NtRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). Treatment of HIV and AIDS consists of Highly Active Antiretroviral Therapy (HAART), which is a combination of at least three medications belonging to at least two types of antiretroviral drugs (5, 7) and slows the progression of the HIV infection. As of 2012, more than 9.7 million people were taking ARVs in low and middle income countries (3). Treatment of HIV and AIDS also includes the preventive and active treatment of opportunistic infections.

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2.1.2 Limitations to current therapy

Although oral antiretroviral drug therapy has significantly improved the treatment of HIV infection and AIDS, several disadvantages are associated with their use. Many ARV drugs have low bioavailabilities, due to extensive hepatic first pass effects and gastrointestinal degradation. The half-lives for several ARVs are short, thus requiring frequent administration of doses which can decrease patient compliance. With frequent dosing, patients are exposed to the risk of severe dose-dependent side-effects. Some ARV drugs also experience formulation problems due to their physicochemical properties such as poor solubility (2,5).

2.1.3 Approaches to overcome limitations

Strategies being explored to overcome these limitations include assessing various dosing regimens, chemical modification of current drugs (8,9), as well as the development of novel drug delivery systems (NDDS) and alternate routes of drug administration for current and new ARV drugs (5). ARVs that are susceptible to the above mentioned limitations (see 2.1.2) will benefit from these proposed strategies, leading to effective ARV treatment and increased patient compliance (2,5). NDDS being explored for ARV drugs include polymeric micelles (10), enteric coated and extended release tablets (11), nanoparticles (12,13), liposomes and niosomes (14,15). The alternate routes for ARV drug administration being explored are transdermal (16), vaginal (17), nasal (18), rectal (19) as well as the buccal (20) routes. The focus of this study is the buccal route of drug administration, and is described in the following sections.

2.2 BUCCAL ROUTE OF DRUG DELIVERY

Most drugs are conventionally introduced into the systemic circulation via the oral route (21). However certain drugs exhibit low bioavailability as many of these drugs are subjected to pre-systemic drug elimination within the gastrointestinal tract and the hepatic system. Drug candidates with this property for the treatment of either local or systemic diseases are emerging and therefore novel / alternative routes of drug administration are becoming more necessary (21,22).

There has been much interest in the use of the mucosal lining of the body cavities as alternative routes of drug administration. As indicated above, there are a number of potential sites for the alternative route of drug delivery. The mucosa lining of the oral cavity comprises of three different categories for drug delivery, i.e. sublingual, buccal and local (e.g. gingival) (23). The oral mucosal sites differ significantly from each other in terms of structure, the ability to retain a delivery system as well as permeability. The sublingual route is by far the most widely studied routes, while the sublingual area is not suited for sustained/controlled drug release as it generally provides rapid absorption and high bioavailabilities. The buccal mucosa, which is found lining the inside of the cheek, has been explored as an alternative route for drug delivery (21-24). Although the buccal mucosa is less permeable than the sublingual mucosa, it is not able to give a rapid onset of absorption although it is more suitable for a sustained/controlled release formulation (21).

2.2.1 Structure of the buccal mucosa

The buccal mucosa refers to the mucosa that lines the inside of the cheeks, and is made up of approximately 40-50 cell layers, giving it a thickness of between 500 and 800 μm (25). The primary role of the buccal mucosa is to protect the underlying tissue from foreign agents. The

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buccal mucosa is a lining mucosa and therefore is nonkeratinized, as compared to the keratinized masticatory mucosa lining the oral cavity. Anatomically, the buccal mucosa consists of a basement membrane that supports the outermost layer of stratified squamous epithelium.

The basement membrane provides the required adherence between the connective tissue and the epithelium (22, 25). The epithelial cells at the basement membrane are actively mitotic, and these serve to replenish damaged/dead cells as they advance through the intermediate layers to the superficial layer. This ability, coupled with the fast turnover time of 3-8 days, lends to the 'rebound' characteristic of the buccal mucosa. . The epithelial cells increase in size and become progressively flatter as they travel from the basal cell layers to the superficial layers (25). The basal cells have characteristically larger intercellular spaces between them, compared to cells closer to the surface, which are more closely packed. The buccal epithelium is composed of many loose intercellular links, such as desmosomes, hemidesmosomes and gap junctions, which is similar to nasal and intestinal mucosa.

Beneath the basement membrane lies the lamina propria, followed by the submucosa as the innermost layer. The lamina propria serves as a support for the basement membrane and the epithelial cells that lie on it (26, 27). This region is comprised mainly of connective tissue, such as collagen and smooth muscle. The lamina propria is richly supplied with capillaries and small vessels that drain directly into the internal jugular. This capillary network also serves to provide nutrition for all layers of the mucosa. The stratified squamous epithelium of the buccal mucosa is relatively thicker than the rest of oral mucosa, which therefore makes the buccal mucosa more resistant to permanent damage during the application of a dosage form (25,28). (Figure 2.1)

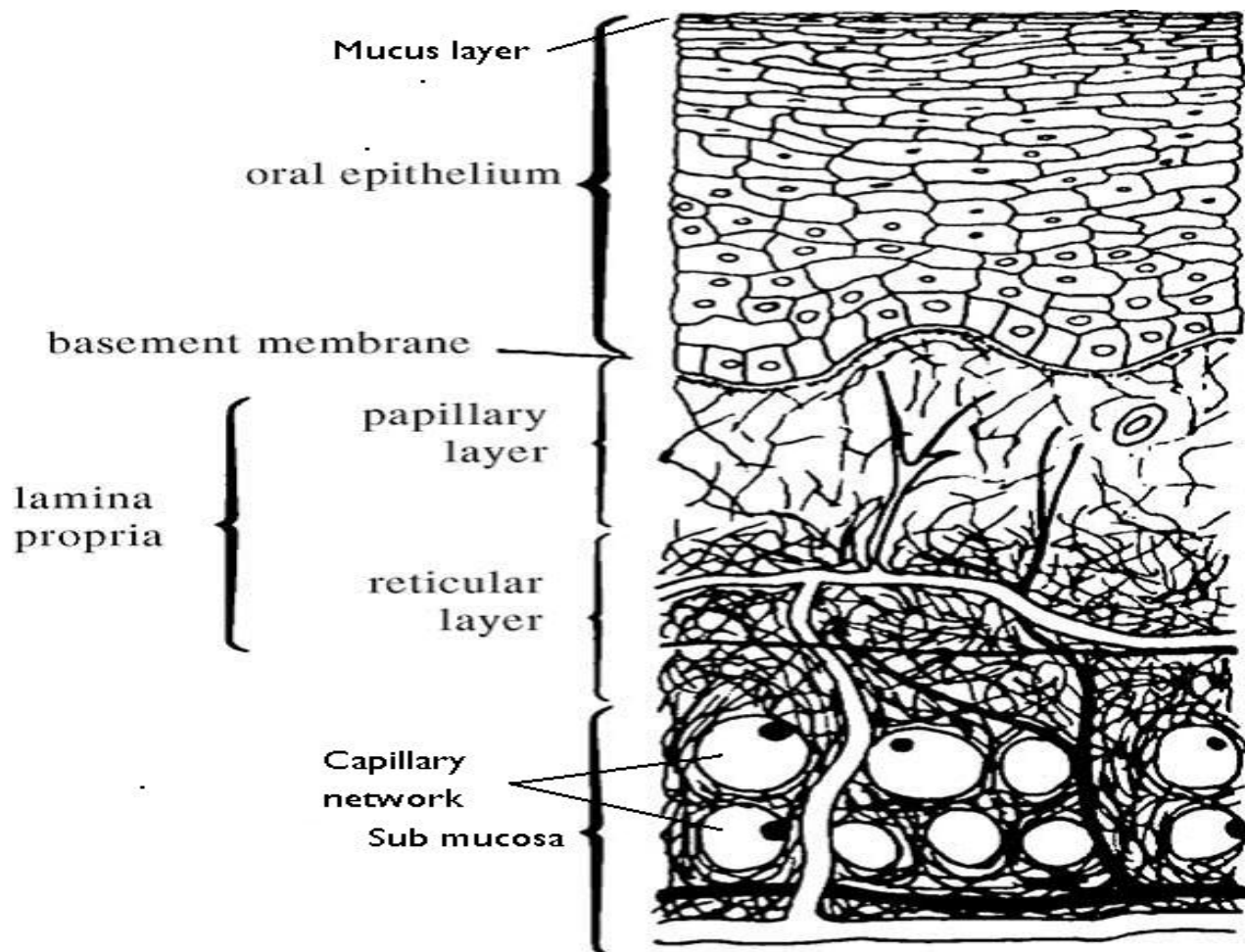


Figure 2.1: Cross section of the buccal mucosa (29)

2.2.2 Advantages of the buccal route

Drug administration via the buccal route has the following advantages:

- The bioavailability of drugs administered via this route is increased as degradation in the gastrointestinal tract and hepatic portal system is avoided. As compared to orally administered drugs, drugs administered via this route are protected from degradation due to the digestive enzymes and pH of the gastrointestinal tract (28,30).
- The buccal mucosa also has an expanse of smooth muscle and relatively immobile mucosa that makes it a more desirable region for retentive systems used for oral trans-

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mucosal drug delivery, making it more suited morphologically for sustained drug delivery applications (28).

- Drugs can be administered in unconscious or incapacitated patients, demonstrating convenience of administration as compared to oral medications (30).
- There is a relatively rapid onset of action compared to the oral route, and the formulation can be removed if therapy is required to be discontinued (25).
- Although less permeable than the sublingual area, the buccal mucosa is well vascularized, and drugs can be rapidly absorbed into the venous system underneath the oral mucosa (21).
- Oral mucosal drug delivery is less variable between patients, resulting in lower inter-subject variability as compared to transdermal patches (30).
- There is rapid cellular recovery of the localized site on the smooth surface of the buccal mucosa (21).

2.2.3 Disadvantages of the buccal route

Although the buccal mucosa presents an attractive site for alternate route of drug delivery, there are however some limitations to this selection.

- There is low permeation across the buccal mucosal membrane due to its barrier properties. It also has a relatively smaller absorptive surface area in comparison to the small intestine (21,25).
- Drugs that are unstable at buccal pH cannot be administered by this route (23).
- For systemic delivery, the relative impermeability of oral cavity mucosa, with regard to drug absorption, especially for large hydrophilic drugs, is a major concern (27).

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However, the advantages of this route render these disadvantages less significant, which have led to substantial research in controlled-release buccal drug delivery systems internationally (25).

2.2.4 Barriers to drug permeation across the buccal mucosa

Comparatively, the major limitation of the buccal route is its low mucosal permeability, especially to drugs with larger molecular weights (31), which has been attributed to many factors. The mucosal lining all the regions of the oral cavity has a lower permeability than that of the intestinal mucosa, which is due to factors such as anatomical differences in the compositions of these tissues, as well as the considerable difference in the surface area available for permeation (23,32). When compared to the permeability between the various regions within the oral cavity, although the buccal is not the most permeable, it is best suited for a sustained drug delivery system (25). The permeability order of the mucosa in the oral cavity indicates the palatal mucosa to be less permeable than the buccal mucosa, which in turn is less permeable than the sublingual mucosa. These differences in permeability have been largely attributed to the degree of keratinization, as well as to the thickness of the mucosa in the different regions (28).

The barrier properties of the buccal mucosa are due to the upper one-third to one-quarter layer of the buccal epithelium. As basal cells migrate towards the surface of the epithelium, they differentiate and become more flattened, with the subsequent accumulation of lipids and proteins. This further culminates in a portion of the lipid becoming concentrated into small organelles called membrane-coating granules (MCGs) (22, 33), which are found in almost all stratified squamous epithelium. The MCGs fuse with the intercellular spaces close to the surface of the mucosa and release the lipid lamellae. These released lamellae subsequently

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fuse from end to end to form broad lipid sheets in the extracellular matrix, creating the main barrier to buccal permeation (23).

When crossing the cellular membranes, this transcellular route involves crossing the polar and lipid domain, whereas the paracellular route implicates diffusion through the extracellular lipid domain. The lipid matrix of the extracellular spaces therefore play a role in the barrier function of the buccal mucosa, especially if the compounds are hydrophilic and have high molecular weights (23-28). Macromolecules and polar compounds traverse the buccal mucosa, utilizing the paracellular route of transport. Intercellular tight junctions are one of the major barriers to this route of transport (21, 25).

The mucosa also consists of an enzymatic barrier that is present at the surface as well as within the mucosa. Although this barrier is less effective than that of the GIT, drugs administered via the buccal route need to overcome this barrier in order to reach the systemic circulation. A drug will only encounter some of the enzymes of the enzymatic barrier. This is dependent on the physicochemical properties of the drug, which dictates its route of transport across the mucosa (28, 30). The surface epithelial cells of the buccal mucosa are surrounded by mucus, which, although it serves as an excellent delivery vehicle by acting as a lubricant, can form a strongly cohesive gel structure that binds to the epithelial surface as a gelatinous layer, posing as a barrier to the permeation of drugs (34). While the superficial layers of the oral epithelium are the primary barrier to the permeation of drugs; the basement membrane may also present some resistance to permeation, limiting the passage of materials across the junction between epithelium and connective tissue (28, 29, 35).

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2.2.5 Factors affecting drug absorption

Various factors of the drug molecule influence the permeation of the molecule across the buccal mucosa. These factors include the molecular weight and size of the drug, the lipid solubility, pKa of the drug, degree of ionization and pH of the drug solution and the presence or absence of permeation enhancers, all affect the absorption and the permeation of drugs through the oral mucosa. These factors are additional to the biochemical and anatomical characteristics of the buccal mucosa that are responsible for the barrier function and permeability, (28,30,36). These factors need to be considered when designing a drug formulation for buccal delivery, so as to ensure that the combination of the chosen drug and excipients in the formulation allow for optimum permeation across the mucosal membrane.

2.2.6 Routes of permeation across the buccal mucosa

Transporting the substances across the epithelial membranes can occur either by simple passive diffusion, active transport or carrier mediated diffusion in addition to other specialized mechanisms (21). Simple passive diffusion is the major mechanism involved in transporting drugs across the buccal mucosa, with the two major routes being the intercellular (paracellular) and the intracellular (transcellular) pathways. The mechanisms, pathways and efficiency of drug permeation through the mucosa can be influenced by the physicochemical properties of the drug (27,28). Depending on these properties, and as the buccal epithelium is stratified, a permeant can use both these routes simultaneously, with one route being usually preferred over the other, that being the one that provides the least amount of hindrance to passage (29,37). The cell membrane acts as the major barrier to permeation of hydrophilic compounds, as it is lipophilic in nature, and the intercellular spaces are the major barrier to the permeation of lipophilic compounds, as it is hydrophilic in nature (35).

2.3 METHODS USED TO EVALUATE MUCOSAL PERMEATION

Identifying permeability properties of a drug and permeation enhancer are critical prior to formulation development, with the ability of a drug to permeate the barrier being assessed by *in vitro*, *ex vivo*, or *in vivo* methods (21). The low availability of human buccal tissue for experimental use has led to the application of various animal models that closely resemble human tissue. The main concern of choosing a particular animal model is the resemblance of its oral mucosa to that of the human, both in enzymatic activity and ultra-structure. Porcine (pig) buccal mucosa has been considered to be a representative model for human buccal mucosa. The porcine mucosa resembles that of human more closely than any other animal model in terms of composition and structure, and being non-keratinized, its membrane morphology, lipid content and composition and permeability barrier functions (21,23,25,38).

Rapid and efficient evaluation of drug permeation across the buccal mucosa has been facilitated by the development of cultured normal human cells. Stratified cultured TR146 cell layers have been recommended as a valuable new *in vitro* model for permeability studies, as they are equivalent to normal human buccal epithelium. These cells resemble stratified human tissue and have originated from a buccal carcinoma. This cell line has been shown to have similar permeability properties, morphology and ultra structure compared to the buccal mucosa. Studies on bidirectional permeability have displayed close correlation to the data obtained using human, monkey and porcine buccal mucosa (30,34).

The permeability of various drugs across the oral mucosa has been determined using various commercially available diffusion devices such as the vertical and horizontal Franz cells, continuous flow perfusion chambers, Ussing chambers, Sweetana-Grass diffusion chambers and a choice of various side-by-side flow through cells (25,39). Table 2.1 shows a summary of

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recent studies that performed *in vitro* diffusion experiments across the buccal mucosa of animal models. The most common diffusion device being used for *in vitro* buccal permeation studies is the Franz diffusion cell, and the most common animal model utilized is the porcine (Table 2.1).

Table 2.1 Recent *in vitro* diffusion studies across the buccal mucosa using animal models.

DIFFUSION DEVICE	DRUG/PERMEANT	ANIMAL MODEL	REFERENCE	Year of Publication
Franz diffusion cell	Carmabazepine, triamcinolone acetonide	Porcine	(40)	2011
Horizontal permeation cells	Buspirone, bupivacaine, antipyrine, caffeine	Porcine	(41)	2011
Franz diffusion cell	Omeprazole	Porcine	(42)	2010
Franz diffusion cell	Ondansetron HCL	Porcine	(43)	2011
Franz diffusion cell	Glibenclamide	Sheep	(44)	2011
Franz diffusion cell	Naltrexone HCL	Porcine	(45)	2011
Ussing chambers	Metoprolol	Porcine	(46)	2013
Franz diffusion cell	Phenylephrine	Porcine	(47)	2011
Franz diffusion cell	Opioid peptide (DPDPE)	Porcine	(48)	2011
Franz diffusion cell	5-Fluorouracil	Guinea Pig	(49)	2009
Franz diffusion cell	Didanosine	Porcine	(20)	2012
Franz diffusion cell	Ropinirole	Porcine	(50)	2012
Franz diffusion cell	Salmon Calcitonin	Porcine	(51)	2011
Franz diffusion cell	Didanosine	Porcine	(52)	2013

2.4 BUCCAL PERMEATION ENHANCERS

Diffusion is solely responsible for the non-enhanced delivery of a drug across the buccal mucosa, which could have inadequate permeability of certain drugs, depending on their physicochemical properties (21-24). The lower permeability of the buccal mucosa, coupled with the limited surface area available for permeation, leads to inadequate absorption of drug into the systemic circulation. Therefore, various enhancement strategies are required to deliver therapeutically relevant amounts of the drug into the systemic circulation. One of the limiting factors for many drugs in the development of a buccal delivery device is membrane permeation (25-28,30,35). Permeation enhancers are substances that promote the absorption of a drug

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through the buccal mucosa. They could promote the permeation of drugs through the epithelium of the buccal mucosa by decreasing their barrier properties by different mechanisms. Generally, enhancement in buccal drug delivery is sub-divided into physical or chemical methods (53), and finding a safe and effective permeation enhancer is a priority.

2.4.1 Physical methods

A number of physical methods of permeation enhancement have been explored, with supersaturation, thermal enhancement, iontophoresis, electroporation and sonophoresis being explored further.

2.4.1.1 Supersaturation

This method does not alter the physicochemical properties of the drug, nor does it modify the structure of the mucosal membrane. In a supersaturated dosage form, the concentration of the drug in a vehicle is greater than the solubility i.e. the vehicle contains more dissolved material than could be dissolved in normal conditions (54). In these formulations, the drug has a high tendency to leave the vehicle, which increases the concentration gradient and flux rates across the mucosal membrane. However, this method of enhancement is dependent on the stability of the formulations, which is currently limited (55).

2.4.1.2 Thermal enhancement

The key parameter for this process is the activation energy of diffusion which, in turn, is dependent on temperature (56). This method is useful for drugs with high lipophilicity, as they have lower activation energies of diffusion. It has been suggested that an accurate increase in temperature may be used in drug delivery devices as a means of increasing

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permeation across the mucosal membrane (57,58). This method is however limited by the range of temperature and requires complex delivery devices that are able to regulate temperature (53).

2.4.1.3 Iontophoresis

This is an electrical assisted method used to overcome the barrier properties of the buccal mucosa, and has been extensively considered to enhance the buccal permeability of drugs from different therapeutic classes with a variety of physicochemical properties. This method utilizes an electric field to assist the passage of molecules through the biological tissue, with small amounts of physiologically acceptable electric current forcing the passage of charged drugs into the tissue. Drug permeation increases when the current density grows (24). This method has been extensively studied for transdermal delivery, with several devices marketed for this purpose. However, it has been reported that in order to improve transbuccal transport, iontophoresis should be combined with chemical penetration enhancers (59). This method is restricted by the complexity of the devices required for its application, and the limit to the current densities that can be applied, with the method being considered to be minimally invasive (53).

2.4.1.4 Electroporation

This is another electrical method used to promote the permeation of molecules across the mucosal membrane, and has been extensively studied for transdermal application (60). This method applies high electric field pulses for a very short period of time, which induce the movement of particles and perturbation of the lipid bilayers inside the membrane. As a result, the structural rearrangements lead to the formation of new aqueous pathways (60). Drug flux can be controlled by adjustment of the voltage, and time of exposure and total

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number of pulses delivered (61). This method is limited by the complexity of the delivery device required, as well as careful monitoring of the applied voltage. This method is also considered to be minimally invasive, with the application of high currents leading to possible membrane damages (62).

2.4.1.5 Sonophoresis

Ultrasound is classified based on three frequencies: high, medium and low, with low frequency (sonophoresis) being found to induce drug transport enhancement rather than high frequencies. While ultrasound application has been used for transdermal drug delivery, this technique may also be used for drug enhancement across the mucosal membrane (63). The application of ultrasound on biological tissues implies several concurrent phenomena including: convection, cavitation, thermal and mechanical effects. The latter three thermal and mechanical effects have the greatest influence on increasing the permeability enhancement, which is mainly brought about by disrupting the lipid bilayer and the formation of water channels. The extent of the enhancement induced by sonophoresis depends on frequency and intensity as well as duty cycle and application time. This approach also requires the use of a complex delivery device and is also classified as minimally invasive (53, 63, 64).

2.4.2 Chemical methods

The use of chemical permeation enhancers is the most widely used approach to enhance buccal absorption, and can be classified into many categories depending on their chemistry and their mechanism of action (65). The detailed chemical classification is tabulated in Table 2.2. An ideal chemical enhancer to be considered for formulation into a buccal drug delivery dosage form should have the following characteristics (28, 30, 65):

- Non-toxic, non-irritating and non-allergenic.
- Pharmacologically and chemically inert.
- The absorption enhancement action should be immediate and unidirectional.
- The effect of the permeation enhancer should be reversible i.e. after removal of the dosage from the mucosal surface; the tissue should immediately fully recover its normal barrier properties.
- The enhancer should be compatible with a wide range of drugs and pharmaceutical excipients.

Factors that can affect the selection of the permeation enhancer and the efficacy of the enhancer are (28):

- Physicochemical properties of the drug to be incorporated into the formulation.
- Nature of the vehicle.
- Other excipients added in the formulation.

A combination of permeation enhancers generally shows a greater effect than the use of individual permeation enhancers (28). Depending on the site of administration, the efficacy of permeation enhancers may vary due to differences in functional and structural properties, such as membrane thickness, lipid composition, enzymatic activity, cellular morphology and potential

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protein interactions. Penetration enhancement via the buccal membrane is also drug specific (66).

Table 2.2 Classes of permeation enhancers and their mechanisms of action (adapted from Hassan et al., 2010).

Class of chemical enhancer	example	Mechanism of action
Bile salts	Sodium-taurocholate, sodium fusidate, sodium taurodihydro fusidate, sodium-deoxycholate, sodium-glycocholate	Reduction of mucus viscosity Peptidase inhibition Denaturation of proteins High concentration – disrupt cell membrane lipids
Surfactants	Sodium lauryl sulphate, polyoxyethylene-9-laurylether, laureth-9, polyethylene glycol-8 laurate, sorbitan laurate, glycerol monolaurate, polysorbate 20 and 80	Phospholipids acyl chain disruption Membrane fluidization Producing reverse micellization in the membrane and creating aqueous channels Extraction of membrane proteins and lipids Solubilization of peptides
Fatty acids	Oleic acid, caprylic acid, lauric acid, palmitoylcarnitine	Phospholipid acyl chain perturbation Disruption of intercellular lipid packing thereby increasing membrane fluidity
Polymers	Chitosan salts, trimethyl chitosan	Ionic interactions with negatively charged groups of glycocalyx
Inclusion complexes	α -, β - and γ -cyclodextrins, methylated β -cyclodextrins	Inclusion of membrane compounds Increase solubility Enzyme inhibition
Chelators	Ethylenediaminetetraacetic acid (EDTA), citric acid, salicylates, polyacrylates	Complexation of Ca^{2+} to maintain intercellular spaces, thereby permitting paracellular transport. Opening of tight junctions

2.4.2.1 Bile salts

Bile salts are the natural or synthetic salts of cholanic acid, play an important role in the absorption of lipids and lipid-soluble vitamins, with all those excreted from the liver being conjugated with either glycine or taurine. Their physiological role is to enable fat digestion and absorption through the intestinal walls by emulsifying lipids in food that passes

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through the intestine (28). These compounds are believed to act due to a number of reasons, such as by extracting lipid components of the mucosa, extracting proteins, membrane fluidization, reverse micellization in the membrane and creating aqueous pores (65). Studies have shown that bile salts at lower concentrations are affecting only the paracellular route of transport. However, at higher concentrations, both the paracellular as well as the transcellular routes are affected (30). At lower concentrations, bile salts possibly solubilize the intercellular lipids and thereby enhance the paracellular route. Apart from the extraction of mucosal lipids, bile salts have also been shown to cause uncoiling and extension of protein helices, which also encourages transport via the paracellular route (34). At higher concentrations, bile salts disrupt the cell membrane lipids, thus enhancing the transcellular route. Bile salts have been extensively used to enhance the absorption of drugs through the oral mucosa, and it is generally considered that changes caused to the oral mucosa by this method are reversible (65).

2.4.2.2 Surfactants

Anionic surfactants are used in many cleaning and hygiene products, and being a detergent, these molecules have amphiphilic properties (34), including being predominantly water soluble and forming associations (micelles) in aqueous solution. The cationic and nonionic surfactants have also proven to be effective permeation enhancers. The actions of surfactants are very similar to those of the bile salts described above (28), namely the swelling of tissues, protein denaturation and extraction of lipids (30). Studies have also shown that like bile salts, surfactants are able to increase both the transcellular as well as paracellular routes of transport at higher concentrations. Their action is mainly brought about by the solubilization of membrane components, which leads to changes in cellular membrane permeability (65).

2.4.2.3 Fatty acids

Fatty acids are the most abundant lipids in biological cell membranes, and although there are numerous reports of fatty acids being used as transdermal permeation enhancers, very few reports are available on their buccal permeation enhancement potential. Medium chain length fatty acids have been shown to be more effective than longer chain, or shorter chain length fatty acids. The administrations of cis-unsaturated fatty acids have been reported to increase membrane permeability by disruption of intercellular lipid packing and increasing fluidity (34). Saturated fatty acids have been shown to be less disruptive than unsaturated fatty acids with the same chain length. It has also been demonstrated that the extent of enhancement using fatty acids are dependent on a few factors, such as the chain length, degree of branching, presence and position of double bonds, fatty acid concentration and contact time (30). Studies have also shown that fatty acids are less irritating than bile salts and surfactants. Furthermore, long chain fatty acids and their analogs are suited to the enhancement of lipophilic drugs, whereas medium chain length fatty acids and their derivatives can be used for both hydrophilic and lipophilic drugs (34, 67).

2.4.2.4 Chitosan and derivatives

In recent years, chitosan and its derivatives have received much attention in the literature for its absorption-enhancing effects, having been proven that these compounds are potential absorption enhancers for mucosal delivery systems. The literature shows that chitosan has a significant effect on enhancing permeation of various therapeutic agents and peptides across the buccal mucosa (68). Various mechanisms have been proposed for the mode of permeation enhancement of chitosan and its derivatives. Studies suggest that the enhancement effect may be related to the direct fluidization effect on intercellular

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lamellae, and the bioadhesive nature of chitosan, which ensures a greater residence time (28,65). It has also been reported that chitosan can open the tight junctions between epithelial cells, thereby enhancing permeation via the paracellular route. Chitosan exhibits several favorable properties, such as its permeation enhancement effect, biocompatibility, biodegradability, bioadhesion, antifungal and antimicrobial properties, which makes it an ideal candidate for incorporation as a multifunctional excipient into a buccal drug delivery formulation (28, 34).

2.4.2.5 Cyclodextrins

Cyclodextrins have been used as complexing agents in an effort to increase membrane permeability, being enzymatically modified starches, forming rings of 6-8 units. The outer surface of the ring is polar, whereas the internal surface is non-polar. The formation of inclusion complexes is therefore possible whereby the the center of the cyclodextrin can be used to carry water-insoluble molecules in an aqueous environment (30). Drug absorption and bioavailability across the buccal mucosa is increased by first solubilizing poorly water-soluble drugs via complexation with cyclodextrins. The opening of tight junctions and the alteration of mucosal permeability is the most probable mechanism of action of cyclodextrins as absorption enhancers (65).

2.4.2.6 Azone

Azone is used extensively as a transdermal permeation enhancer, and it has been proposed that it was able to form ion pairs with anionic drugs to promote their permeation. Azone has also found use in buccal drug delivery to enhance molecules across the membrane (34), and was shown to interact with the lipid domains and alter the molecular moment on the surface of the bilayers. The enhancing effect of Azone on buccal mucosa

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has been attributed to it being able to increase the reservoir capacity of the buccal epithelium and to also increase of the fluidity of lipids in the mucosal membrane (35).

2.4.2.7 Vehicles

To improve transport, drugs can be dissolved or dispersed in a solvent with the mechanism being categorized as follows (28):

- Increasing the degree of saturation in the vehicle resulting in a change in the thermodynamic activity.
- Facilitate partitioning of drug from the vehicle in the mucosa.

2.4.2.8 Enzyme inhibitors

Although the oral cavity is not as enzymatically active as the gastrointestinal tract, the environment of the oral cavity and oral epithelium is relatively enzymatic. This causes reduced bioavailability of some drugs that are susceptible to this enzymatic degradation before they are absorbed (28). In order to overcome this short-coming, research has been directed into the use of enzyme inhibitors, with the literature showing that co-administration of a drug with enzyme inhibitors improves the buccal absorption of drugs. Bile salts and protease inhibitors and have also been shown to stabilize peptides against buccal mucosal enzymes (34).

2.4.3 Mechanisms of action of buccal permeation enhancers

Buccal mucosal absorption is increased by the use of permeation/penetration enhancers. Although various classes of enhancers are available, many may exhibit similar mechanism of action, which have been classified as changing mucus rheology, increasing the fluidity of lipid

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bilayer membrane, acting on the components at tight junctions, by overcoming the enzymatic barrier and increase in the thermodynamic activity of drugs.

2.4.3.1 Changing mucus rheology (28,65)

Buccal drug permeation is largely hindered by the thickness of mucus viscoelastic layer, while saliva covering the mucus layers also hinders the permeation. Some permeation enhancers diminish the viscosity of the mucus and saliva, overcomes this barrier.

2.4.3.2 Increasing the fluidity of lipid bilayer membrane (28,30,65)

The intracellular route is the most preferred route of buccal permeation. Permeation enhancers using this mechanism perturb the intracellular lipid packing by interaction with lipid or protein components.

2.4.3.3 Acting on the components at tight junctions (28,65)

Larger molecules pass through the buccal mucosa via the intracellular route, with tight junctions and similar interconnections hindering the movement of mainly macromolecules across the buccal mucosa. In this mechanism, permeation enhancer's act on desmosomes, a foremost component at the tight junctions, which enhances drug absorption via the intracellular route.

2.4.3.4 By overcoming the enzymatic barrier (28,30)

These permeation enhancers achieve their effect by altering the action of various peptidases and proteases inside buccal mucosa, in this way they are able to overcome the enzymatic barrier. Additionally the enzymatic activity is indirectly altered by the modification in membrane fluidity.

. 2.4.3.5 Increase in the thermodynamic activity of drugs

Some permeation enhancers alter the partition coefficient of the drug and thus increase the solubility. The escalation in the thermodynamic activity results in improved drug permeation across the buccal mucosal membrane.

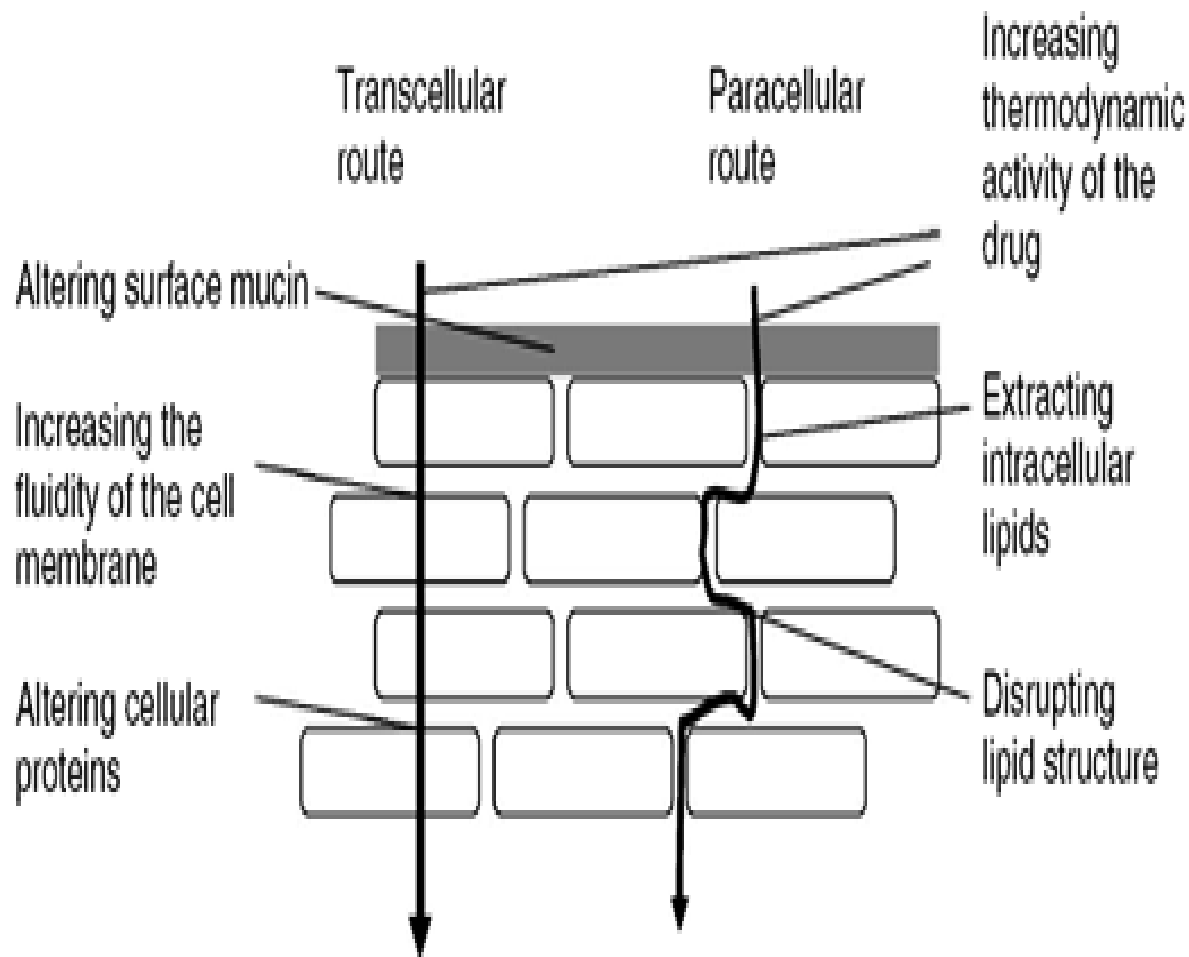


Figure 2.2: Schematic representation of penetration routes in buccal drug delivery, showing the mechanisms of action of chemical permeation enhancers (30).

2.5 EVALUATION OF MUCOSAL INTEGRITY

With the availability of human buccal mucosal tissue being very limited to perform *in-vitro* permeation experiments, scientists have explored the use mucosal cell culture as well as animal models for this purpose. The porcine buccal mucosa has been considered to be a representative model, as it resembles human tissue more closely than any other animal model (21, 25). In order to perform the perfusion experiments required for such studies, authors have used both fresh and frozen membranes (25). Studies have shown that there are negligible differences between experiments that compared frozen membrane to fresh (40). However, the uses of integrity markers are vital as positive controls in such studies (25). These tests should be performed before and after the experiment to ensure the integrity of the membrane during the experiment. Transepithelial electrical resistance (TEER) is a method commonly used to assess membrane integrity. TEER is able to detect minute tears in the membrane, which is represented by alterations in the electrical resistance between the donor and receptor cells. Performing the test before the experiment ensures that there was no damage to the membrane during the harvesting, excising or the storage processes of the membrane prior to the experiment. The second test after completion of the experiment confirms that the drug treatments did not compromise the integrity of the membrane during the experimental duration (25).

2.6 CONCLUSION

The literature indicates the advantages of the buccal route of drug administration, with ARV drugs that are susceptible to degradation in the gastrointestinal tract and/or hepatic first pass metabolism show potential for delivery via this route. With limited studies available on the delivery of ARVs through the buccal mucosa, further drugs from this class need to be identified for administration. With the major limitation of this route being the low membrane permeability, the literature shows various approaches adopted to overcome them. Permeation enhancers have proven to be critical in delivering adequate amounts of the drug via the buccal mucosa, therefore necessitating the identification of more buccal permeation enhancers. These enhancers will serve to increase the selection available to formulation scientists for incorporation into buccal drug delivery systems. The chemical approach to permeation enhancement is at the forefront of buccal permeation enhancement studies; however, there has recently been an increased investigation of alternate approaches to enhancing permeability across the buccal mucosa. The development of new approaches is vital to increase the pool of available strategies for the enhancement of drug permeation across the buccal mucosa.

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CHAPTER 3 – MANUSCRIPT OF PUBLISHED ARTICLE

Comparative Buccal Permeability Enhancement of Didanosine and Tenofovir by Potential Multifunctional Polymeric Excipients and their Effects on Porcine Buccal Histology

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ABSTRACT

This study identified and compared the buccal permeability properties of antiretroviral drugs; didanosine (ddl) and tenofovir (TNF), and the permeability effects of polymeric excipients i.e. carboxymethyl cellulose (CMC), sodium alginate (SA), polyacrylic acid (PAA) and polyethylene glycol (PEG) as potential multifunctional excipients for buccal drug delivery. Permeation studies across porcine buccal mucosa were performed and the drug was quantified using UV spectrophotometry. The mean flux for both ddl (113-181 $\mu\text{g}/\text{cm}^2\text{hr}$) and TNF (40-102 $\mu\text{g}/\text{cm}^2\text{hr}$) increased linearly with increasing donor concentration. All polymeric excipients improved permeability of TNF while only PEG was effective for ddl. Permeability enhancement ratios at 20 mg/mL for ddl and TNF were 1.63 and 1.74 respectively, using PEG (0.5% w/v) and CMC (0.5% w/v), respectively. The maximum enhancement ratio of 2.13 for TNF was achieved with 4% w/v PEG. Light and transmission electron microscopy revealed no significant loss in cellular integrity for mucosa treated with either TNF or ddl alone or when coupled with PEG as a polymeric enhancer. Histomorphological observations correlated with flux values obtained for TNF and ddl alone, as well as with PEG's effects on drug mass flux. TNF and ddl have demonstrated buccal delivery potential. Selective polymeric excipients provide an effective means to increase their penetration and may serve as potential formulation multifunctional excipients in a delivery system for delivery via the buccal route.

Keywords: buccal permeability, didanosine; tenofovir, polymers, permeation enhancer, histology

1. INTRODUCTION

Drug delivery via the buccal route is receiving increasing interest as an alternative to oral and other conventional routes of administration for the following reasons: It is able to bypass enzymatic degradation and hepatic first pass metabolism, improving bioavailability (1, 2) and is not influenced by variations in gastric emptying rate or the presence of food. As compared to the skin for transdermal delivery, the permeability of the buccal mucosa is higher and cell recovery is also more rapid, and it has easier access than nasal, rectal and vaginal mucosae. The buccal mucosa is also more resistant to irritation, and has greater patient acceptability than other non-oral routes. Buccal delivery systems are also advantageous for pediatrics and patients with a swallowing problem (3, 4). Although oral antiretroviral drug (ARV) therapy has significantly improved the treatment of HIV (Human Immunodeficiency Virus) infection and AIDS (Acquired Immune Deficiency Syndrome), one of the most serious public health diseases (5, 6); several disadvantages are associated with their use. Many ARV drugs have a low bioavailability, as well as short half-lives, and therefore require frequent administration that decreases patient compliance and leads to severe dose dependent side-effects. Furthermore, some may lead to formulation difficulties due to poor solubility (7). These ARV drugs may therefore benefit from buccal drug delivery. While ARV drugs have been more widely studied for the transdermal route to overcome the above disadvantages (8-11), data on their buccal permeability potential appear to be limited (12, 13). Tenofovir (TNF), a nucleotide analog, is widely used in the treatment of HIV/AIDS. Due to its hydrophilicity and poor intestinal permeability it is currently administered orally as the ester prodrug; tenofovir disoproxil fumarate. Buccal delivery of TNF in its base form will allow for increased bioavailability and will obviate the need for synthesizing and using the current prodrug. Furthermore, while TNF has been investigated for transdermal (14) and vaginal routes (15), its buccal delivery potential still

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remains to be identified. Didanosine (2'3'-dideoxyinosine; ddl) is a dideoxy analogue of the purine nucleoside inosine (16) and is also used in the treatment of HIV AIDS. However, it is characterized by a short half-life of 1.3-1.6 hours, extensive first pass metabolism (23-40% bioavailability), gastrointestinal pH degradation and severe dose dependent side-effects (14, 17). ddl is therefore also an ideal ARV candidate for controlled release buccal delivery.

The advantages of the buccal route has led to the design and evaluation of various buccal delivery systems such as tablets, films, wafers, patches, gels and pastes (18). The benefit of these buccal delivery systems can be enhanced by ensuring that the drug is released in a controlled manner. Various polymers both alone or in combination, are used in buccal delivery systems to modify drug release profiles via several mechanisms, such as erosion, swelling and hydration (19).

One of the main disadvantages of the buccal route is the low mucosal permeability, necessitating the use of various classes of permeation enhancers. These enhancers alter mucosal permeability by various mechanisms, such as changing the mucus rheology, disturbing the intracellular lipid packing in the bilayer and the loosening of tight junctions (1). Substances such as surfactants, fatty acids, cyclodextrins, chelators and positively charged polymers have been identified in the literature as buccal permeation enhancers (7). The search and identification of new drugs and permeation enhancers via *in vitro* permeation studies with drug and potential enhancer solutions is currently a major focus of research in the literature to widen the available pool of drug and excipients and consequently inform the selection of excipients for formulation into future buccal delivery systems.

To facilitate prolonged retention on the mucosae allowing for sustained delivery and permeation; buccal delivery systems also need to display mucoadhesivity i.e. they need to adhere to the

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mucus layer covering the buccal mucosae. In order to meet the challenges of buccal delivery, the key properties therefore needed for a buccal drug delivery system are: 1) drug permeation enhancement; 2) mucoadhesivity and 3) controlled drug release profiles. The identification of formulation excipients to provide these properties is therefore critical. Various polymers such as cellulose derivatives, and those based on polymethacrylic acid, polysaccharides and alginic acid have successfully shown mucoadhesivity and sustained drug release properties (20).

The identification of excipients with potential multifunctional properties such as mucoadhesivity, drug permeation enhancement and controlled drug release profiles, will significantly contribute to the optimization of the design of buccal delivery systems. The multifunctional properties required for buccal delivery systems has led investigators to study, separately, different classes of excipients as permeation enhancers and polymers that have either or both mucoadhesivity and controlled release properties. Clearly, a need exists for the use of multifunctional pharmaceutical excipients in the design of buccal delivery systems i.e. excipients that could simultaneously provide mucoadhesivity, sustained drug release and permeation enhancement. The use of multifunctional excipients for buccal drug delivery will decrease the number and quantity of excipients needed in product formulation. Advantages include decreased manufacturing costs, adverse or allergic reactions and excipient incompatibilities. To date, to the best of our knowledge, the only excipient thus far reported in separate studies to have mucoadhesive, sustained release and permeation enhancing properties; has been chitosan (20, 21). Hence the need exists for the identification of excipients that could have multifunctional properties such as mucoadhesivity, sustained drug release and drug permeation enhancement capabilities. While various excipients such as poly(acrylic acid) (21), sodium alginate (21), poly(ethylene glycol) (19, 20) and carboxymethylcellulose (21, 22) are recognized as having

both sustained drug release and mucoadhesivity properties, their buccal permeability enhancement potential still remains to be investigated.

The aim of this study was therefore to firstly identify and compare the buccal permeability potential of two ARV drugs i.e. didanosine (ddl) and tenofovir (TNF). The second aim was to determine the effects of poly (acrylic acid), sodium alginate, poly (ethylene glycol) and carboxymethylcellulose on the permeation of these drugs as well as their histomorphological effects on the buccal mucosae as potential multifunctional formulation excipients for buccal delivery.

PEG was particularly chosen in this study to further investigate its potential as an enhancer as it proved to be the only polymer that was able to increase the permeability of both TNF and ddl. Also PEG has been reported to be strongly mucoadhesive due to hydrogen bonding between either oxygen atoms in PEG and sugars on glycosylated mucins, and/or interpenetrating polymer network (IPN) effects between PEG chains and the mucus mesh (42). This interaction with the mucus to enable PEG's mucoadhesivity could also be beneficial in decreasing the barrier properties displayed by the mucus to the movement of molecules across the mucosa.

2. MATERIALS

The buccal mucosal tissue was obtained from Baynesfield Abattoir (Pietermaritzburg, South Africa), from pigs weighing approximately 60-80 kg. Phosphate buffered saline (PBS), pH 7.4, was prepared using sodium chloride, potassium dihydro-orthophosphate and disodium hydrogen orthophosphate (Associated Chemical Enterprises, South Africa). Excipients (Carboxymethyl Cellulose (CMC) MW = 90000 Da, Sodium Alginate (SA), Poly acrylic acid (PAA) MW = 1800 Da, and Polyethylene Glycol (PEG) MW = 4000 Da) were purchased from

Sigma-Aldrich (USA). Tenofovir (TNF) MW = 287.21 g/mol was obtained from Gilead Sciences, Inc. (USA) and Didanosine (ddl) MW = 236.23 g/mol was donated by Aspen Pharmacare (South Africa).

3. METHODS

3.1 Ethical Clearance

Ethical clearance was obtained from the UKZN Ethics committee in 2010 (010/10/Animal) and renewed in 2011 (24/11/Animal).

3.2 Tissue preparation

Porcine buccal tissue as obtained from the pigs immediately after slaughter. The tissues were excised, using surgical scissors, to remove all underlying tissue, to a thickness of between 500-700 μm , leaving the basal lamina intact. The tissues were transported in cold, normal saline (NS), snap frozen in liquid nitrogen, stored in a biofreezer (-80°C) and used within three months (23). Cold PBS (pH 7.4) was used to defrost the buccal mucosal tissue at room temperature before it was used in permeation studies.

3.3 Permeation Measurement across Porcine Buccal Mucosa

In vitro permeation studies were conducted at $37\pm 1^{\circ}\text{C}$ using modified vertical Franz type diffusion cells (PermeGear, Inc., Bethlehem, USA) with a diffusional area of 0.786 cm^2 . A circular section of the buccal mucosa was mounted onto the diffusional area between the donor and receptor cells and was equilibrated with PBS (pH 7.4) at 37°C for 30 minutes. The donor compartment solution (1 mL) contained either drug (TNF/ddl) with PBS alone (20 mg/mL) or drug (TNF/ddl) (20 mg/mL) in the presence of the various excipients (PAA, SA, CMC and PEG) at a concentration of 0.5% w/v in respective experiments. The concentration dependent effect of

PEG, specifically, on the enhancement of drug (TNF/ddl) permeation, was performed with the donor compartment solution (1 mL) containing drug (TNF/ddl) (20 mg/mL) in the presence of various concentrations of PEG (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0% w/v). The receptor compartments were filled with PBS and stirred with a teflon-coated magnetic bar. Samples were removed from the receptor compartments at predetermined time intervals and replaced with the same volume of ddl/TNF-free (fresh) PBS. Each experiment represents minimum of three replicates. The drugs were quantified by a validated UV spectrophotometry method at a λ_{\max} of 262 nm and 250 nm, for TNF and ddl, respectively (UV Spectrophotometer 1650, Shimadzu, Japan) which are similar to other buccal permeability studies.

3.4 Permeability data analysis

The cumulative amount of drug (TNF/ddl) permeated per unit surface area was plotted against time. The steady state flux (J_{ss}) was determined from the linear part of the permeability curve by linear regression analysis (Microsoft Excel 2007, USA). The permeability coefficient (P) was calculated as follows (24):

$$P = (dQ/dt)/A \times C_d = J_{ss}/C_d \quad [1]$$

dQ/dt is the cumulative amount permeated per unit time, A is the diffusion area and C_d is the drug concentration in the donor compartment. The enhancement ratio (ER) was calculated using the following equation (24):

$$ER = \frac{\text{Permeability coefficient of drug in the presence of enhancer}}{\text{Permeability coefficient of drug in the absence of enhancer}} \quad [2]$$

3.5 Light Microscopy and Transmission Electron Microscopy

Histological evaluations were performed on freshly harvested, excised buccal mucosa. Untreated buccal mucosa was transferred directly after excision from normal saline into 10% buffered formalin without any equilibration in PBS and served as the control. Treated samples comprised of buccal mucosa that were exposed to PBS only, or 20 mg/mL TNF dissolved in PBS alone or 20 mg/mL ddl dissolved in PBS alone, or 20 mg/mL TNF dissolved in PBS with 4% w/v PEG, or 20 mg/mL ddl dissolved in PBS with 0.5% w/v PEG. Permeation experiments were carried out with these solutions in the donor compartment; and with the freshly excised buccal mucosa placed between donor and receptor compartments as described in 3.3 above, without drug quantification (12, 25). At the end of the experiment the buccal mucosa was removed from the Franz diffusion cells, cut into cross sections and fixed in 10% buffered formalin. Both the control and treated buccal mucosa were fixed in formalin for 7 days at room temperature. Buccal mucosa was dehydrated using an ethanol gradient ranging from 50% up to 96% and embedded in paraffin wax. The mucosae sections were collected on slides, dried and stained with hematoxylin and eosin (H&E). Sections were examined using a light microscope (Nikon 80i, Japan) and bright field images were digitally captured using NIS Elements D software and a camera (Nikon U2, Japan). The samples for transmission electron microscopy (TEM) were obtained after the above mentioned permeation experiments. The samples were then cut into pieces not exceeding 0.5 mm³, and fixed for 24 hours (4°C) using Karnovsky's fixative (26) buffered to pH 7.2. For TEM, each sample was processed and embedded in epoxy resin using standard protocols. Ultrathin sections (90 nm) were cut and contrasted with uranyl acetate and lead citrate and viewed with a transmission electron microscope (JEOL 1010, Japan). All experiments were performed using a minimum of three replicates.

3.6 Statistical Analysis

The results, expressed as mean \pm standard deviation (SD), were analyzed using one-way analysis of variance (ANOVA) followed by the Mann Whitney test using GraphPad Prism® (Graph Pad Software Inc., Version 5., USA).

4. RESULTS AND DISCUSSION

4.1 Buccal permeability profiles of TNF and ddl

The permeability potential of TNF and ddl, were first investigated in the absence of an enhancer. Increasing the donor concentrations (1 to 20 mg/mL) led to an increase in the cumulative amount permeated across the buccal mucosa for both drugs. The flux of TNF and ddl at the varying donor concentrations are shown in Figure 1. Both TNF and ddl were able to permeate the buccal mucosa at increasing concentrations. The results obtained displayed concentration dependant permeability for both ARV drugs. For TNF, as the donor concentration increased from 1 mg/mL to 20 mg/mL, the steady state flux increased from 40.18 ± 8.85 to $102.1 \pm 19.80 \mu\text{g}/\text{cm}^2\text{hr}$ (Table 1). While the increase in flux at lower concentrations from 1 to 7.5 mg/mL were not significant ($p > 0.05$), statistically significant differences were seen at the higher concentrations of 10 and 20 mg/mL ($P < 0.05$). One-way ANOVA for the various concentrations of TNF were also significantly different ($P < 0.05$). For ddl, as the donor concentration increased, flux values increased from 113.4 ± 6.85 to $181.62 \pm 23.62 \mu\text{g}/\text{cm}^2 \text{hr}$. The increases in flux values from 1 mg/mL to 20 mg/mL were statistically different ($P < 0.05$) and one-way ANOVA for the various concentrations of ddl studied were also significantly different ($P < 0.05$). An apparent linear relationship between the donor concentrations and flux values for both TNF ($R^2 = 0.94$) and ddl ($R^2 = 0.82$) was observed (Figure 2). These findings suggest passive diffusion of both the drugs across the mucosa (4, 7, 27).

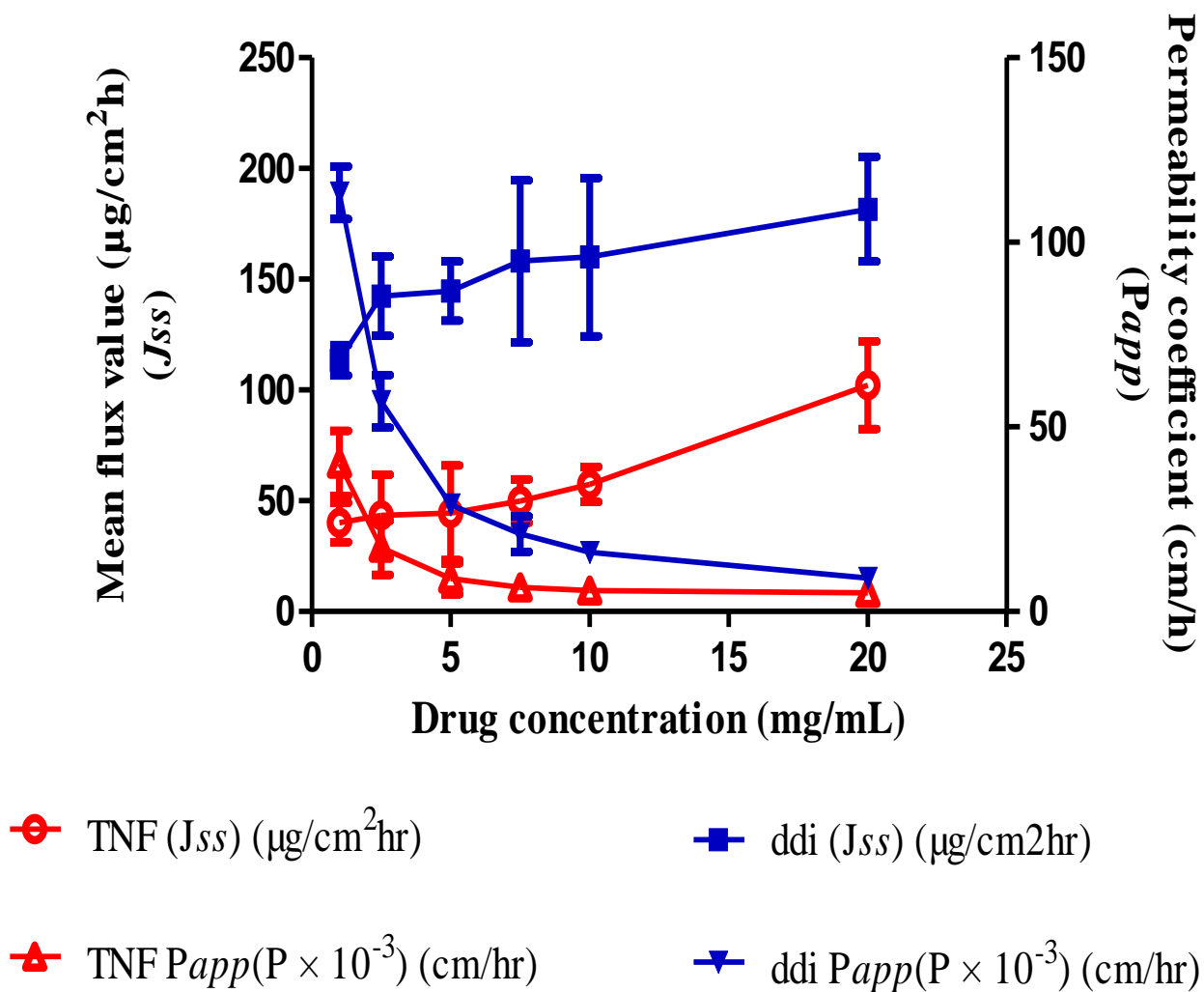


Figure 1: Effect of drug concentration on mean flux and mean permeability coefficients for TNF and ddi across buccal mucosa.

Table 1 : Effect of donor concentration on the permeability parameters of TNF and ddl.

Drug concentration (mg/mL)	Flux (Jss) ($\mu\text{g}/\text{cm}^2\text{hr}$)		Permeability coefficient ($P \times 10^{-2}$) (cm/hr)	
	TNF	ddl	TNF	ddl
1	40.18 \pm 8.85	113.4 \pm 6.85	4.01 \pm 0.88	11.34 \pm 0.71
2.5	43.41 \pm 18.35	*142.4 \pm 17.84	1.73 \pm 0.73	5.69 \pm 0.71
5	44.51 \pm 21.38	*144.62 \pm 13.36	0.89 \pm 0.42	2.89 \pm 0.26
7.5	49.95 \pm 9.83	*158.19 \pm 36.53	0.66 \pm 0.13	2.10 \pm 0.48
10	*57.43 \pm 7.88	*160.02 \pm 35.69	0.57 \pm 0.07	1.60 \pm 0.14
20	*102.10 \pm 19.80	*181.62 \pm 23.62	0.51 \pm 0.09	0.90 \pm 0.11

* Indicates significant difference i.e P < 0.05 (All values compared to 1mg/mL)

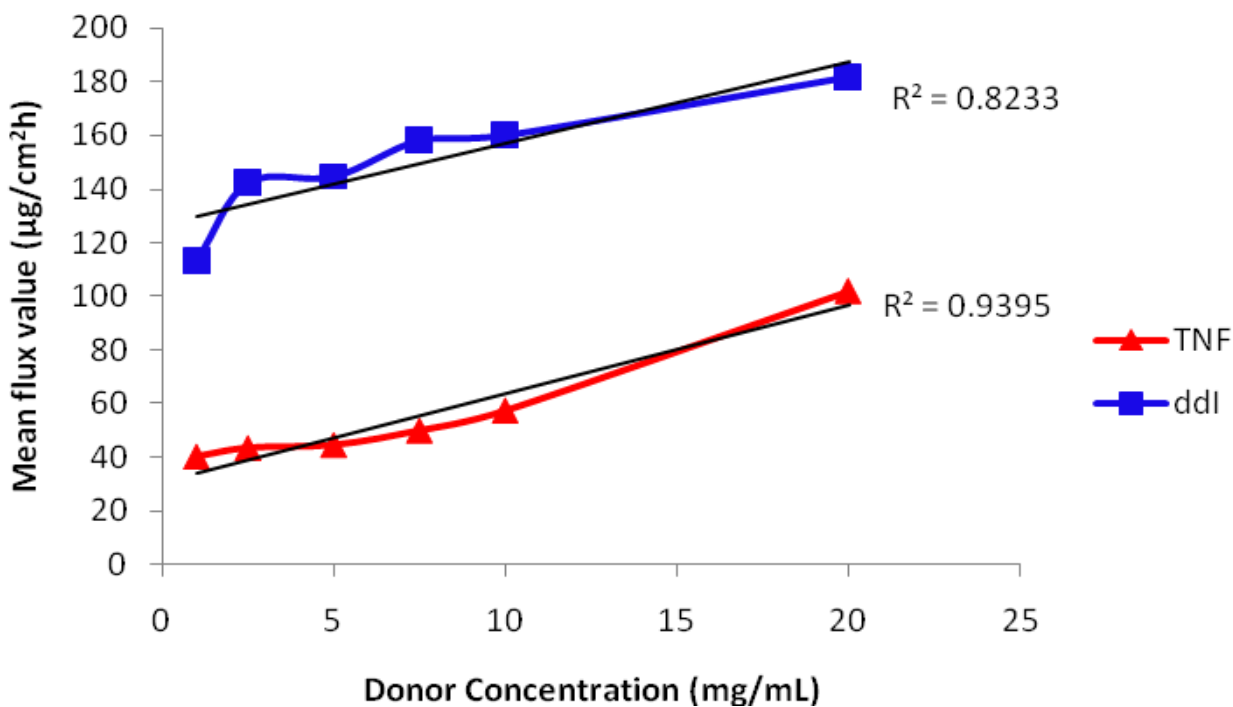


Figure 2: Effect of donor concentration on the steady state flux.

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Since the buccal epithelium is stratified, a permeant can use either the transcellular or paracellular route or both the routes simultaneously. However one route is usually preferred over the other, favoring the one that provides the least amount of hindrance to passage. The physicochemical properties of a drug can also influence the mechanisms of transport across the buccal mucosa. The permeation of many hydrophilic drugs across the buccal mucosa is thought to be via the paracellular route using passive diffusion (7, 22, 28-30). These hydrophilic drugs are therefore able to dissolve more readily in the aqueous fluid of the intercellular spaces; however, the lipid matrix of the extracellular spaces plays a role in the barrier function of the buccal mucosa especially if the compounds have high molecular weights (28, 29). Tight junctions act as major barriers to paracellular transport of macromolecules and polar compounds (2, 7, 31). Hence both of these hydrophilic drugs may have used the paracellular route, however a higher ionization at pH 7.4 of ddl ($pK_a=9.12$) as compared to that of TNF ($pK_a=7.9$) suggests that ddl is the more capable permeant via this route (32, 33). This is illustrated by higher mean flux and permeability coefficient observed for ddl (Table 1). Although both drugs displayed increased permeability with increasing concentrations, ddl displayed greater flux values than TNF at similar concentrations. This could be also attributed to ddl's lower molecular weight as compared to TNF, thus allowing greater permeability. The donor concentration range of the drug solutions of this study was in keeping with previous such permeability studies (13) and the flux values obtained in this study for TNF and ddl are similar to, or greater than flux values that have been reported for other drugs identified as having potential for buccal delivery also via in vitro permeation studies (13, 34). TNF and ddl may be considered as having the potential for improving HIV/AIDS drug therapy when administered via the buccal mucosa instead of the oral route.

4.2 Effects of polymeric excipients on permeability enhancement

Polymeric excipients of varying ionic charges were employed as potential permeation enhancers for TNF (as an anionic drug) and ddl (as a cationic drug). Permeation/penetration enhancers promote the absorption of a drug across the buccal mucosa by decreasing its barrier properties, delivering therapeutically relevant amounts of the drug into the systemic circulation. Hydrophilic drugs, such as TNF and ddl, could both show increased permeability if they are coupled with enhancers that are able to either decrease the interaction of the drug with the lipid matrix of the extracellular spaces and/or temporarily alter tight junction or both. To identify their multifunctional capabilities, polymeric excipients with previously reported controlled release and mucoadhesive properties (23, 25, 35); were used as potential enhancers for the buccal delivery of TNF and ddl, respectively. The respective enhancers were added to the donor chamber at 0.5% w/v which is in keeping with previous studies (13, 36). The results of this study showed that the permeability of TNF was increased in the presence of all the excipients (Figure 3). CMC displayed the greatest increase in mean flux value, which increased from $102.10 \pm 19.80 \mu\text{g}/\text{cm}^2\text{hr}$ to $178.12 \pm 12.87 \mu\text{g}/\text{cm}^2$ with an enhancement ratio of 1.74 (Table 2). All of the excipients displayed permeation enhancement for TNF, however the anionic excipients displayed the greatest enhancement. These findings may be attributed to the anionic polymers having strong hydrogen bonding with mucin, altering the mucus rheology and enhancing permeability (23, 35). While the increases in flux values were statistically significant ($p < 0.05$) for CMC, PEG and PAA, CMC displayed the greatest enhancement ratio. The permeability of ddl was increased in the presence of the nonionic excipient PEG, whereas the addition of all the anionic excipients (PAA, SA and CMC) caused a decrease in the flux values obtained (Table 2). Changes in the mean flux and permeability coefficient may have resulted from the anionic excipients' complexation with the positively charged drug, ddl, forming an aggregate which hindered movement across the buccal mucosa. In this study the effect of permeant modifiers

appears to be dependent on the ionic charge of the enhancer and drug. The permeation enhancement ratios obtained using PAA, SA, CMC and PEG for TNF and ddI in this study are similar to ratios of other permeants reported as promising enhancers in the literature (36, 37). This study has confirmed that in addition to their previously reported controlled-release and mucoadhesive properties in the literature, these polymeric excipients can also enhance buccal drug permeability and may therefore be regarded as promising multifunctional excipients in the field of buccal drug delivery.

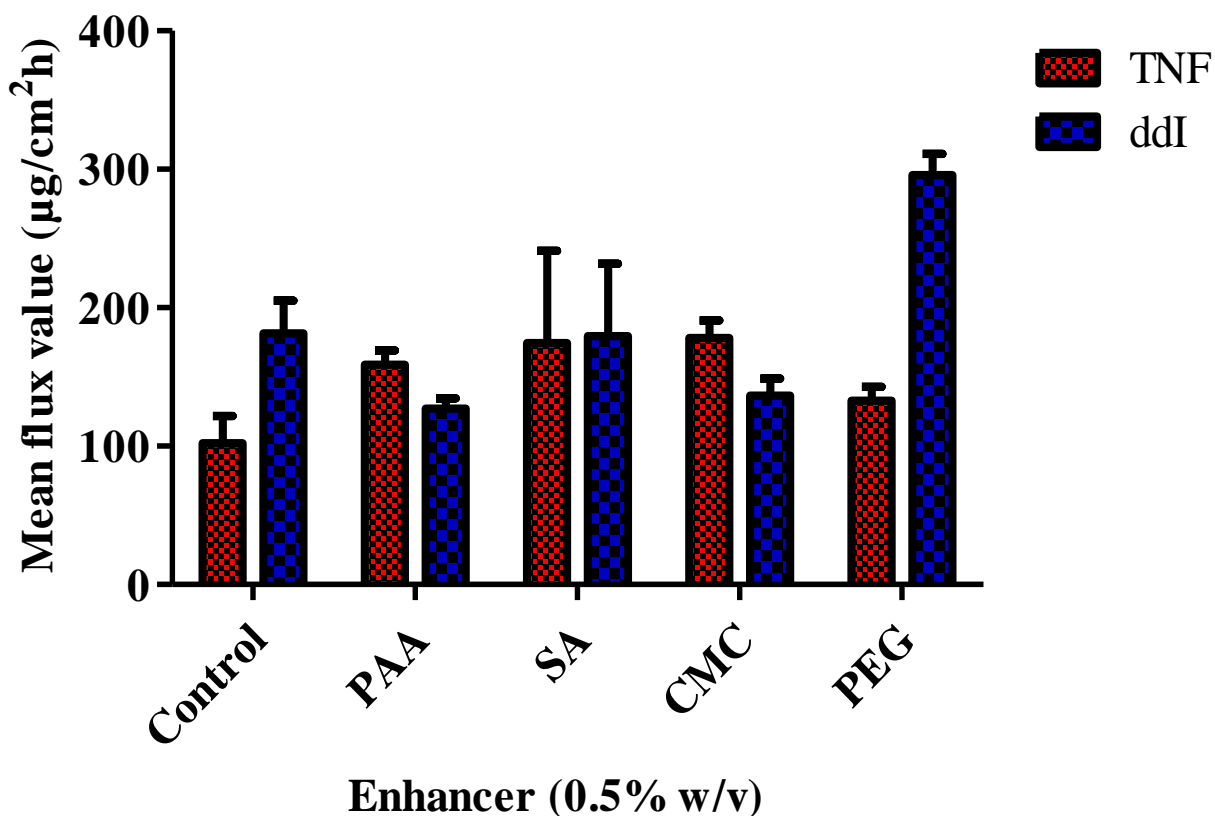


Figure 3: Effect of various multifunctional excipients on the mean flux of TNF and ddI across buccal mucosa.

Tables 2 : Effect of multifunctional excipients on the permeability parameters of TNF and ddl.

Excipient concentration (% w/v)	Flux (Jss) ($\mu\text{g}/\text{cm}^2\text{hr}$)		Permeability coefficient ($P \times 10^{-2}$) (cm/hr)		Enhancement ratio	
	TNF	ddl	TNF	ddl	TNF	ddl
Control	102.10 \pm 19.80	181.62 \pm 23.62	0.51 \pm 0.09	0.90 \pm 0.11	1	1
PAA (0.5%)	*158.80 \pm 10.19	*127.07 \pm 7.30	0.79 \pm 0.05	0.63 \pm 0.03	1.56	0.7
SA (0.5%)	174.46 \pm 66.56	179.61 \pm 52.24	0.87 \pm 0.33	0.90 \pm 0.26	1.71	0.99
CMC (0.5%)	*178.12 \pm 12.87	*136.45 \pm 12.29	0.89 \pm 0.06	0.68 \pm 0.06	1.74	0.75
PEG (0.5%)	*132.82 \pm 9.95	*295.94 \pm 15.24	0.66 \pm 0.04	1.48 \pm 0.07	1.3	1.63

* Indicates significant difference i.e $P < 0.05$ (All values compared to Control (20 mg/mL TNF/ddl))

4.3 Effect of Polyethylene Glycol concentrations on permeability

PEG was the only excipient that displayed enhanced permeation for both TNF (ER = 1.3) and ddl (ER = 1.63) (Table 2), and was selected to evaluate its effects on the permeability of TNF and ddl. The effect of PEG, within a concentration range of 0.25 – 6% w/v on the permeability of TNF and ddl was investigated. This concentration range is in keeping with previous reports (13, 36). The mean flux values obtained for TNF was increased, with the addition of PEG from 0.25 to 4% w/v (Figure 4). PEG, at 4% w/v, displayed the greatest enhancement ratio (ER = 2.13) and the greatest increase in mean flux value viz. from 102.1 \pm 19.80 to 217.49 \pm 18.88 $\mu\text{g}/\text{cm}^2\text{hr}$ (Table 3). An increase in PEG concentration above 4% w/v, resulted in a decrease in the mean flux values, from 217.49 \pm 18.88 to 195.54 \pm 17.57 $\mu\text{g}/\text{cm}^2\text{hr}$ (ER = 1.91) (Figure 4). The mean flux values obtained for ddl was increased with the addition of PEG from 0.25 to 4% w/v. PEG at 0.5% w/v displayed the greatest enhancement ratio (ER = 1.63) and the greatest increase in mean flux value which increased from 181.62 \pm 23.62 to 295.94 \pm 15.24 $\mu\text{g}/\text{cm}^2\text{hr}$. A further increase in PEG concentration from 0.5 to 6% w/v resulted in a decrease in mean flux values

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obtained, which decreased from 295.94 ± 15.24 to 155.85 ± 11.25 $\mu\text{g}/\text{cm}^2\text{hr}$ (ER = 0.86) (Table 3). Similar trends have been reported, where an enhancer (Aloe vera gel or propylene glycol) at varying concentrations, was coupled with a permeant. These studies reported an initial increase in flux values with an increase in enhancer concentration and with a subsequent decrease in flux values as the enhancer concentration was further increased (13, 38).

PEG's mucoadhesion property has been attributed to its ability to increase hydrogen bonding and facilitating adherence to the mucosae (39). One of the mechanisms of buccal permeation enhancement results from the alteration of the mucus rheology (23, 35), which could account for PEG increasing the permeation of both TNF and ddl. PEG, at a lower concentration 0.5% w/v, achieved the greatest enhancement of the lower molecular weight drug ddl, whereas for the higher molecular weight drug TNF, maximal enhancement was achieved at a higher concentration of PEG (viz. 4% w/v). Possible differences in drug interactions could have resulted in the decreased movement across the buccal mucosae at higher concentrations than the aforementioned. The permeation enhancement ratios achieved with the addition of PEG in this study is similar to other studies that identified promising enhancers (32, 36-38). In addition to its mucoadhesive and controlled release properties, PEG also showed permeation enhancement properties. PEG displayed the greatest enhancement potential for TNF and ddl at a concentration of 4% and at 0.5% w/v, respectively, and could be considered when formulating a buccal delivery system.

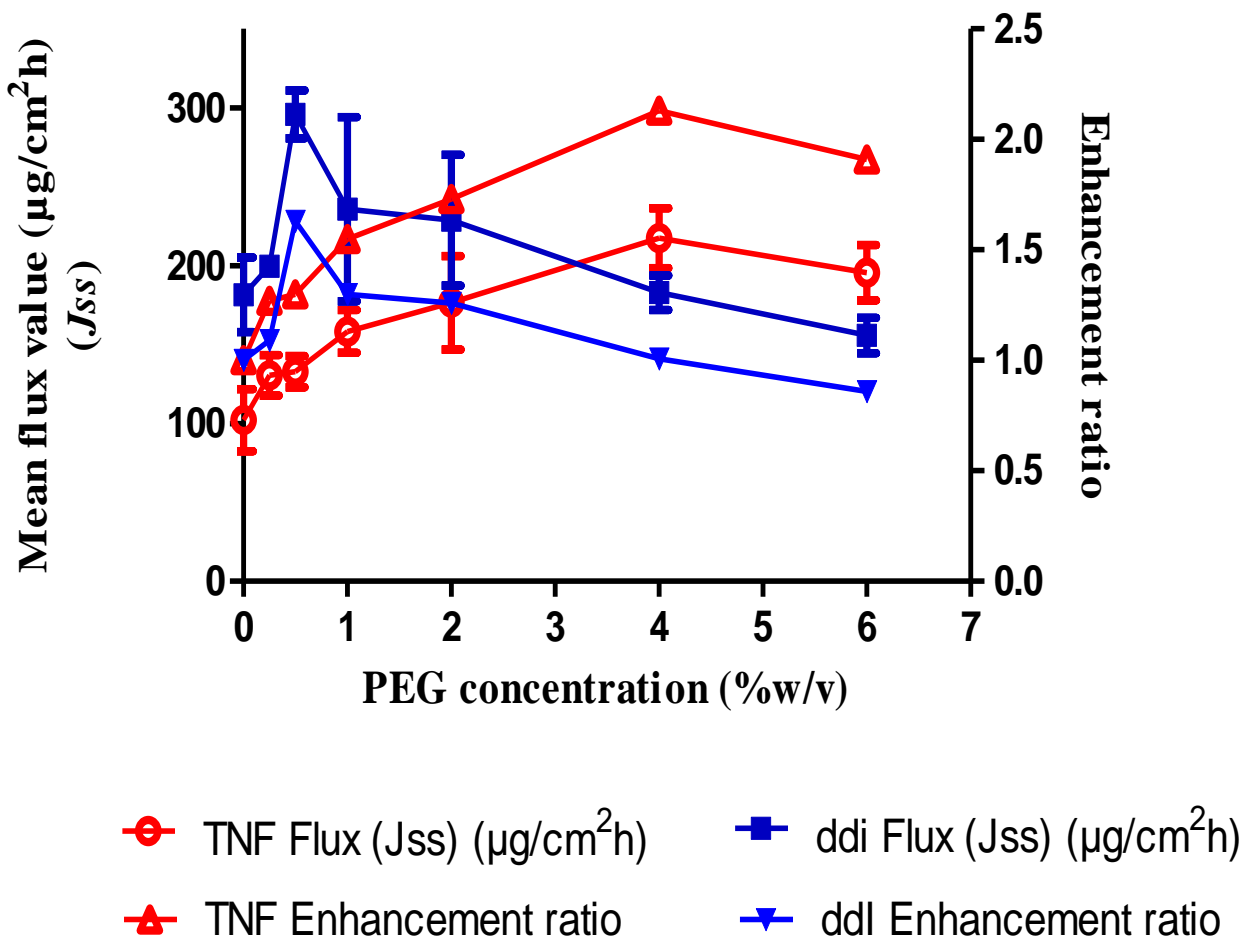


Figure 4: Effect of PEG concentration on permeation enhancement ratio, mean mass flux and mean permeability coefficients for TNF and ddi across buccal mucosa

Tables 3 : Effect of PEG concentration on the permeability parameters of TNF and ddl.

Excipient concentration (% w/v)	Flux (Jss) ($\mu\text{g}/\text{cm}^2\text{hr}$)		Permeability coefficient ($P \times 10^{-2}$)(cm/hr)		Enhancement ratio	
	TNF	ddl	TNF	ddl	TNF	ddl
Control	102.10 \pm 19.80	181.62 \pm 23.62	0.51 \pm 0.09	0.90 \pm 0.11	1	1
0.25	130.43 \pm 12.83	*199.66 \pm 4.54	0.65 \pm 0.07	0.99 \pm 0.02	1.27	1.09
0.50	*132.82 \pm 9.95	*295.94 \pm 15.24	0.66 \pm 0.05	1.48 \pm 0.07	1.3	1.63
1	158.34 \pm 13.63	235.81 \pm 58.36	0.79 \pm 0.06	1.17 \pm 0.29	1.55	1.3
2	*176.50 \pm 29.70	*228.79 \pm 41.41	0.88 \pm 0.14	1.14 \pm 0.20	1.73	1.26
4	*217.49 \pm 18.88	183.01 \pm 10.93	1.08 \pm 0.09	0.91 \pm 0.05	2.13	1.007
6	*195.54 \pm 17.57	*155.85 \pm 11.25	0.97 \pm 0.08	0.77 \pm 0.05	1.91	0.86

* Indicates significant difference i.e $P < 0.05$ (All values compared to Control (20 mg/mL TNF/ddl))

4.4 Histomorphological evaluations

The effect of the ARV drugs (TNF and ddl) and the optimal enhancer identified in this study i.e. PEG on the buccal mucosa was investigated histomorphologically. These studies were performed to assess whether or not the expected changes following permeation, are of a nature that is deleterious to cell i.e. whether cellular integrity has been compromised. Also, these studies may indicate to a possible transport mechanism for these drugs and enhancer. The criteria for the use of permeation enhancers are that they should be non-irritant and nontoxic, and allow the mucosa to rebound to its former integrity (7). In this study we are able to comment on the level of perturbation, however, whether or not the cells would regain their former morphology requires further investigation. Two routes have been proposed for movement of molecules across the buccal mucosae i.e. paracellular and transcellular (2). Although limited, from this study, inferences can be made on the mode of transport employed by the molecule (drug) and the permeation enhancer, PEG. The mucosa of the buccal lining has been

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extensively described (25). Briefly, histologically, the mucosal layer is made of basal cells which are actively mitotic and represent the germinal layer which gives rise to newer cells; a mid-region where cells acquire a more polygonal shape and surface cells which appear more flattened. This description matched our control sample which was not subjected to any experimental procedure (Figure 5a).

When compared to the controls, mucosae exposed to the drugs only, ddl and TNF, at 20 mg/mL (Figure 5b and 5c respectively) also retained relatively normal histomorphology i.e. no major loss of cellular integrity. TNF treatment, however, resulted in mild distortion of cellular outlines and desquamation, and an increase in the size of intercellular spaces was observed in electron micrographs. The ddl treatment resulted in a vacuolated appearance inside some of the cells which was observed both in LM and TEM images (Figure 5e and 6g respectively) unlike those of TNF (5b and 6b respectively). These vacuoles may indicate a possible transcellular mode of transport (40). These histomorphological observations appear to correlate with the permeation data (Table 1,3 and Figure 2,4) where ddl displayed greater permeation in comparison to TNF, both in the presence and absence of PEG (Table 1 and Table 2). Combining both the permeability data and histomorphological evaluations, it can be speculated that TNF permeation may follow the paracellular route while ddl may use both the paracellular and transcellular routes. Substances may use many several modes of transport for movement, including carrier-mediated transport across the epithelium lining; therefore further investigations are needed to confirm how ddl, TNF and PEG move across the mucosal barrier, including an investigation on possible endosomal transport mechanisms. Furthermore, the vacuolated appearance may not be considered deleterious and these findings are similar to Dhiman et al., (2009) who also found a vacuolated appearance in the buccal mucosa after permeation experiments using sodium tauroglycocholate as an enhancer.

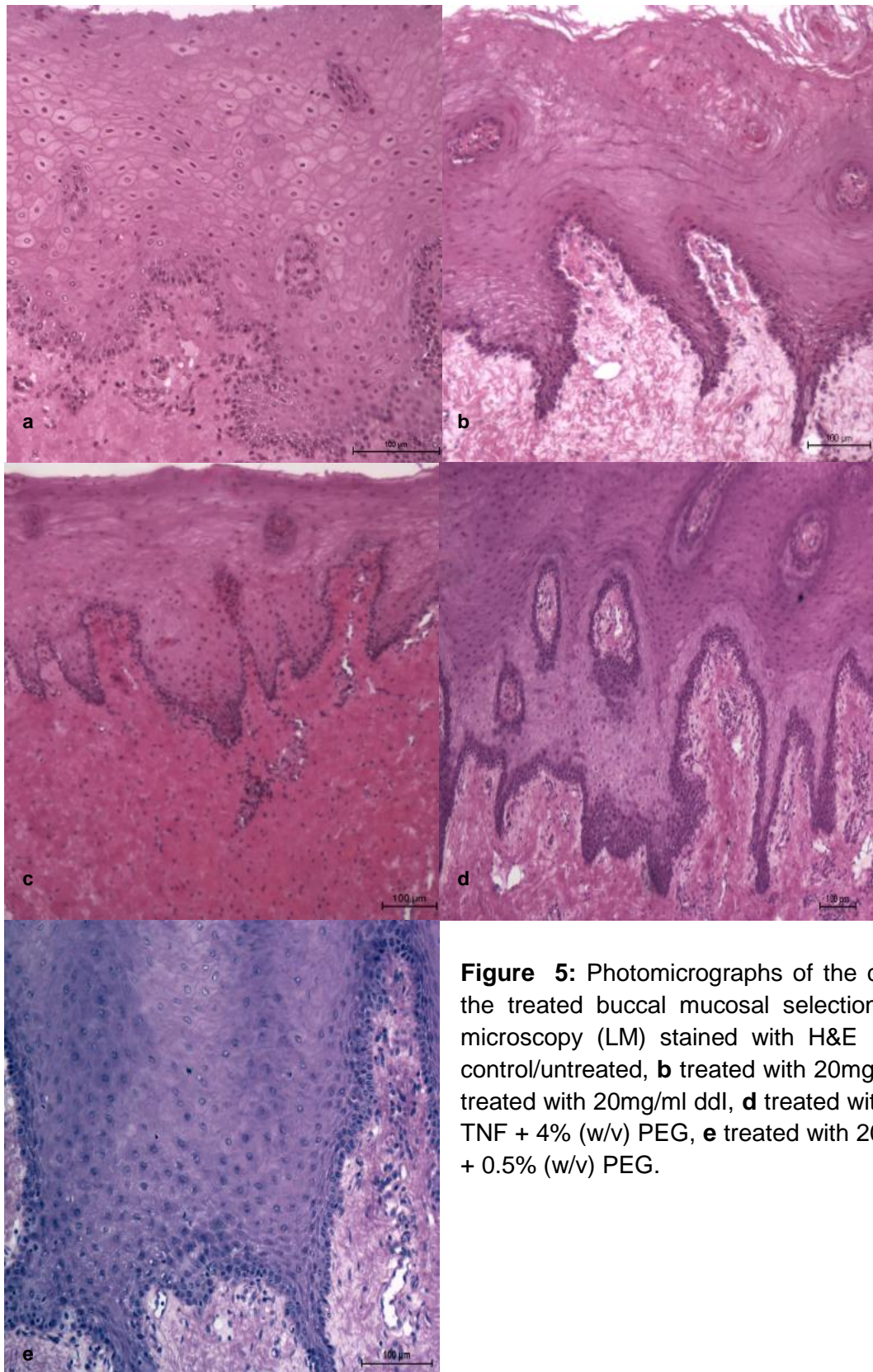
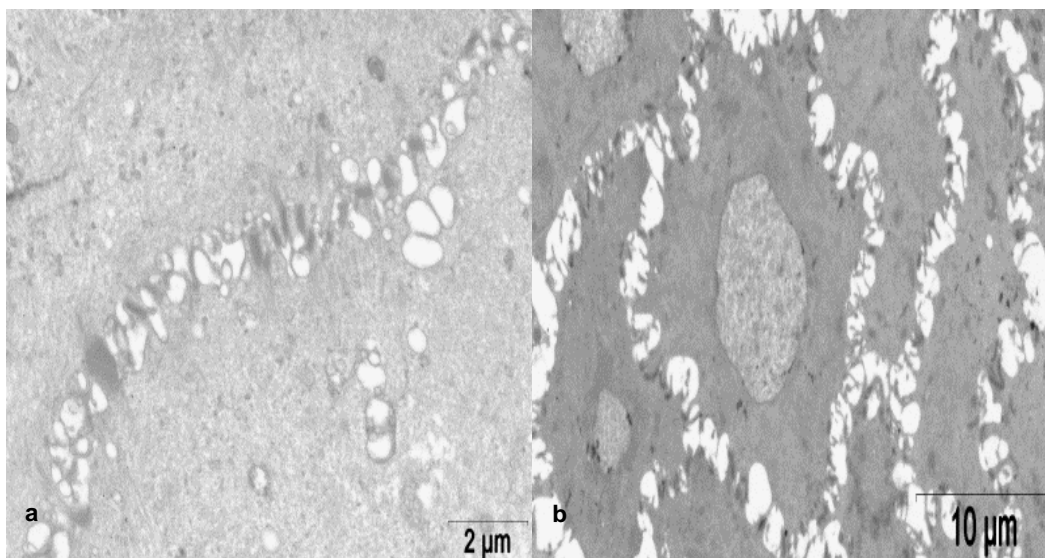


Figure 5: Photomicrographs of the control and the treated buccal mucosal selections for light microscopy (LM) stained with H&E ; ($\times 40$) **a** control/untreated, **b** treated with 20mg/ml TNF, **c** treated with 20mg/ml ddl, **d** treated with 20mg/ml TNF + 4% (w/v) PEG, **e** treated with 20mg/ml ddl + 0.5% (w/v) PEG.

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In addition electron micrographs, compared to control (Figure 6a), also revealed an increase in the size of intercellular spaces when exposed to both drugs (Figure 6b and 6c respectively). The distinct expanded intercellular spaces observed for both drug treatments can be corroborated by the assertion that hydrophilic drugs could dissolve in the aqueous intercellular space, and therefore favor the paracellular route of absorption (25, 33).

Using LM we were unable to establish a loss in cellular integrity when PEG was coupled with both ddi and TNF at 0.5% and 4% w/v respectively (Figure 5d and 5e). However, using TEM, larger intercellular spaces were observed with the addition of PEG to both drugs (Figure 6d and 6e respectively). These larger intercellular spaces may be responsible for the increased permeability observed with the addition of PEG (36). We also observed an increase in nuclear invaginations and changes in the nuclear envelopes; (TNF + 4% w/v PEG electron micrographs) (Figure 6f). The mechanisms and functional significance of this is not really known but studies suggest the involvement of the cytoskeleton (41). We speculate that, in this study, a combination of factors such as mechanical stress, ionic changes and increased cellular activity may have led to these effects; in response to the permeation experiments. These changes observed by TEM for PEG with both drugs may not be considered deleterious.



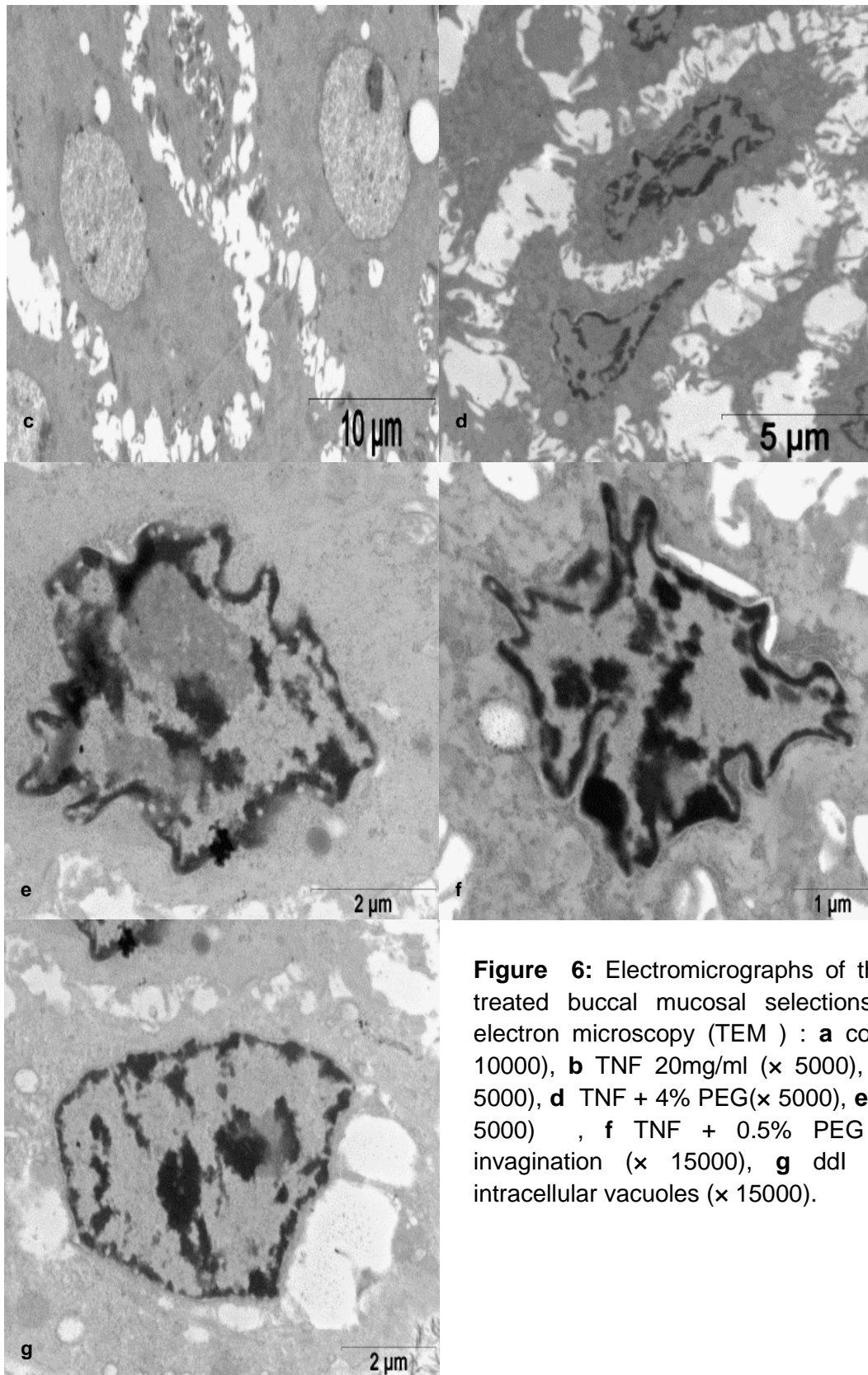


Figure 6: Electromicrographs of the control and the treated buccal mucosal selections for transmission electron microscopy (TEM) : **a** control/untreated ($\times 10000$), **b** TNF 20mg/ml ($\times 5000$), **c** ddi 20mg/ml ($\times 5000$), **d** TNF + 4% PEG($\times 5000$), **e** ddi + 0.5% PEG($\times 5000$) , **f** TNF + 0.5% PEG showing nuclear invagination ($\times 15000$), **g** ddi 20mg/ml showing intracellular vacuoles ($\times 15000$).

5. CONCLUSIONS

This study investigated the buccal delivery potential of two ARV drugs, ddl and TNF. It further demonstrated the effects of polymeric excipients on the buccal permeation of ddl and TNF. Both TNF and ddl were able to permeate the buccal mucosa in a concentration dependent manner at similar concentrations, with ddl having the greater permeability. The permeation of these drugs used alone can be attributed to passive diffusion via the paracellular route with transcellular route, being an additional possibility for ddl. The addition of all polymeric excipients, i.e. PAA, SA, CMC and PEG, used in this study increased the permeability of TNF, however only PEG was able to increase the permeability of ddl. The effect of these polymeric excipients and that of the drug appeared to be dependent on their ionic charge. Histological investigations revealed no significant loss in cellular integrity for mucosa treated with either TNF or ddl, alone, or when coupled with PEG as an enhancer. The differences in histomorphological changes in response to TNF and ddl alone, could support the greater permeation observed with ddl.

Selective polymeric excipients provide an effective means to increase the penetration of ddl and TNF. Their previously reported mucoadhesive and controlled release properties coupled with their permeation enhancing effects shown in this study highlight their potential use as multifunctional excipients for the design of buccal drug delivery systems.

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CHAPTER 4 – MANUSCRIPT OF ACCEPTED ARTICLE

High-energy Ball Milling of Saquinavir Increases Permeability across the Buccal Mucosa

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ABSTRACT

Saquinavir (SQV), a candidate for buccal drug delivery, is limited by poor solubility. This study identified the effects of high energy ball milling on the buccal permeability of SQV and compared it to the effects of chemical enhancers i.e. Ethylenediaminetetraacetic acid (EDTA), Sodium lauryl sulphate (SLS), Polyethylene glycol (PEG) and Beta Cyclodextrin (β -cyclodextrin). SQV was ball milled using a high energy planetary mill (1, 3, 15 and 30 hr) and permeation studies across porcine buccal mucosa were performed using franz diffusion cells. Drug was quantified by UV spectrophotometry. Both unmilled and milled SQV samples were able to permeate the buccal mucosa. Milled samples of 15 hr displayed the greatest flux of $10.40 \pm 1.24 \mu\text{g}/\text{cm}^2\text{hr}$ and an enhancement ratio of 2.61. All enhancers were able to increase the buccal permeability of unmilled SQV, with SLS achieving the greatest flux ($6.99 \pm 0.7 \mu\text{g}/\text{cm}^2$) and an enhancement ratio of 1.75. However, all the milled SQV samples displayed greater permeability than SLS, the best chemical enhancer for unmilled SQV. Enhanced permeability by ball milling was attributed to reduction in particle size, formation of solid dispersions and an increase in solubility of milled samples. Microscopical evaluation revealed no significant loss in mucosal cellular integrity treated with either unmilled or milled SQV. Histological studies suggest that SQV uses both the paracellular and transcellular route of transport across the mucosa, with drug treatment having no permanent affects. High energy ball milling was superior to the chemical enhancers studied for enhancement of SQV buccal permeation.

Keywords: buccal permeability, saquinavir, ball milling, permeation enhancer, histology

1. INTRODUCTION

HIV (Human Immunodeficiency Virus) infection and AIDS (Acquired Immune Deficiency Syndrome) is one of the most serious public health diseases and the main cause of death in sub-Saharan Africa^{1,2}. Although oral antiretroviral (ARV) drug therapy has significantly improved the treatment of HIV/AIDS, several disadvantages are associated with their use. HIV/AIDS drugs administered via the conventional oral route are exposed to the risk of pre-systemic degradation in the gastrointestinal tract, therefore decreasing bioavailability^{3,4}. Short half-lives require frequent administration of doses, which decrease patient compliance, and severe dose dependent side-effects may also occur, and some may display formulation problems due to poor solubility⁵. Alternate routes of drug administration over the oral route have therefore been widely studied to help overcome these limitations.

The buccal route of drug administration has received significant attention in the literature, enabling them to bypass enzymatic degradation and hepatic first pass metabolism, thereby improving the systemic bioavailability of various drugs⁶⁻⁸. In addition, absorption following buccal route administration is not influenced by potential variations in the gastric emptying rate or the presence of food⁹. Another route of administration is the skin, but despite the ease of application, permeability of the buccal mucosa is higher and cell recovery is also more rapid¹⁰. The buccal mucosa also has a larger area for drug application, is more easy accessibility compared to the nasal, rectal and vaginal mucosa¹¹, and is more resistant to tissue damage or irritation¹². In addition, buccal delivery systems can be easily applied and removed, has a high patient acceptability compared to other non-oral routes of drug administration¹³, will be advantageous for pediatrics and patients with swallowing problems. The epithelium found lining the buccal mucosa of the pig is stratified squamous epithelium, and resembles that of the

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human more closely than any other animal in terms of structure and composition. With its main function being to withstand abrasion due to mastication and to remain lubricated to protect against mechanical abrasion, this epithelial lining remains unkeratinized and therefore an attractive site for drug delivery^{8, 14}. ARV drugs may benefit from buccal drug delivery, and the literature indicates that Tenofovir, Didanosine and Zalcitabine have been studied for their buccal permeation properties. While ARV drugs have been more widely studied for their transdermal route of administration¹⁵⁻¹⁸, research on their buccal permeability properties appear to be limited^{19,20}, necessitating further studies on optimizing their delivery via this route.

Saquinavir (SQV) an ARV drug, belongs to the protease inhibitor class of ARV's, and is one of the new and effective classes of first-line therapies for HIV/AIDS, and inhibits both HIV-1 and HIV-2 proteases. SQV has low aqueous solubility, and has a very low systemic bioavailability when administered orally, this being a function of incomplete absorption and high first pass metabolism in the gut and liver^{3, 4, 21-23}. While SQV may therefore be an ideal candidate for buccal drug delivery, this route of administration for may be limited by its poor solubility. Strategies that enhance the solubility of SQV will therefore be required for delivery via the buccal route.

Common approaches used to enhance low aqueous solubility of drugs include methods that reduce particle size (including microsizing and nanosizing), increase their solubilization in co-solvents, improve their complexation with cyclodextrins, or for the delivery of lipophilic drugs, use lipid-based vehicles as well as solid dispersion technology^{21,24-26}. Although some of these techniques have been effective at enhancing oral bioavailability for specific compounds, success is usually marginal and highly dependent on the physicochemical properties of the drug. Poorly soluble drugs, including some poorly soluble ARVs, have been studied in the

literature using a number of these strategies to enhance their aqueous solubility. Chiappetta et al. conducted a study that encapsulated Efavirenz in polymeric micelles to improve its aqueous solubility with positive results²⁷. However, it has been highlighted in the literature that concerns over storage stability, nanotoxicity and a limited number of polymers available for clinical use for this type of formulation may be limiting factors to this approach²⁸. Further research should therefore be done on other strategies to enhance the solubility of poorly water-soluble drugs.

With the addition of polymeric micelles, Chen et al. describes in detail the various strategies used for the nanonization of poorly soluble drugs such as the formation of nanoemulsions, as well as drug nanocrystals. This is the most widely used strategy to increase the oral bioavailability of hydrophobic drugs, with nanoprecipitation, high-pressure homogenization and media milling being the most preferred preparations²⁸. These strategies have shown significant promise, with many water-insoluble drugs approved for clinical use or under clinical trials^{28,29}. The 'top down' technologies, such as media milling to produce drug nanocrystals, are preferred to the 'bottom up' (precipitation) methods. Media milling can process milligram quantities in a rapid screening mode in an early discovery stage, and can also facilitate the possibilities of large-scale production for market, therefore offering a promising alternative to enhance solubility of poorly soluble drugs²⁹.

The formation of amorphous solids or solid dispersions also presents a promising approach to overcome limited solubility of poorly soluble drug^{24, 30-32}. Although milling is an approach widely used to reduce particle size, it is not commonly used for making amorphous dispersions, despite being shown to be suitable for this purpose³³. A recent study by Caron et al. showed that ball milling is a viable process for producing amorphous dispersions of sulfonamide³³, while Al-Obaidi et al. indicated the advantages of using ball milling to prepare solid dispersions of

griseofulvin³⁴. High energy ball milling has the advantage of generating an amorphous system while simultaneously promoting the formation of reduced particle size²¹.

In a previous study by our group, high energy ball milling was used to prepare solid dispersions of SQV with optimal surface area and porosity in order to overcome its limited solubility. Milled samples were extensively characterized using nuclear magnetic resonance spectroscopy (NMR), attenuate total reflectance-infrared spectroscopy (ATR-IR), differential scanning calorimetry/thermogravimetric analysis (DSC/TGA), X-ray power diffraction (XRD) and BET surface area and porosity. It was also characterized using field emissions scanning electron microscopy (FESEM) and solubility studies to determine the effects of ball milling on the solid-state characteristics and aqueous solubility of SQV²¹. The results showed that the milled SQV samples displayed a decrease in particle size, the formation of solid dispersions and an increased solubility in simulated saliva. High energy ball milling displayed a 9-fold increase in the solubility of SQV in simulated saliva at pH 6.8²¹, which may promote its permeation across the buccal mucosa. However, the subsequent effect of these ball milled samples on buccal permeation parameters such as steady state flux and permeability coefficient have not been reported, and neither has the permeation of SQV through the buccal route been studied. Therefore, based on the suitability for ARV delivery, there is a need to investigate whether the ball milling can improve the buccal permeation of SQV.

One of the main disadvantages of the buccal route is the low mucosal permeability that impedes optimal permeation of drug, necessitating use of permeation enhancers for the drug to permeate across the mucosa⁸. These permeation enhancers alter mucosal permeability by various mechanisms, such as changing the mucus rheology, disturbing the intracellular lipid packing in the bilayer membrane and loosening the tight junctions between cell^{6,8}. There are various

classes of permeation enhancers, namely surfactants, fatty acids, cyclodextrins, chelators and others. Ethylenediaminetetraacetic acid (EDTA) belongs to the chelator class of buccal permeation enhancers, this class being known to increase buccal permeability by interfering with Ca^{2+} to maintain the intercellular spaces, thereby permitting paracellular transport. Sodium lauryl sulphate (SLS) belongs to the surfactant class of enhancers that act by extracting membrane protein or lipids, assisting membrane fluidization, or producing reverse micellization in the membrane and creating aqueous channels, thereby increasing the permeability of the drug/compound. β cyclodextrin belongs to the cyclodextrin class of enhancers and acts via the inclusion of membrane compounds to increase the membrane permeability. PEG, recently identified as a multifunctional excipient in our laboratory, has shown promise as a buccal permeation enhancer^{6,35}. The effect of these various classes of enhancers on SQV have not been reported, and a study comparing the effects of high energy ball milling as a strategy for permeation enhancement, compared to typical examples from the various classes of chemical enhancers, has not been undertaken.

The aims of this study were therefore to investigate the buccal permeability potential of SQV, and to evaluate the effects of high energy ball milling on the permeability parameters of SQV. The effects of various classes of commonly used permeation enhancers on the permeability of SQV were also investigated and compared to high energy ball milling as an approach to enhancing buccal permeability. In addition, it evaluated the histomorphological effects of both milled and unmilled samples of SQV on the buccal mucosa.

2. METHODS

2.1 Ethical Clearance

Ethical clearance was obtained from the University of KwaZulu-Natal Ethics committee in 2010 (010/10/Animal), and renewed in 2011 (24/11/Animal) and 2012 (08/12/Animal).

2.2 Materials

The buccal mucosal tissue was obtained from Baynesfield Abattoir (Pietermaritzburg, South Africa), from pigs weighing approximately 60-80 kg. Phosphate buffered saline (PBS) with a pH 7.4 was prepared using sodium chloride, potassium dihydro-orthophosphate and disodium hydrogen orthophosphate (Associated Chemical Enterprises, South Africa). Ethylenediaminetetraacetic acid (EDTA), Sodium lauryl sulphate (SLS), Polyethylene glycol (PEG) and Beta Cyclodextrin (β -cyclodextrin) was purchased from Sigma-Aldrich. Saquinavir mesylate (MW = 767.0 g/mol) was kindly donated by Hoffmann- La Roche Ltd. (Basel, Switzerland) and analyzed both before and after milling for 1–30 hr.

2.3 Preparation of nanoporous saquinavir: high-energy ball milling

The ball milling was performed in a high-energy planetary mill (Retsch PM 400) at room temperature under compressed air. Stainless steel milling jars (250 mL) containing an appropriate mass of stainless steel balls (15 mm) were used to mill the samples. One gram of SQV was milled for 1, 3, 15, and 30 hr in each jar, with a ball/sample weight ratio of 30:1. The speed of the solar disk was set to 200 rpm. The milled and unmilled samples were characterized in terms of solubility, melting point, density, particle size, BET surface area, pore volume and pore size, and is presented in Table 1, as previously reported by our group²¹.

Table 1: Saquinavir mesylate solid state characteristics (21).

Ball milling time (hr)	0	1	3	15	30
Solubility (mg/ml)	0.293 ± 0.07	1.349 ± 0.03	2.986 ± 0.02	2.279 ± 0.01	2.979 ± 0.01
Density (g/ml)	1.149	1.197	1.168	1.210	1.172
Particle size (nm)	1030	912	355	3400	n/d
BET surface area (m²/g)	5.069 ± 0.17	5.470 ± 0.15	14.43 ± 0.36	1.455 ± 0.092	n/d
Pore volume (μL/g)	20.0	29.0	41.0	11	n/d
Pore size (nm)	15.59	23.29	11.29	30.58	n/d

2.4 Tissue preparation

The porcine buccal tissue was harvested using surgical scissors from the pigs immediately after slaughter. The tissues were excised, using surgical scissors, to remove all underlying excess tissue to a thickness of between 500-700 μm, leaving the basal lamina intact. The excised tissues were transported in cold, normal saline (NS), snap frozen in liquid nitrogen within 30 minutes after excision, stored in a biofreezer (-80°C) and used within three months^{19,35}. Cold PBS (pH 7.4) was used to defrost the buccal mucosal tissue at room temperature for 45 minutes before it was used in permeation studies.

2.5 Permeation Measurement across Porcine Buccal Mucosa

In vitro permeation studies were conducted at 37±1°C using modified vertical Franz type diffusion cells (PermeGear, Inc., Bethlehem, USA) with a diffusional area of 0.786 cm². A circular section of the buccal mucosa was mounted onto the diffusional area between the donor and receptor cells, and was equilibrated with PBS (pH 7.4) at 37°C for 30 minutes. The donor

compartment solution contained either SQV unmilled or milled samples dissolved in PBS (20 µg/ml), or SQV unmilled in the presence of the various enhancers (BCD, EDTA, PEG and SLS) at a concentration of 0.5% w/v in respective experiments. The receptor compartments were filled with PBS and stirred with a teflon-coated magnetic bar. Samples were removed from the receptor compartments at predetermined time intervals and replaced with the same volume of SQV-free (fresh) PBS. Each experiment represents a minimum of three replicates. The drug was quantified by a validated UV spectrophotometry method at a λ_{max} of 239 nm (UV Spectrophotometer 1650, Shimadzu, Japan), which are similar to other buccal permeability studies^{19, 35}.

2.6 Permeability data analysis

The cumulative amount of drug (SQV) permeated per unit surface area was plotted against time. The steady state flux (J_{ss}) was determined from the linear part of the permeability curve by linear regression analysis (Microsoft Excel 2007, USA). The permeability coefficient (P) was calculated as follows^{19, 35}:

$$P = (dQ/dt)/A \times C_d = J_{ss}/C_d \quad [1]$$

dQ/dt is the cumulative amount permeated per unit time, A is the diffusion area and C_d is the drug concentration in the donor compartment. The enhancement ratio (ER) was calculated using the following equation^{19, 35}:

$$ER = \frac{\text{Permeability coefficient of drug in the presence of enhancer}}{\text{Permeability coefficient of drug in the absence of enhancer}} \quad [2]$$

2.7 Transepithelial electrical resistance (TEER) studies

The integrity of the buccal mucosa was assessed by transepithelial electrical resistance (TEER) measurements using a Millicell ERSmeter (Millipore, USA) connected to a pair of chopstick electrodes (STX01). TEER measurements were taken across the mucosa prior to, and at the end of the permeation experiment³⁷. TEER values at time zero were used as 100%. The rebound effect of the mucosa post drug treatment was measured by removing the drug solution from the donor compartment after a period of six hours and replacing it with fresh PBS for a period of two hours with subsequent TEER measurements³⁸.

2.8 Light Microscopy and Transmission Electron Microscopy for Histomorphological evaluations

Histological evaluations were performed on freshly harvested, excised buccal mucosa. Untreated buccal mucosa was transferred directly after excision from normal saline into 10% buffered formalin without any equilibration in PBS, and served as the control. Treated samples consisted of buccal mucosa that were exposed to PBS only, or 20 µg/mL SQV (unmilled) dissolved in PBS or 20 µg/mL SQV (1 hr milled) dissolved in PBS or 20 µg/mL SQVM (15 hr milled). Permeation experiments were carried out with these solutions in the donor compartment, and with the freshly excised buccal mucosa placed between donor and receptor compartments as described in 2.5 above, without drug quantification^{19, 20, 35}. At the end of the experiment, the buccal mucosa was removed from the Franz diffusion cells, cut into cross sections and fixed in 10% buffered formalin. Both the control and treated buccal mucosa were fixed in formalin for seven days at room temperature. Buccal mucosa was dehydrated using an ethanol gradient ranging from 50% up to 96% and embedded in paraffin wax. The mucosae sections were collected on slides, dried and stained with hematoxylin and eosin (H&E). Sections were examined using a light microscope (Nikon 80i, Japan), and bright field images were

digitally captured using NIS Elements D software and a camera (Nikon U2, Japan). The samples for transmission electron microscopy (TEM) were obtained after the above mentioned permeation experiments. The samples were then cut into pieces not exceeding 0.5 mm³, and fixed for 24 hours (4°C) using Karnovsky's fixative³⁹ buffered to pH 7.2. For TEM, each sample was processed and embedded in epoxy resin using standard protocols. Ultrathin sections (90 nm) were cut and contrasted with uranyl acetate and lead citrate, and were viewed with a transmission electron microscope (JEOL 1010, Japan). All experiments were performed using a minimum of three replicates.

2.9 Statistical Analysis

The results, expressed as mean \pm standard deviation (SD), were analyzed using one-way analysis of variance (ANOVA) followed by the Mann Whitney test using GraphPad Prism® (Graph Pad Software Inc., Version 5., USA). A *p* value of less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Buccal permeability of SQV

The permeability potential of unmilled SQV was first investigated, with the results displaying a maximal cumulative amount of $28.78 \pm 2.18 \mu\text{g}/\text{cm}^2$ (Figure 1) and a steady state flux value of $3.99 \pm 0.11 \mu\text{g}/\text{cm}^2\text{hr}$ (Table 2). These buccal permeability parameters were comparable to other drugs such as risperidone, ondansetron HCl and 5-aza-2-deoxycytidine which have been investigated for buccal drug delivery⁴⁰⁻⁴². A series of milled SQV samples (1, 3, 15 and 30 hours milled) were then studied to determine their effects on the permeability parameters. Milling the SQV for one hour led to a significant increase ($p < 0.05$) in the steady state flux, as compared to

the control, which increased from $3.99 \pm 0.11 \mu\text{g}/\text{cm}^2\text{hr}$ to $10.30 \pm 0.85 \mu\text{g}/\text{cm}^2\text{hr}$ (Table 2), and represents an enhancement ratio (ER) of 2.58. This decreased particle size (912 nm), displayed by the one hour milled sample compared to the control (1030 nm), is responsible for the increase in solubility and resulting increased permeability²¹. Raising the milling time to three hours also led to a significant increase ($p < 0.05$) in the flux value $7.99 \pm 0.34 \mu\text{g}/\text{cm}^2\text{hr}$ compared to the control. However, this increase was lower (ER= 2) than those obtained using the one hour milled sample (Table 2). These findings can be explained by the physicochemical properties of the three hour sample, which displayed a lower particle size of 355 nm compared the control (Table 1).

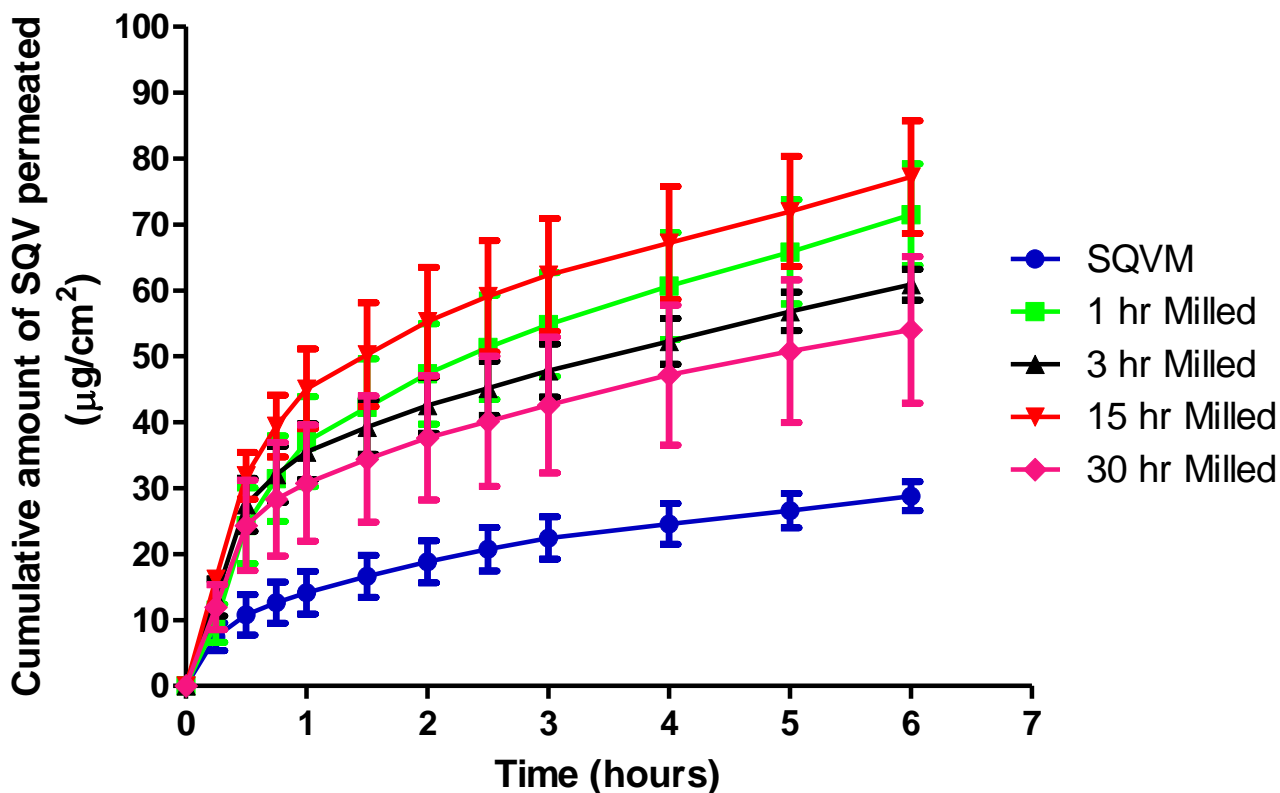


Figure 1: Effect of milling time on the cumulative amount of SQV permeated across the buccal mucosa.

Table 2: Effect of ball milled SQV samples on buccal permeability properties

Donor (20µg/ml)	Flux (Jss) (µg/cm ² h)	Permeability coefficient (P×10 ⁻²) (cm/h)	E.R
unmilled SQV	3.99 ± 0.11	19.95 ± 0.57	
1 hour milled	*10.30 ± 0.85	51.53 ± 4.25	2.58
3 hour milled	*7.99 ± 0.34	39.95 ± 1.71	2
15hour milled	*10.40 ± 1.24	52.01 ± 6.21	2.61
30 hour milled	*7.18 ± 1.38	35.93 ± 6.92	1.8

* Indicates significant difference i.e $p < 0.05$ (all values compared to unmilled SQV)

Although the reduction in particle size was responsible for the increased solubility, there was an increase in surface area to 14.43 ± 0.36 nm compared to the 5.47 ± 0.15 nm of the one hour milled sample (Table 1). The increase in surface area combined with the reduced particle size of the three hour sample could have increased the interaction between drug molecules, and resulted some of these particles possibly forming complexes, thereby slightly hindering the permeation of the drug across the mucosa and reducing the flux values. Increasing the milling time to 15 hours displayed a highest ER of 2.61 with a cumulative amount of 77.21 ± 4.53 µg/cm² (Figure 1) and steady state flux value of 10.40 ± 1.24 µg/cm²hr (Table 2). This resulted in an increase in the particle size and a decrease in the surface area when compared to the control and the one and three hour samples (Table 1).

Despite this increase in the particle size, the solubility was not reduced when compared to the three hour sample, which displayed the highest solubility (Table 1). A previous paper showed that this was attributed to the formation of solid dispersions in samples milled for at least 15 hours²¹. Furthermore, SQV exhibiting extended pseudo-polymorphism undergoes polymorphic changes during ball milling. At 15 and 30 h period of ball milling, new but unstable polymorphic

structures of SQV are formed²¹. These polymorphic conversions and generation of amorphous mixture due to loss of water of hydration during ball milling results in materials referred as solid dispersions⁴³⁻⁴⁶. The loss of crystalline water upon extensive grinding results in very hygroscopic drug solids⁴⁵⁻⁴⁷ and many researchers have shown previously that amorphous drug requires lower activation energy for dissolution than its crystalline form⁴⁸⁻⁵⁰. Therefore, it can be assumed that formation of more soluble polymorphic forms and hygroscopic drug solids together have contributed to highest ER of 15 h milled samples. The 15 hour milled sample has a higher solubility than the one hour milled sample (Table 1), which explains the slightly higher values obtained using the former. The 15 hour sample also displayed the largest pore size of all the milled samples (Table 1), indicating that milled samples with a larger pore size (i.e. 1 and 15 hour) have a greater permeability.

A further increase in milling time from 15 hours to 30 hours produced a sample that also displayed enhanced permeability compared to the control, being able to attain a cumulative amount of $54 \pm 6.10 \mu\text{g}/\text{cm}^2$ and an increase in mean steady state flux from $3.99 \pm 0.11 \mu\text{g}/\text{cm}^2\text{hr}$ to $7.18 \pm 1.38 \mu\text{g}/\text{cm}^2\text{hr}$ (Table 2) with an ER of 1.8. Although these values are greater than that of the control, the values obtained for the 30 hour milled sample were the lowest of the milled samples. Branham et al., 2012 reported that the greatest increases in solubility of SQV was observed at the lower end of the milling range, and as the milling time increased from 30 and 60 hours, the solubility of SQV decreased, with drug aggregates forming after 15 hour milling. These results support the data observed in this study, with the decrease in solubility and aggregate formation beyond 15 hours possibly being responsible for the decrease in permeability observed with 30 hour milled SQV sample. In previous studies will ball milled SQV, XRD diffractograms indicated a gradual crystalline to amorphous transition with milling time. Also, surface micrographs of pellets of SQV before and after milling indicated differences.

Unmilled SQV consisted of fused plate-like crystalline structures whilst milled SQV showed a smooth glassy microstructure²¹. Therefore these changes in crystallinity and morphology may have also resulted in the enhanced permeation of SQV with ball milling. The maximal enhancement ratio obtained using the milled samples compares favourably with the enhancement ratios reported by previous studies using chemical permeation enhancers, such as sodium tauroglycocholate, carboxymethyl cellulose, sodium alginate and methyl-beta-cyclodextrin, for enhancement across the buccal mucosa^{35, 51, 52}.

Transepithelial electrical resistance (TEER) measurements were taken prior to and after permeation experiments using the milled samples to identify the mucosal integrity during permeation experiments. TEER measurements were also taken two hours post permeation, with the drug solution in the donor compartment being replaced with fresh PBS. TEER measurements reflect the tightness of intercellular junctions between epithelial cells, and can be used as an indicator of epithelial viability for mucosal permeation experiments^{37, 53}. A decrease in TEER measurements is also an indication of the opening of tight junctions between adjacent epithelial cells, which indicates an enhancement of the paracellular permeability across the epithelial cell layer³⁸. However, the reported values in the limited studies on porcine buccal mucosa permeation varying widely (144 ± 12 to $950 \pm 392 \text{ } \Omega/\text{cm}^2$), with standardization for this type of experiment still needing to be developed^{37,54}.

The TEER values across the buccal mucosa prior to the permeation experiment (control) in this study was found to be between 322 ± 17 to $458 \pm 21 \text{ } \Omega/\text{cm}^2$ (Table 3), which compare favorably with the range reported for such studies in the literature^{37,54}. TEER values at time zero (control) were used as 100%. After six hours of permeation, these values decreased to a range of 5.70 ± 1.4 to $11.4 \pm 2.8 \%$ (Figure 2) from the initial values recorded. These reductions in the TEER

values are indicative of the opening/widening of intercellular junctions, which facilitates the permeation of SQV across the buccal mucosa. As there was a decrease in all samples tested (Figure 2 and Table 3), this indicates that SQV utilizes the paracellular route of transport across the buccal mucosa. It was observed that the sample with the greatest decrease in TEER values after the permeation, the 15 hour sample, also displayed the greatest enhancement ratio of 2.61. Two hours after the replacement of the drug solution from the donor chamber with fresh PBS, the TEER values increased within a range of 319 ± 18 to $435 \pm 23 \Omega /\text{cm}^2$ from the values measured after permeation (Table 3). There was also a difference of only 3.04 ± 0.8 to $6.73 \pm 1.9 \%$ between initial TEER values measured and those two hours post permeation (Figure 2). These values are a reflection of the recovery of the mucosa post drug treatment and signified a return towards the initial measured integrity. The TEER values obtained in this study appear to be within the reported range, and the overall percentage change also indicates that mucosal integrity was not irreversibly affected^{37, 55}. The results of the TEER study correlated with the histomorphological studies (Section 3.3), which further confirmed that integrity and viability of the tissue was maintained.

Table 3: TEER measurements across the buccal mucosa after permeation with milled SQV samples

SQV hrs milled (hrs)	Control (0 hrs)(Ω/cm^2)	6 hr permeation (Ω/cm^2)	*% reduction in TEER after 6hr permeation	2 hrs post permeation (Ω/cm^2)
1	458 ± 21	424 ± 24	7.42 %	435 ± 23
3	394 ± 12	368 ± 21	6.59 %	382 ± 9
15	386 ± 22	342 ± 27	11.39 %	360 ± 27
30	332 ± 17	313 ± 21	5.72 %	319 ± 18

* % change compared to control (0 hrs)

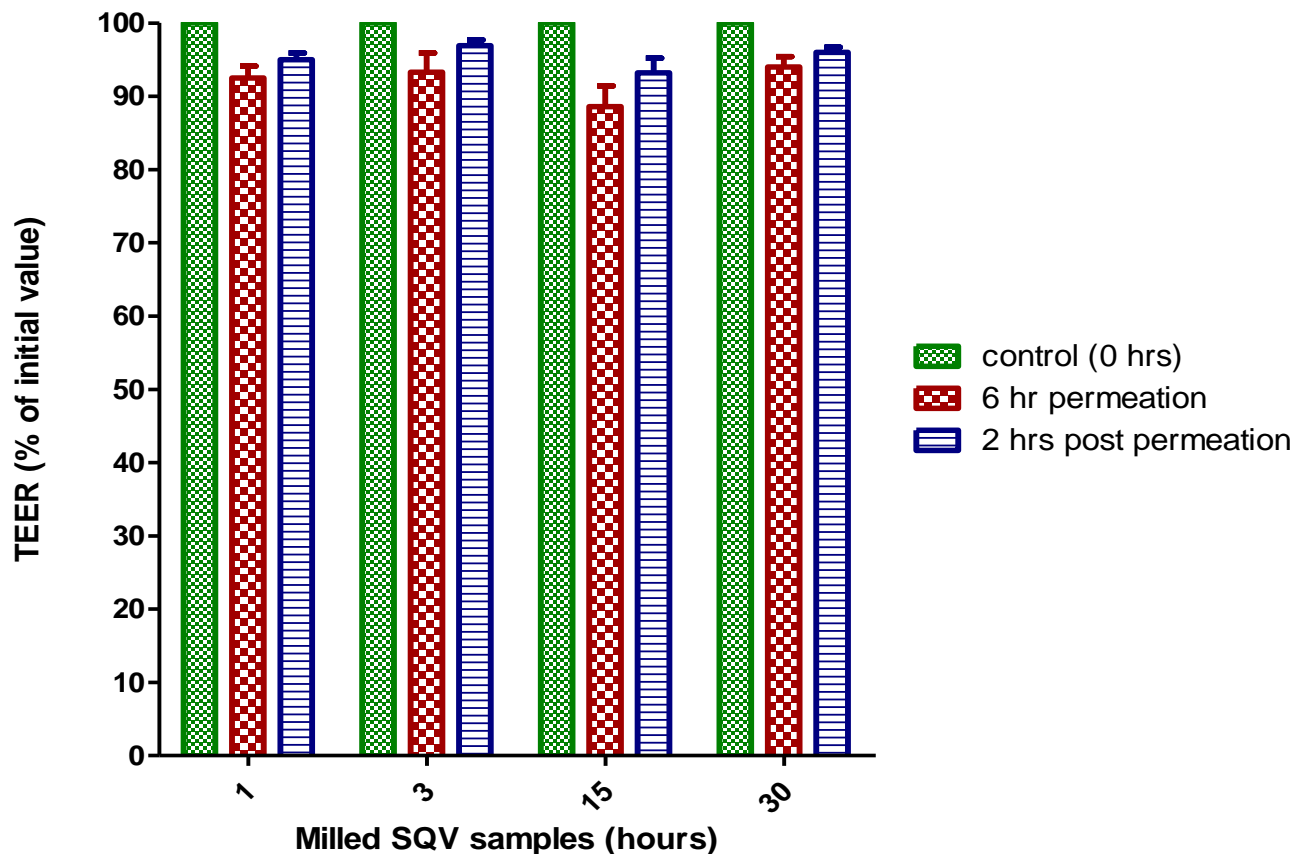


Figure 2: % change in TEER values after permeation with milled SQV samples.

This study has demonstrated that high energy ball milling is an effective strategy for enhancing SQV permeation across the buccal mucosa, and that the enhanced permeability is due to a contribution of various factors such as solubility, particle size, surface area, and the formation of solid dispersions.

3.2 Effects of permeation enhancers on SQV permeability

Low mucosal permeability has been reported as one of the disadvantages associated with the buccal route of drug administration. The lipid matrix of the extracellular spaces plays a role in

the barrier function, particularly if the permeating compounds have higher molecular weights^{12,56}, and paracellular transport of macromolecules are restricted by tight junctions or similar interconnections that exist between cells^{5,7,57,58}. To help alleviate this, permeation/penetration enhancers have been used to promote absorption of a drug across the buccal mucosa by decreasing its barrier properties, thereby delivering therapeutically relevant amounts into the systemic circulation. SQV could show increased permeability across the buccal mucosa if it were coupled with permeation enhancers. High energy ball milling of SQV displayed enhanced permeability of SQV (Figure 1 and Table 2), and the increased permeability to enhancement the ratio's obtained for SQV using commonly employed chemical enhancers in the literature was investigated.

Buccal permeation enhancers from the different classes (surfactants, fatty acids, inclusion complexes and chelators) were used as permeation enhancers for SQV. EDTA, SLS, β -cyclodextrin and PEG were chosen as they have been reported to have significant enhancement potential^{6, 35}. The respective enhancers were added to the donor chamber at 0.5% w/v. This concentration was chosen as it has been widely used in previous studies that incorporated different enhancers within their donor solutions for permeability studies^{19, 35, 51}. The results of this study showed that the permeability of SQV was increased in the presence of all the enhancers (Figure 3 and Table 4), these increases being statistically significant ($p < 0.05$). The addition of SLS displayed the greatest increase in steady state flux value, which increased from $3.99 \pm 0.11 \mu\text{g}/\text{cm}^2\text{hr}$ to $6.99 \pm 0.7 \mu\text{g}/\text{cm}^2$ with an enhancement ratio of 1.75 (Table 4), which was statistically significant. These enhancement ratios are in keeping with those achieved by these classes of enhancers and regarded as effective in the literature^{6, 35}. Changes in the steady state flux values and permeability coefficient could have resulted from the enhancers increasing the transport pathways through the buccal mucosa as described in the introduction.

The greatest enhancement obtained using the chemical enhancers (ER = 1.75) was lower than the lowest enhancement ratio obtained by milling SQV ie. 30 hour milled with an ER of 1.8. The flux values obtained in this study are similar or greater than flux values reported for other ARV drugs, as well those identified as having potential for buccal delivery e.g. Didanosine, Tenofovir, Ondansetron HCl, Risperidone and Decitabine^{19, 35, 40-42}. As the enhancement ratios obtained using the milled samples are comparable or greater than those obtained when using chemical methods for permeation enhancement, as previously described, these results confirm that at an equivalent concentration ball milling of SQV is more effective for permeability enhancement than the addition of EDTA, SLS, β -cyclodextrin and PEG at 0.5% w/v.

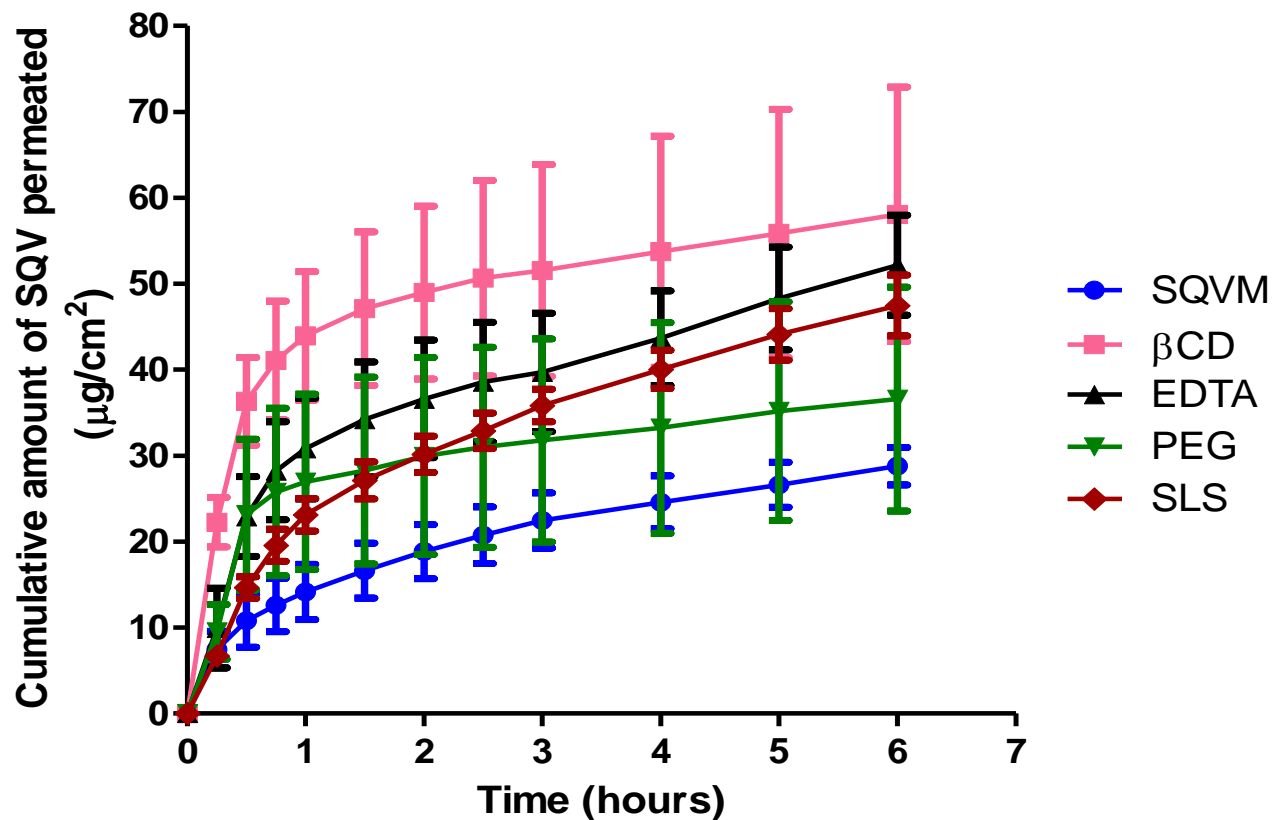


Figure 3: Effect of enhancers on the cumulative amount of unmilled SQV permeated across the buccal mucosa.

Table 4: Effect of permeation enhancers on buccal permeability properties

Donor (20µg/ml)	Flux (Jss) (µg/cm ² h)	Permeability coefficient (P×10 ⁻²) (cm/h)	E.R
unmilled SQV	3.99 ± 1.15	19.95 ± 0.57	
unmilled SQV + 0.5%βCD	*6.35 ± 2.37	31.75 ± 11.91	1.59
unmilled SQV + 0.5%EDTA	*6.79 ± 0.9	33.99 ± 4.50	1.7
unmilled SQV + 0.5%PEG	4.20 ± 1.52	21.0 ± 7.61	1.05
unmilled SQV + 0.5%SLS	*6.99 ± 0.7	34.95 ± 3.54	1.75

* Indicates significant difference i.e $p < 0.05$ (all values compared to unmilled SQV)

3.3 Histomorphological evaluations

The effect of the ARV drug SQV in its unmilled and milled form on the buccal mucosa was investigated histomorphologically to determine any deleterious effects of SQV on the cells of the buccal mucosa, with Light Microscopy (LM) and Transmission Electron Microscopy (TEM) being used to determine any loss of tissue viability^{35, 37}. The one hour and 15 hour milled samples were chosen as they displayed the greatest increase in SQV permeability. Assessing the impact of the drug on the histomorphology of the tissue included toxic effects upon exposure. It must be noted the barrier function of the stratified epithelium lends itself to 'rebound' effect after prolonged exposure to a drug, which was not assessed in this study. However, adequate information can be obtained from LM and TEM images to determine whether the tissue suffered permanent/irreversible damage after exposure to the drug treatment.

The buccal mucosa is multilayered, consisting of a changing morphology from base to apex, originating at the basal lamina, which is superficial to the lamina propria. The basal lamina is

lined by regenerative basal cuboidal cells that stain very darkly in LM preparations, depicting its highly mitotic activity³⁷. As the cells transgress closer to the surface, they become flattened and more closely packed, with less distinguishable intercellular spaces, which mainly present between cells closer to the basement membrane in TEM preparations. Any molecule that enters the circulation via the buccal mucosa must first traverse through these layers before it enters the circulation present in the lamina propria.

The control images (Figure 4b) of the H&E preparations closely resembled the description given above, indicating that the animal tissue used in the study was normal and healthy. The H&E micrographs of the treated samples were similar to that of the controls (Figure 4c and 4d), with cells showing normal morphology and darkly stained basal cells, depicting no decrease in the activity of these regenerative cells compared to their counterparts in the control image. There were also no observable cellular distortions and the basal lamina remained intact, preserving its supportive role and barrier function. Any damage observed to the superficial layers, caused by the drug treatment of the mucosa, should not be considered permanent, as the buccal mucosa has a high recovery rate¹⁴. However, no such observations were made in this study, the data from the H&E preparations suggesting that SQV permeation for a duration of six hours has no permanent effects on the buccal mucosa.

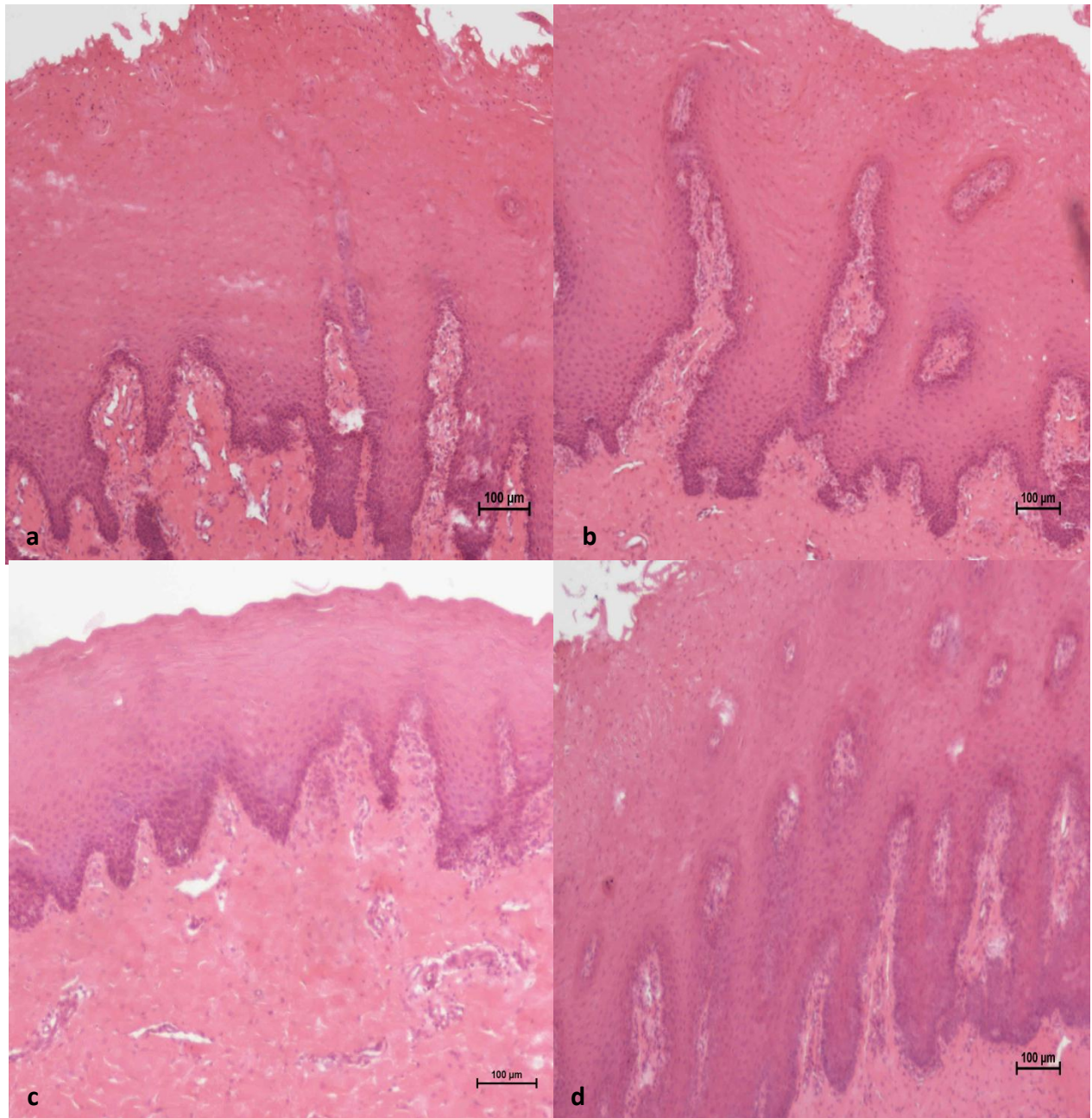
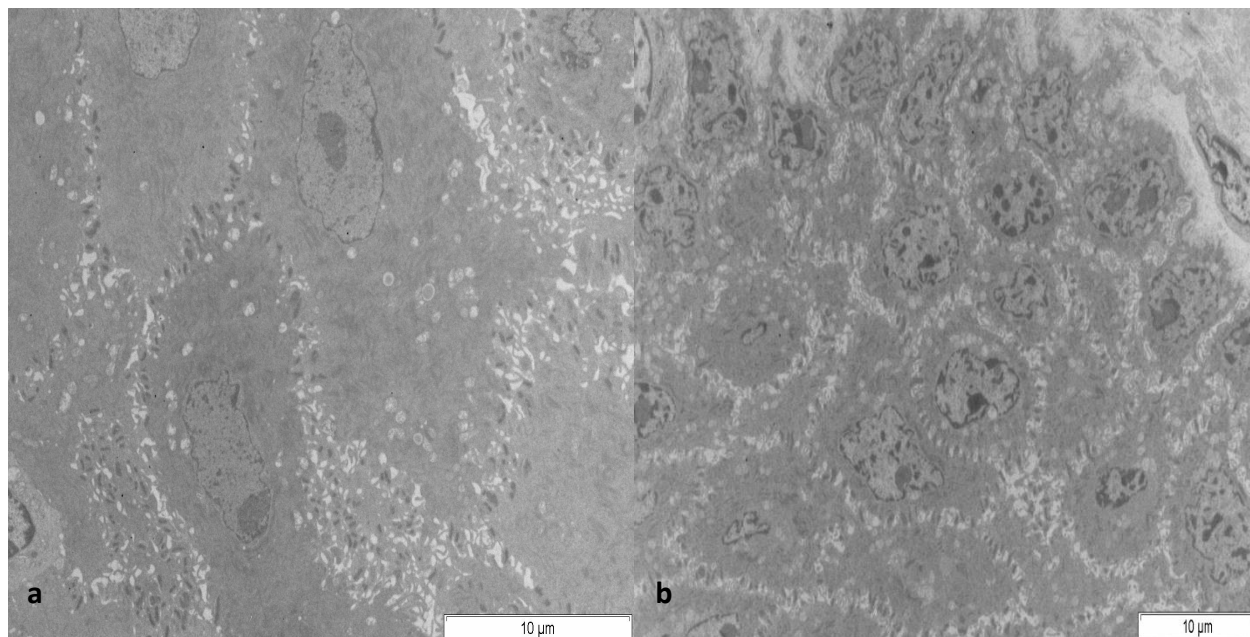


Figure 4: Photomicrographs of the control and the treated buccal mucosal selections for light microscopy (LM) stained with H&E ; ($\times 10$) **a** control/untreated, **b** treated with unmilled SQV, **c** treated with 1 hr milled SQV, **d** treated with 15 hr milled SQV.

TEM allows for a more in depth analysis of the tissue sample at a cellular level, and was therefore used to confirm the observations made with the H&E preparations, and the condition of the cellular components needed for the proper functionality of individual cells was assessed. Although limited, the TEM micrographs may provide clues to possible modes of transport across the cell, as the intercellular spaces between neighbouring cells was evaluated, being used as a route of paracellular transport across the mucosa. The conditions of the tight junctions that link these neighboring cells together were simultaneously observed, with their damage possibly leading to the tissue losing its 'rebound' affect. A permeant is able to use either the transcellular, paracellular or both the routes simultaneously to transgress through the multiple layers of the buccal epithelium¹⁴. The physicochemical properties of a drug can also influence the mechanisms and pathway of transport across the buccal mucosa. The paracellular mode may be one of the mechanisms that SQV uses to permeate across the mucosa, as shown by the increased intercellular spaces between the cells in the treated samples (Figure 5b and 5c) when compared to the control (figure 5a). This therefore suggests that although SQV is lipophilic and would most likely use the transcellular route of transport, it also utilizes the paracellular route. Its use of the paracellular route as indicated in these TEM images correlates with the TEER values. It was observed that the samples with the greatest extent of TEER reduction after the permeation (Table 3), i.e. 1 and 15 hour samples, also displayed the greatest permeation enhancement ratio of 2.58 and 2.61 respectively (Table 2). These findings therefore are in agreement with the TEM results which describes the larger intercellular spaces observed for these samples.

The TEM micrographs of the control displayed all the characteristics of healthy cells (figure 5a), and while the treated samples (figure 5b and 5c) also appeared to have no cellular damage, mild convolutions in the nuclear membranes (figure 5d) were observed. Although these may not

necessarily be deleterious to the cells, as no signs of necrosis, such as destruction of the nuclear membrane and nucleus, or damage to the cellular membrane were observed. An increase in the size of the intercellular spaces in the treated samples (figure 5b and 5c) was observed, these could have aided the transport of the drug via the paracellular route across the mucosa. The TEM Micrographs also displayed intact tight junctions in both the control as well as the treated samples (figure 5e and 5f respectively), which indicated that although the size of the intercellular spaces have been increased in the treated samples, they will not lead to permanent changes within the tissue.



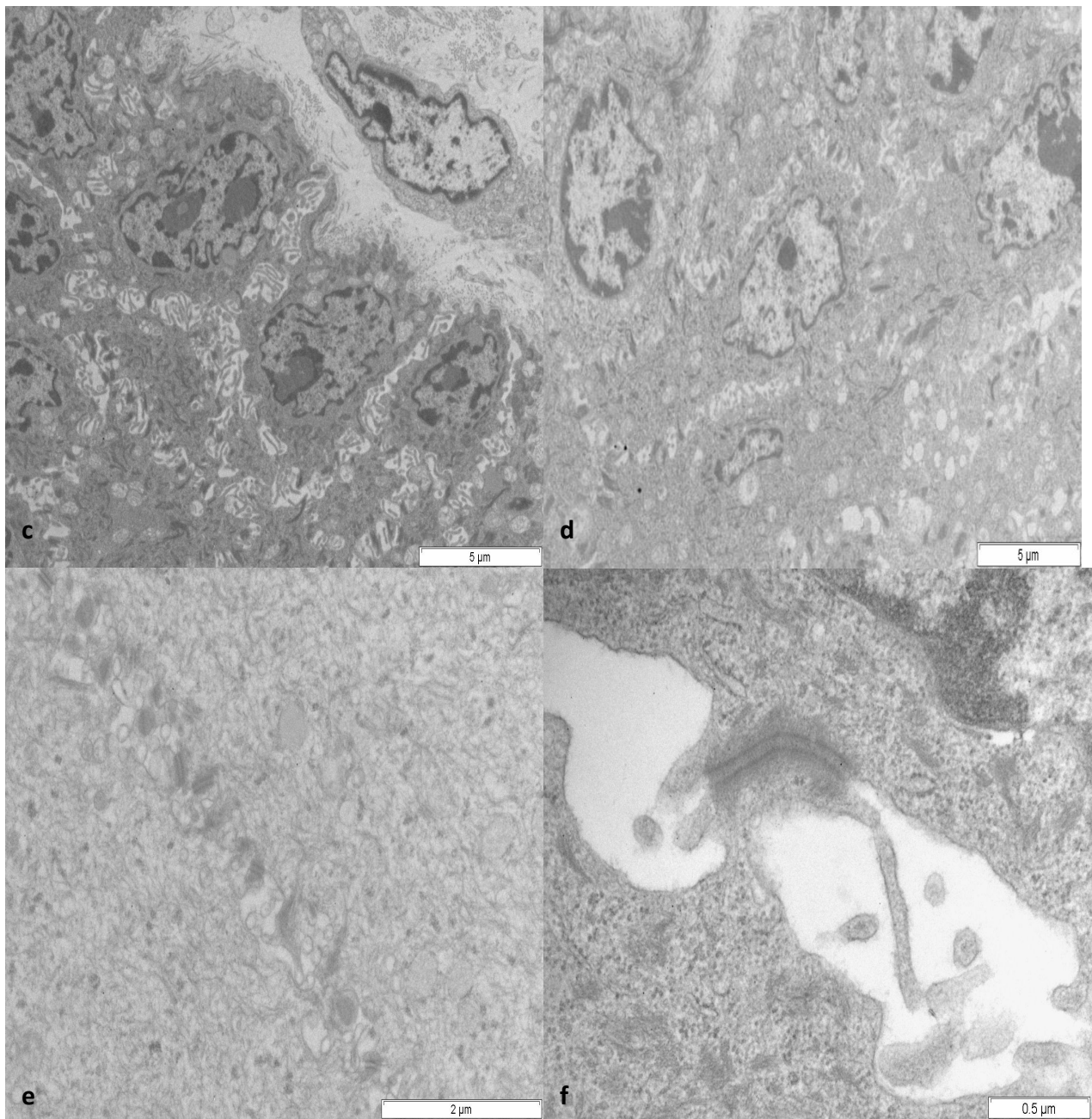


Figure 5: Electromicrographs of the control and the treated buccal mucosal selections for transmission electron microscopy (TEM) : **a** control/untreated ($\times 4000$), **b** 1 hr milled SQV ($\times 2500$), **c** 15 hr milled ($\times 6000$), **d** unground SQV showing nuclear distortions ($\times 5000$), **e** control/untreated showing tight junctions ($\times 20000$), **f** unground SQV showing tight junctions ($\times 50000$).

LM and TEM images supported the assessment of no irreversible tissue damage to the mucosa after exposure to the drug treatment for six hours. TEM assessments further provided a possible mode of transport for SQV across the mucosa, and both LM and TEM observations provided reasons to believe that any changes brought about by the drug treatment would be temporary. These studies therefore confirmed that exposure to SQV did not have any adverse effects on the buccal mucosa.

4. CONCLUSIONS

Enhancing permeation across the buccal mucosa is a major challenge and therefore an area of great interest in the literature, with electrical mechanisms and chemical enhancers being the most widely studied approaches. In light of the need for studies to widen the pool of available strategies to increase buccal permeation, this study investigated the buccal delivery potential of an ARV drug, SQV, and the effect of ball milling as a strategy to enhance permeation across the buccal mucosa compared to commonly used chemical permeation enhancers. The results indicated that both unmilled and milled samples were able to permeate the buccal mucosa, with the optimal sample being the 15 hour milled displaying the greatest permeability with an enhancement ratio of 2.61. This enhanced permeability of the milled SQV samples was attributed to a contribution of various factors such as solubility, particle size, surface area, crystallinity, morphology and the formation of solid dispersions. All of the permeation enhancers studied were also able to increase the permeability of SQV across the buccal mucosa, with SLS achieving the greatest enhancement ratio of 1.75. However, all of the milled samples displayed greater permeability than the best permeation enhancer SLS, and are therefore more effective as a means of permeation enhancement. Histological investigations revealed no significant loss in cellular integrity for mucosa treated with either unmilled or milled SQV samples. The presence of larger intercellular spaces in the treated tissue suggests that SQV also uses the

paracellular route of transport in combination with the transcellular route across the mucosa. Therefore high energy ball milling of SQV is an effective approach of increasing buccal permeability when formulating SQV for a buccal delivery system, when compared to incorporating the enhancers studied at 0.5% w/v into the delivery system for this purpose. SQV milled samples may therefore be considered as having the potential for improving HIV/AIDS drug therapy when administered via the buccal mucosa instead of the oral route.

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CHAPTER 5 – CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1 CONCLUSIONS

Buccal delivery of drugs, including ARVs, can overcome several limitations associated with the oral route thereby improving drug therapy and patient outcomes. Due to the small mucosal surface area and poor membrane permeability, strategies to improve drug permeation across the buccal mucosa are a major research area. The aims of this study were therefore: 1) To identify and compare the buccal permeability potential of tenofovir (TNF) and didanosine (ddl). 2) To identify the buccal permeation effects of potential multifunctional excipients ie. carboxymethylcellulose (CMC), sodium alginate (SA), polyacrylic acid (PAA) and polyethylene glycol (PEG) for TNF and ddl, and 3) To identify the buccal permeation potential of saquinavir (SQV) and assess the effect of high-energy ball milling on its permeability. The following conclusions can be drawn from the various investigations in this study.

- Both TNF and ddl were able to permeate the buccal mucosa in a concentration-dependent manner with ddl having a higher permeability (Flux = $181.62 \pm 23.62 \mu\text{g}/\text{cm}^2\text{h}$) as compared to TNF (Flux = $102.10 \pm 19.80 \mu\text{g}/\text{cm}^2\text{h}$). The permeation of these drugs in the absence of enhancers was attributed to passive diffusion via the paracellular route with transcellular route being an additional possibility for ddl.
- Polymeric excipients previously reported to have mucoadhesive and controlled release properties were shown in this study to additionally display buccal drug permeation enhancing effects. The addition of PAA, SA, CMC and PEG increased the permeability of TNF whilst only PEG was able to increase the permeability of ddl. Furthermore PEG

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was identified as the optimal permeation enhancer for TNF and ddl. The effect of these polymeric excipients appeared to be dependent on their ionic charges as well as that of the respective drugs.

- Histological investigations revealed no significant loss in cellular integrity for mucosa treated with either TNF or ddl, alone or when coupled with PEG as an enhancer. The differences in histomorphological changes in response to TNF and ddl alone could support the greater permeation observed with ddl. The histological findings proved useful in assessing the effects on mucosal integrity and provided insight into permeation pathways across the mucosa.
- Selective polymeric excipients therefore provide an effective means to increase the penetration of ddl and TNF. Their previously reported mucoadhesive and controlled release properties coupled with their permeation enhancing effects shown in this study highlight their potential use as multifunctional excipients for the design of buccal drug delivery systems.
- Unmilled SQV was able to permeate through the buccal mucosa with a flux of $3.99 \pm 0.11 \mu\text{g}/\text{cm}^2\text{h}$. Ball milling of SQV at all the time periods led to its increase in permeability with optimal enhancement obtained at 15 hrs with an enhancement ratio of 2.61. The enhanced permeability of the milled SQV samples was attributed to a contribution of various factors such as solubility, particle size, surface area, crystallinity, morphology and the formation of solid dispersions.

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- Common chemical permeation enhancers ie. PAA, SA, CMC and PEG were also able to increase the permeability of unmilled SQV across the buccal mucosa, with SLS achieving the greatest enhancement ratio of 1.75. However, ball milling of SQV without any chemical permeation enhancers led to a greater enhancement ratio (2.61) as compared to that of the best permeation enhancer SLS at 0.5 %w/v with unmilled SQV (1.75). Ball milling of SQV therefore proved to be an effective strategy for permeation enhancement of SQV.
- Histological investigations revealed no significant loss in cellular integrity for mucosa treated with either unmilled or milled SQV samples. The presence of larger intercellular spaces in the treated tissue suggests that SQV also uses the paracellular route of transport in combination with the transcellular route across the mucosa.
- High energy ball milling of SQV is therefore an effective approach for increasing buccal permeability of SQV as compared to the conventional approach of incorporating common chemical enhancers at 0.5 %w/v with unmilled SQV. These findings offer the possibility of also obviating the need for additionally adding chemical permeation enhancers into a buccal drug delivery system.

This study has identified the buccal permeability properties of three ARV drugs TNF, ddl and SQV. It also identified new chemical permeation enhancers that can provide multifunctional properties in a buccal drug delivery system. Furthermore, ball milling an ARV drug is an additional strategy for enhancing permeation across the buccal mucosa. These findings will provide formulation scientists with effective strategies for optimizing the development of novel ARV buccal delivery systems for improving the treatment of patients with HIV and AIDS. Buccal

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drug delivery is currently acknowledged as one of the delivery mechanisms significant for improving therapeutic outcomes of current drugs. This study has therefore made a significant contribution to addressing a challenge within this important field in the pharmaceutical sciences.

5.2 RECOMMENDATIONS FOR FUTURE WORK

Modifying the route of administration of an existing drug, whose biological and physicochemical characteristics are well understood, is recognised as an alternative cost effective approach for improving therapeutic outcomes as compared to only developing new chemical entities. In light of this, significant focus is placed on improving the delivery of currently marketed drugs. This study has therefore addressed important challenges in this field prior to formulation of these ARVs into a buccal drug delivery system. In this regard, further studies are essential to optimize the efficient and convenient delivery of these ARVs via the buccal route of administration. Future work should therefore be in the direction of:

- Performing further transport and mechanistic studies, on TNF, ddi and SQV, such as fluorescent and/or laser confocal fluorescent microscopy. These techniques are able to characterize the exact route and also to establish the most preferred route of transport across the mucosa by the drug. These findings will further confirm the assertions made from the light and transmission electron micrographs in this study and will provide further insight for formulation scientists to consider when developing new strategies to enhance the permeation of drugs across the buccal mucosa.
- Exploring the use of novel derivatives of existing chemical enhancers as buccal permeation enhancers for these model ARV drugs. Recent literature shows an emerging trend of synthesising novel derivatives of well known and safe chemical permeation

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enhancers that are superior to their parent compound. Synthesising and indentifying buccal permeation enhancing potential of new derivatives such as fatty acids will increase the number of approaches available to enhance the permeation of drugs across the buccal mucosa.

- Designing and manufacturing of delivery systems (buccal films, buccal patches etc.) for the delivery of TNF, ddl and SQV with optimal formulation excipients such as polymers and enhancers being taken into consideration to ensure adequate release and delivery of therapeutically relevant doses into the systemic circulation.
- Performing in-depth characterization studies on the performance of the prepared buccal delivery systems such as drug content, dissolution studies as well as mechanical and mucoadhesive assessments to optimize the formulation and obtain a mechanistic understanding of the delivery system.
- Performing *in vivo* evaluations of the buccal delivery system in a suitable animal model such as the pig to confirm its *in vivo* performance prior to scale up for commercialization.

APPENDIX A – PUBLISHED ARTICLE

This is an original research article published in an international ISI journal: Journal of Pharmaceutical Development and Technology, titled: “Comparative Buccal Permeability Enhancement of Didanosine and Tenofovir by Potential Multifunctional Polymeric Excipients and their Effects on Porcine Buccal Histology”

Comparative buccal permeability enhancement of didanosine and tenofovir by potential multifunctional polymeric excipients and their effects on porcine buccal histology

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Abstract

This study identified and compared the buccal permeability properties of antiretroviral drugs, didanosine (ddI) and tenofovir (TNF), and the permeability effects of polymeric excipients – i.e. carboxymethylcellulose (CMC), sodium alginate (SA), polyacrylic acid (PAA) and polyethylene glycol (PEG) – as potential multifunctional excipients for buccal drug delivery. Permeation studies across porcine buccal mucosa were performed and the drug was quantified using UV spectrophotometry. The mean flux for both ddI (113–181 µg/cm²h) and TNF (40–102 µg/cm²h) increased linearly with increasing donor concentration. All polymeric excipients improved permeability of TNF while only PEG was effective for ddI. Permeability enhancement ratios at 20 mg/mL for ddI and TNF were 1.63 and 1.74, respectively, using PEG (0.5% w/v) and CMC (0.5% w/v), respectively. The maximum enhancement ratio of 2.13 for TNF was achieved with 4% w/v PEG. Light and transmission electron microscopy revealed no significant loss in cellular integrity of mucosa treated with either TNF or ddI alone or when coupled with PEG as a polymeric enhancer. Histomorphological observations correlated with flux values obtained for TNF and ddI alone, as well as with PEG's effects on drug mass flux. TNF and ddI have demonstrated buccal delivery potential. Selective polymeric excipients provide an effective means to increase their penetration and may serve as potential formulation multifunctional excipients in a delivery system for delivery via the buccal route.

Keywords

Buccal permeability, didanosine, histology, permeation enhancer, polymers, tenofovir

History

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Introduction

Drug delivery via the buccal route is receiving increasing interest as an alternative to oral and other conventional routes of administration for the following reasons: it is able to bypass enzymatic degradation and hepatic first pass metabolism, improving bioavailability^{1,2} and is not influenced by variations in gastric emptying rate or the presence of food. As compared to the skin for transdermal delivery, the permeability of the buccal mucosa is higher and cell recovery is also more rapid, and it has easier access than nasal, rectal and vaginal mucosae. The buccal mucosa is also more resistant to irritation, and has greater patient acceptability than other non-oral routes. Buccal delivery systems are also advantageous for pediatrics and patients with a swallowing problem^{3,4}. Although oral antiretroviral (ARV) drug therapy has significantly improved the treatment of human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS), one of the most serious public health diseases^{5,6}, several disadvantages are associated with their use. Many ARV drugs have a low bioavailability, as well as short half-lives, and therefore require frequent administration that decreases patient compliance and leads to severe dose-dependent side-effects. Furthermore, some may lead to formulation

difficulties due to poor solubility⁷. These ARV drugs may therefore benefit from buccal drug delivery. While ARV drugs have been more widely studied for the transdermal route to overcome the above disadvantages^{8–11}, data on their buccal permeability potential appear to be limited^{12,13}. Tenofovir (TNF), a nucleotide analog, is widely used in the treatment of HIV/AIDS. Due to its hydrophilicity and poor intestinal permeability, it is currently administered orally as the ester prodrug, tenofovir disoproxil fumarate. Buccal delivery of TNF in its base form will allow for increased bioavailability and will obviate the need for synthesizing and using the current prodrug. Furthermore, while TNF has been investigated for transdermal¹⁴ and vaginal routes¹⁵, its buccal delivery potential still remains to be identified. Didanosine (2'3'-dideoxyinosine, ddI) is a dideoxy analog of the purine nucleoside inosine¹⁶ and is also used in the treatment of HIV/AIDS. However, it is characterized by a short half-life of 1.3–1.6 h, extensive first pass metabolism (23–40% bioavailability), gastrointestinal pH degradation and severe dose-dependent side-effects^{14,17}. ddI is therefore also an ideal ARV candidate for controlled release of buccal delivery.

The advantages of the buccal route has led to the design and evaluation of various buccal delivery systems such as tablets, films, wafers, patches, gels and pastes¹⁸. The benefit of these buccal delivery systems can be enhanced by ensuring that the drug is released in a controlled manner. Various polymers, both alone or in combination, are used in buccal delivery systems to modify drug release profiles via several mechanisms, such as erosion, swelling and hydration¹⁹.

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One of the main disadvantages of the buccal route is the low mucosal permeability, necessitating the use of various classes of permeation enhancers. These enhancers alter mucosal permeability by various mechanisms, such as changing the mucus rheology, disturbing the intracellular lipid packing in the bilayer and the loosening of tight junctions¹. Substances such as surfactants, fatty acids, cyclodextrins, chelators and positively charged polymers have been identified in the literature as buccal permeation enhancers⁷. The search and identification of new drugs and permeation enhancers via *in vitro* permeation studies with drug and potential enhancer solutions is currently a major focus of research in the literature to widen the available pool of drug and excipients and consequently inform the selection of excipients for formulation into future buccal delivery systems.

To facilitate prolonged retention on the mucosae allowing for sustained delivery and permeation, buccal delivery systems also need to display mucoadhesivity, i.e. they need to adhere to the mucus layer covering the buccal mucosae. In order to meet the challenges of buccal delivery, the key properties therefore needed for a buccal drug delivery system are (1) drug permeation enhancement, (2) mucoadhesivity and (3) controlled drug release profiles. The identification of formulation excipients to provide these properties is therefore critical. Various polymers such as cellulose derivatives, and those based on polymethacrylic acid, polysaccharides and alginic acid have successfully shown mucoadhesivity and sustained drug release properties²⁰.

The identification of excipients with potential multifunctional properties, such as mucoadhesivity, drug permeation enhancement and controlled drug release profiles, will significantly contribute to the optimization of the design of buccal delivery systems. The multifunctional properties required for buccal delivery systems have led investigators to study, separately, different classes of excipients as permeation enhancers and polymers that have either or both mucoadhesivity and controlled-release properties. Clearly, a need exists for the use of multifunctional pharmaceutical excipients in the design of buccal delivery systems, i.e. excipients that could simultaneously provide mucoadhesivity, sustained drug release and permeation enhancement. The use of multifunctional excipients for buccal drug delivery will decrease the number and quantity of excipients needed in product formulation. Advantages include decreased manufacturing costs, adverse or allergic reactions and excipient incompatibilities. To date, to the best of our knowledge, the only excipient thus far reported in separate studies to have mucoadhesive, sustained release and permeation enhancing properties has been chitosan^{20,21}. Hence the need exists for the identification of excipients that could have multifunctional properties such as mucoadhesivity, sustained drug release and drug permeation enhancement capabilities. While various excipients such as poly(acrylic acid) (PAA)²¹, sodium alginate (SA)²¹, poly(ethylene glycol) (PEG)^{19,20} and carboxymethylcellulose (CMC)^{21,22} are recognized as having both sustained drug release and mucoadhesivity properties, their buccal permeability enhancement potential still remains to be investigated.

The aim of this study was therefore to first identify and compare the buccal permeability potential of two ARV drugs, i.e. ddI and TNF. The second aim was to determine the effects of PAA, SA, PEG and CMC on the permeation of these drugs as well as their histomorphological effects on the buccal mucosae as potential multifunctional formulation excipients for buccal delivery.

PEG was particularly chosen in this study to further investigate its potential as an enhancer as it proved to be the only polymer that was able to increase the permeability of both TNF and ddI. Also PEG has been reported to be strongly mucoadhesive due to hydrogen bonding between either oxygen atoms in PEG and sugars on glycosylated mucins, and/or interpenetrating polymer

network effects between PEG chains and the mucus mesh²³. This interaction with the mucus to enable PEG's mucoadhesivity could also be beneficial in decreasing the barrier properties displayed by the mucus to the movement of molecules across the mucosa.

Materials

The buccal mucosal tissue was obtained from Baynesfield Abattoir (Pietermaritzburg, South Africa), from pigs weighing approximately 60–80 kg. Phosphate buffered saline (PBS), pH 7.4, was prepared using sodium chloride, potassium dihydro-orthophosphate and disodium hydrogen orthophosphate (Associated Chemical Enterprises, Johannesburg, South Africa). Excipients (CMC, MW = 90 000 Da; SA, PAA, MW = 1800 Da and PEG, MW = 4000 Da) were purchased from Sigma-Aldrich (St. Louis, MO). TNF MW = 287.21 g/mol was obtained from Gilead Sciences Inc. (Foster City, CA) and ddI, MW = 236.23 g/mol was donated by Aspen Pharmacare (Durban, South Africa).

Methods

Ethical clearance

Ethical clearance was obtained from the UKZN Ethics committee in 2010 (010/10/Animal) and renewed in 2011 (24/11/Animal).

Tissue preparation

Porcine buccal tissue was obtained from the pigs immediately after slaughter. The tissues were excised, using surgical scissors, to remove all underlying tissue, to a thickness of between 500 and 700 μm , leaving the basal lamina intact. The tissues were transported in cold, normal saline (NS), snap frozen in liquid nitrogen, stored in a biofreezer (-80°C) and used within three months²⁴. Cold PBS (pH 7.4) was used to defrost the buccal mucosal tissue at room temperature before it was used in permeation studies.

Permeation measurement across porcine buccal mucosa

In vitro permeation studies were conducted at $37 \pm 1^\circ\text{C}$ using modified vertical Franz-type diffusion cells (PermeGear, Inc., Bethlehem, PA) with a diffusional area of 0.786 cm^2 . A circular section of the buccal mucosa was mounted onto the diffusional area between the donor and receptor cells and was equilibrated with PBS (pH 7.4) at 37°C for 30 min. The donor compartment solution (1 mL) contained either drug (TNF/ddI) with PBS alone (20 mg/mL) or drug (TNF/ddI) (20 mg/mL) in the presence of the various excipients (PAA, SA, CMC and PEG) at a concentration of 0.5% w/v in respective experiments. The concentration dependent effect of PEG, specifically, on the enhancement of drug (TNF/ddI) permeation, was performed with the donor compartment solution (1 mL) containing drug (TNF/ddI) (20 mg/mL) in the presence of various concentrations of PEG (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0% w/v). The receptor compartments were filled with PBS and stirred with a teflon-coated magnetic bar. Samples were removed from the receptor compartments at predetermined time intervals and replaced with the same volume of ddI/TNF-free (fresh) PBS. Each experiment represents minimum of three replicates. The drugs were quantified by a validated UV spectrophotometry method at a λ_{max} of 262 nm and 250 nm, for TNF and ddI, respectively (UV Spectrophotometer 1650, Shimadzu, Kyoto, Japan) which are similar to other buccal permeability studies.

Permeability data analysis

The cumulative amount of drug (TNF/ddI) permeated per unit surface area was plotted against time. The steady state flux (J_{ss})

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was determined from the linear part of the permeability curve by linear regression analysis (Microsoft Excel 2007, Microsoft, Washington, DC). The permeability coefficient (P) was calculated as follows²⁵:

$$P = \frac{(dQ/dt)}{A} \times C_d = \frac{J_{ss}}{C_d} \quad (1)$$

Where dQ/dt is the cumulative amount permeated per unit time, A is the diffusion area and C_d is the drug concentration in the donor compartment. The enhancement ratio (ER) was calculated using the following equation²⁵:

$$ER = \frac{\text{Permeability coefficient of drug in the presence of enhancer}}{\text{Permeability coefficient of drug in the absence of enhancer}} \quad (2)$$

Light microscopy and transmission electron microscopy

Histological evaluations were performed on freshly harvested, excised buccal mucosa. Untreated buccal mucosa was transferred directly after excision from NS into 10% buffered formalin without any equilibration in PBS and served as the control. Treated samples comprised of buccal mucosa that were exposed to PBS only, or 20 mg/mL TNF dissolved in PBS alone or 20 mg/mL ddi dissolved in PBS alone, or 20 mg/mL TNF dissolved in PBS with 4% w/v PEG, or 20 mg/mL ddi dissolved in PBS with 0.5% w/v PEG. Permeation experiments were carried out with these solutions in the donor compartment and with the freshly excised buccal mucosa placed between donor and receptor compartments as described in the Section ‘‘Permeation measurement across porcine buccal mucosa’’, without drug quantification^{12,26}. At the end of the experiment, the buccal mucosa was removed from the Franz diffusion cells, cut into cross sections and fixed in 10% buffered formalin. Both the control and treated buccal mucosa were fixed in formalin for seven days at room temperature. Buccal mucosa was dehydrated using an ethanol gradient ranging from 50% to 96% and embedded in paraffin wax. The mucosae sections were collected on slides, dried and stained with hematoxylin and eosin (H&E). Sections were examined using a light microscope (Nikon 80i, Nikon, Tokyo, Japan) and bright field images were digitally captured using NIS Elements D software and a camera (Nikon U2). The samples for transmission electron microscopy (TEM) were obtained after the above-mentioned permeation experiments. The samples were then cut into pieces not exceeding 0.5 mm³, and fixed for 24 h (4 °C) using Karnovsky’s fixative²⁷ buffered to pH 7.2. For TEM, each sample was processed and embedded in epoxy resin using standard protocols. Ultrathin sections (90 nm) were cut and contrasted with uranyl acetate and lead citrate and viewed with a transmission electron microscope (JEOL 1010, JEOL, Tokyo, Japan). All experiments were performed using a minimum of three replicates.

Statistical analysis

The results, expressed as mean \pm standard deviation (SD), were analyzed using one-way analysis of variance (ANOVA) followed by the Mann–Whitney test using GraphPad Prism® Version 5 (Graph Pad Software Inc., La Jolla, CA).

Results and discussion

Buccal permeability profiles of TNF and ddi

The permeability potential of TNF and ddi were first investigated in the absence of an enhancer. Increasing the donor concentrations (1–20 mg/mL) led to an increase in the cumulative amount permeated across the buccal mucosa for both drugs. The flux of

Polymeric excipients as permeation enhancers 3

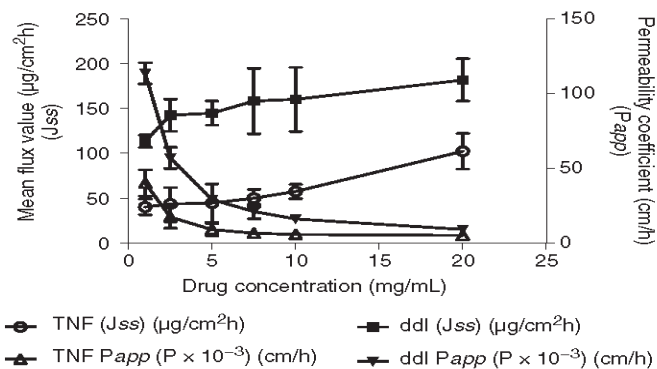


Figure 1. Effect of drug concentration on mean flux and mean permeability coefficients for TNF and ddi across buccal mucosa.

TNF and ddi at the varying donor concentrations are shown in Figure 1. Both TNF and ddi were able to permeate the buccal mucosa at increasing concentrations. The obtained results displayed concentration-dependent permeability for both ARV drugs. For TNF, as the donor concentration increased from 1 to 20 mg/mL, the steady state flux increased from 40.18 ± 8.85 to $102.1 \pm 19.80 \mu\text{g}/\text{cm}^2\text{h}$ (Table 1). While the increase in flux at lower concentrations from 1 to 7.5 mg/mL were not significant ($p > 0.05$), statistically significant differences were seen at higher concentrations of 10 and 20 mg/mL ($p < 0.05$). One-way ANOVA for the various concentrations of TNF were also significantly different ($p < 0.05$). For ddi, as the donor concentration increased, flux values increased from 113.4 ± 6.85 to $181.62 \pm 23.62 \mu\text{g}/\text{cm}^2\text{h}$. The increases in flux values from 1 to 20 mg/mL were statistically different ($p < 0.05$) and one-way ANOVA for the various concentrations of the studied ddi were also significantly different ($p < 0.05$). An apparent linear relationship between the donor concentrations and flux values for both TNF ($R^2 = 0.94$) and ddi ($R^2 = 0.82$) was observed (Figure 2). These findings suggest passive diffusion of both the drugs across the mucosa^{4,7,28}.

Since the buccal epithelium is stratified, a permeant can use either the transcellular or paracellular route, or both the routes simultaneously. However, one route is usually preferred over the other, favoring the one that provides the least amount of hindrance to passage. The physicochemical properties of a drug can also influence the mechanisms of transport across the buccal mucosa. The permeation of many hydrophilic drugs across the buccal mucosa is thought to be via the paracellular route using passive diffusion^{7,22,29–31}. These hydrophilic drugs are therefore able to dissolve more readily in the aqueous fluid of the intercellular spaces; however, the lipid matrix of the extracellular spaces plays a role in the barrier function of the buccal mucosa especially if the compounds have high molecular weights^{29,30}. Tight junctions act as major barriers to paracellular transport of macromolecules and polar compounds^{2,7,32}. Hence both of these hydrophilic drugs may have used the paracellular route; however, a higher ionization at pH 7.4 of ddi ($pK_a = 9.12$) as compared to that of TNF ($pK_a = 7.9$) suggests that ddi is the more capable permeant via this route^{33,34}. This is illustrated by higher mean flux and permeability coefficient observed for ddi (Table 1). Although both drugs displayed increased permeability with increasing concentrations, ddi displayed greater flux values than TNF at similar concentrations. This could also be attributed to ddi’s lower molecular weight as compared to TNF, thus allowing

Table 1. Effect of donor concentration on the permeability parameters of TNF and ddl.

Drug concentration (mg/mL)	Flux (Jss) ($\mu\text{g}/\text{cm}^2\text{h}$)		Permeability coefficient ($p \times 10^{-2}$) (cm/h)	
	TNF	ddl	TNF	ddl
1	40.18 \pm 8.85	113.4 \pm 6.85	4.01 \pm 0.88	11.34 \pm 0.71
2.5	43.41 \pm 18.35	^a 142.4 \pm 17.84	1.73 \pm 0.73	5.69 \pm 0.71
5	44.51 \pm 21.38	^a 144.62 \pm 13.36	0.89 \pm 0.42	2.89 \pm 0.26
7.5	49.95 \pm 9.83	^a 158.19 \pm 36.53	0.66 \pm 0.13	2.10 \pm 0.48
10	^a 57.43 \pm 7.88	^a 160.02 \pm 35.69	0.57 \pm 0.07	1.60 \pm 0.14
20	^a 102.10 \pm 19.80	^a 181.62 \pm 23.62	0.51 \pm 0.09	0.90 \pm 0.11

^aIndicates significant difference, i.e. $p < 0.05$ (all values compared to 1 mg/mL).

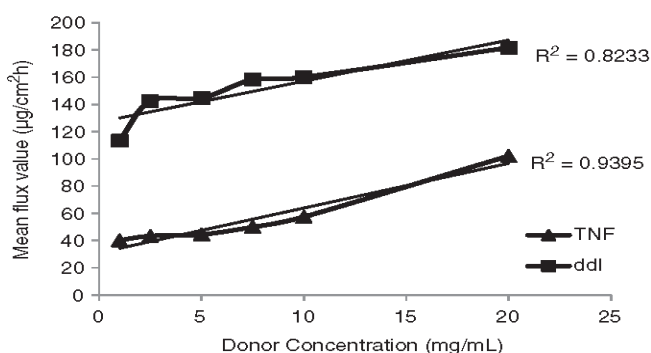


Figure 2. Effect of donor concentration on the steady state flux.

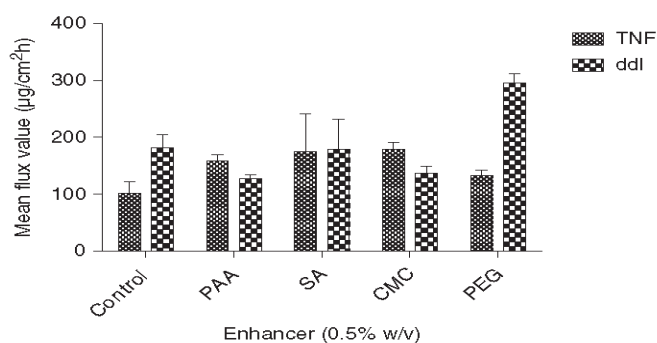


Figure 3. Effect of various multifunctional excipients on the mean flux of TNF and ddl across buccal mucosa.

greater permeability. The donor concentration range of the drug solutions of this study was in keeping with previous such permeability studies¹³ and the flux values obtained in this study for TNF and ddl are similar to or greater than flux values that have been reported for other drugs identified as having potential for buccal delivery also via *in vitro* permeation studies^{13,35}. TNF and ddl may be considered as having the potential for improving HIV/AIDS drug therapy when administered via the buccal mucosa instead of the oral route.

Effects of polymeric excipients on permeability enhancement

Polymeric excipients of varying ionic charges were employed as potential permeation enhancers for TNF (as an anionic drug) and ddl (as a cationic drug). Permeation/penetration enhancers promote the absorption of a drug across the buccal mucosa by decreasing its barrier properties, delivering therapeutically relevant amounts of the drug into the systemic circulation. Hydrophilic drugs, such as TNF and ddl, could both show increased permeability if they are coupled with enhancers that are able to either decrease the interaction of the drug with the lipid matrix of the extracellular spaces and/or temporarily alter tight junction or both. To identify their multifunctional capabilities, polymeric excipients with previously reported controlled release and mucoadhesive properties^{24,26,36} were used as potential enhancers for the buccal delivery of TNF and ddl, respectively. The respective enhancers were added to the donor chamber at 0.5% w/v, which is in keeping with previous studies^{13,37}. The results of this study showed that the permeability of TNF was increased in the presence of all the excipients (Figure 3). CMC displayed the greatest increase in mean flux value, which increased from 102.10 \pm 19.80 $\mu\text{g}/\text{cm}^2\text{h}$ to 178.12 \pm 12.87 $\mu\text{g}/\text{cm}^2\text{h}$ with an enhancement ratio of 1.74 (Table 2). All the

excipients displayed permeation enhancement for TNF; however, the anionic excipients displayed the greatest enhancement. These findings may be attributed to the anionic polymers having strong hydrogen bonding with mucin, altering the mucus rheology and enhancing permeability^{24,36}. While the increases in flux values were statistically significant ($p < 0.05$) for CMC, PEG and PAA – CMC displayed the greatest enhancement ratio. The permeability of ddl was increased in the presence of the nonionic excipient PEG, whereas the addition of all the anionic excipients (PAA, SA and CMC) caused a decrease in the flux values obtained (Table 2). Changes in the mean flux and permeability coefficient may have resulted from the anionic excipients' complexation with the positively charged drug, ddl, forming an aggregate which hindered movement across the buccal mucosa. In this study, the effect of permeant modifiers appears to be dependent on the ionic charge of the enhancer and drug. The permeation enhancement ratios obtained using PAA, SA, CMC and PEG for TNF and ddl in this study are similar to ratios of other permeants reported as promising enhancers in the literature^{37,38}. This study has confirmed that in addition to their previously reported controlled-release and mucoadhesive properties in the literature, these polymeric excipients can also enhance buccal drug permeability and may therefore be regarded as promising multifunctional excipients in the field of buccal drug delivery.

Effect of polyethylene glycol concentrations on permeability

PEG was the only excipient that displayed enhanced permeation for both TNF (ER = 1.3) and ddl (ER = 1.63) (Table 2), and was selected to evaluate its effects on the permeability of TNF and ddl. The effect of PEG, within a concentration range of 0.25–6% w/v on the permeability of TNF and ddl was investigated. This concentration range is in keeping with previous reports^{13,37}.

Table 2. Effect of multifunctional excipients on the permeability parameters of TNF and ddI.

Excipient concentration (% w/v)	Flux (Jss) ($\mu\text{g}/\text{cm}^2\text{h}$)		Permeability coefficient ($p \times 10^{-2}$) (cm/h)		Enhancement ratio	
	TNF	ddI	TNF	ddI	TNF	ddI
Control	102.10 \pm 19.80	181.62 \pm 23.62	0.51 \pm 0.09	0.90 \pm 0.11	1	1
PAA (0.5%)	^a 158.80 \pm 10.19	^a 127.07 \pm 7.30	0.79 \pm 0.05	0.63 \pm 0.03	1.56	0.7
SA (0.5%)	174.46 \pm 66.56	179.61 \pm 52.24	0.87 \pm 0.33	0.90 \pm 0.26	1.71	0.99
CMC (0.5%)	^a 178.12 \pm 12.87	^a 136.45 \pm 12.29	0.89 \pm 0.06	0.68 \pm 0.06	1.74	0.75
PEG (0.5%)	^a 132.82 \pm 9.95	^a 295.94 \pm 15.24	0.66 \pm 0.04	1.48 \pm 0.07	1.3	1.63

^aIndicates significant difference, i.e. $p < 0.05$ (all values compared to control (20 mg/mL TNF/ddI)).

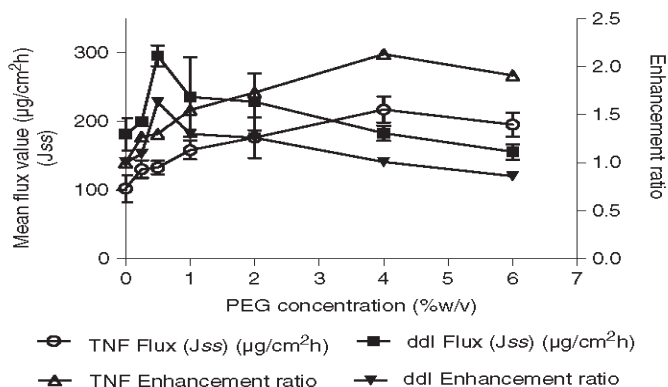


Figure 4. Effect of PEG concentration on permeation enhancement ratio, mean mass flux and mean permeability coefficients for TNF and ddI across buccal mucosa.

The mean flux values obtained for TNF was increased with the addition of PEG from 0.25 to 4% w/v (Figure 4). PEG at 4% w/v displayed the greatest enhancement ratio (ER = 2.13) and the greatest increase in mean flux value, namely from 102.1 ± 19.80 to $217.49 \pm 18.88 \mu\text{g}/\text{cm}^2\text{h}$ (Table 3). An increase in the PEG concentration above 4% w/v resulted in a decrease in the mean flux values, from 217.49 ± 18.88 to $195.54 \pm 17.57 \mu\text{g}/\text{cm}^2\text{h}$ (ER = 1.91) (Figure 4). The mean flux values obtained for ddI was increased with the addition of PEG from 0.25 to 4% w/v. PEG at 0.5% w/v displayed the greatest enhancement ratio (ER = 1.63) and the greatest increase in mean flux value, which increased from 181.62 ± 23.62 to $295.94 \pm 15.24 \mu\text{g}/\text{cm}^2\text{h}$. A further increase in the PEG concentration from 0.5 to 6% w/v resulted in a decrease in mean flux values obtained, which decreased from 295.94 ± 15.24 to $155.85 \pm 11.25 \mu\text{g}/\text{cm}^2\text{h}$ (ER = 0.86) (Table 3). Similar trends have been reported, where an enhancer (Aloe vera gel or propylene glycol) at varying concentrations, was coupled with a permeant. These studies reported an initial increase in flux values with an increase in enhancer concentration and with a subsequent decrease in flux values as the enhancer concentration was further increased^{13,39}. PEG's mucoadhesion property has been attributed to its ability to increase hydrogen bonding and facilitating adherence to the mucosae⁴⁰. One of the mechanisms of buccal permeation enhancement results from the alteration of the mucus rheology^{24,36}, which could account for PEG increasing the permeation of both TNF and ddI. PEG, at a lower concentration of 0.5% w/v, achieved the greatest enhancement of the lower molecular weight drug ddI, whereas for the higher molecular weight drug TNF, maximal enhancement was achieved at a higher concentration of PEG (namely 4% w/v). Possible differences in drug interactions could have resulted in the decreased movement across the buccal mucosae at higher concentrations than

the aforementioned. The permeation enhancement ratios achieved with the addition of PEG in this study is similar to other studies that identified promising enhancers^{33,37-39}. In addition to its mucoadhesive and controlled release properties, PEG also showed permeation enhancement properties. PEG displayed the greatest enhancement potential for TNF and ddI at a concentration of 4% and 0.5% w/v, respectively, and could be considered when formulating a buccal delivery system.

Histomorphological evaluations

The effect of the ARV drugs (TNF and ddI) and the optimal enhancer identified in this study, i.e. PEG on the buccal mucosa, was investigated histomorphologically. These studies were performed to assess whether or not the expected changes following permeation are of a nature that is deleterious to cell, i.e. whether cellular integrity has been compromised. Also, these studies may indicate a possible transport mechanism for these drugs and enhancer. The criteria for the use of permeation enhancers are that they should be nonirritant and nontoxic, and allow the mucosa to rebound to its former integrity⁷. In this study, we are able to comment on the level of perturbation; however, whether or not the cells would regain their former morphology requires further investigation. Two routes have been proposed for movement of molecules across the buccal mucosae, i.e. paracellular and transcellular². Although limited, from this study, inferences can be made on the mode of transport employed by the molecule (drug) and the permeation enhancer, PEG.

The mucosa of the buccal lining has been extensively described²⁶. Briefly, histologically, the mucosal layer is made of basal cells which are actively mitotic and represent the germinal layer that gives rise to newer cells; a mid-region where cells acquire a more polygonal shape and surface cells which appear more flattened. This description matched our control sample which was not subjected to any experimental procedure (Figure 5a).

When compared to the controls, mucosae exposed to the drugs only, ddI and TNF, at 20 mg/mL (Figure 5b and c respectively) also retained relatively normal histomorphology, i.e. no major loss of cellular integrity. TNF treatment, however, resulted in mild distortion of cellular outlines and desquamation, and an increase in the size of intercellular spaces was observed in electron micrographs. The ddI treatment resulted in a vacuolated appearance inside some of the cells which was observed both in LM and in TEM images (Figures 5e and 6g, respectively) unlike those of TNF (Figures 5b and 6b, respectively). These vacuoles may indicate a possible transcellular mode of transport⁴¹. These histomorphological observations appear to correlate with the permeation data (Tables 1 and 3 and Figures 2 and 4) where ddI displayed greater permeation in comparison to TNF, both in the presence and in the absence of PEG (Tables 1 and 2). Combining both the permeability data and histomorphological evaluations, it can be speculated that TNF permeation may follow the paracellular route while ddI may use both the paracellular and

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Table 3. Effect of PEG concentration on the permeability parameters of TNF and ddI.

Excipient concentration (% w/v)	Flux (Jss) ($\mu\text{g}/\text{cm}^2\text{h}$)		Permeability coefficient ($p \times 10^{-2}$)(cm/h)		Enhancement ratio	
	TNF	ddI	TNF	ddI	TNF	ddI
Control	102.10 \pm 19.80	181.62 \pm 23.62	0.51 \pm 0.09	0.90 \pm 0.11	1	1
0.25	130.43 \pm 12.83	^a 199.66 \pm 4.54	0.65 \pm 0.07	0.99 \pm 0.02	1.27	1.09
0.50	^a 132.82 \pm 9.95	^a 295.94 \pm 15.24	0.66 \pm 0.05	1.48 \pm 0.07	1.3	1.63
1	158.34 \pm 13.63	235.81 \pm 58.36	0.79 \pm 0.06	1.17 \pm 0.29	1.55	1.3
2	^a 176.50 \pm 29.70	^a 228.79 \pm 41.41	0.88 \pm 0.14	1.14 \pm 0.20	1.73	1.26
4	^a 217.49 \pm 18.88	183.01 \pm 10.93	1.08 \pm 0.09	0.91 \pm 0.05	2.13	1.007
6	^a 195.54 \pm 17.57	^a 155.85 \pm 11.25	0.97 \pm 0.08	0.77 \pm 0.05	1.91	0.86

^aIndicates significant difference, i.e. $p < 0.05$ (all values compared to control (20 mg/mL TNF/ddI)).

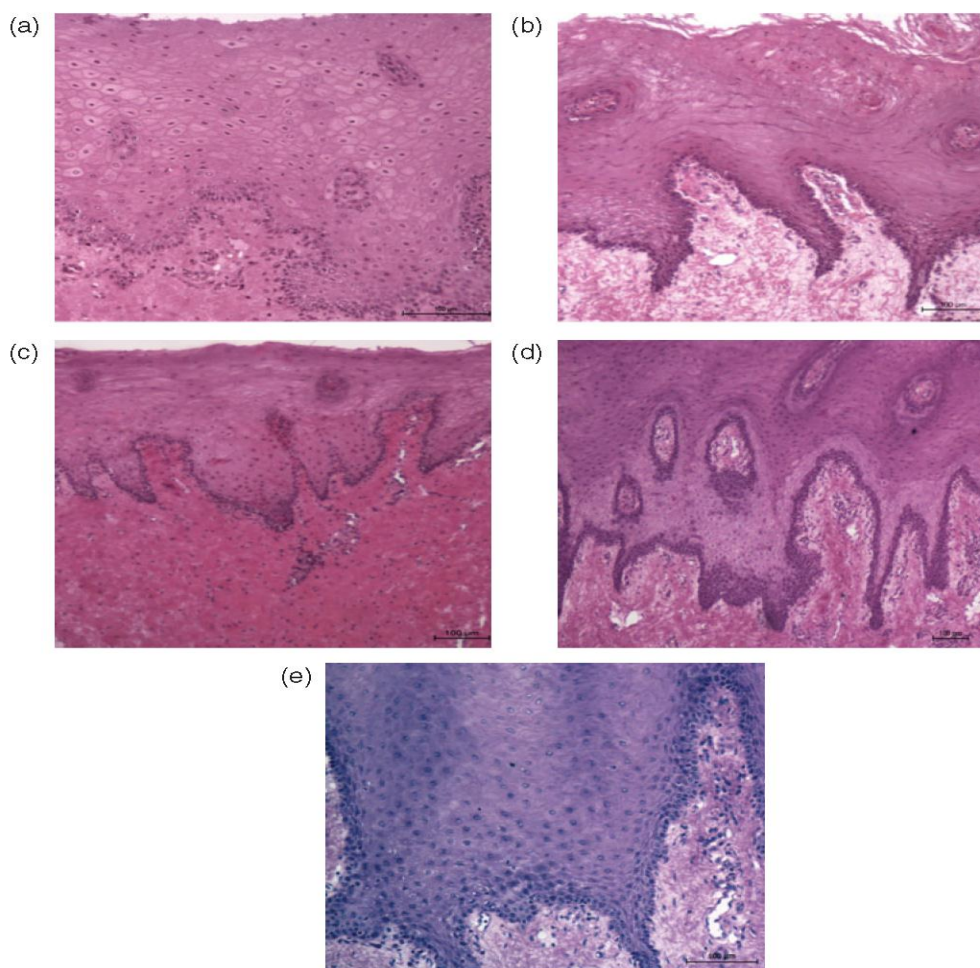


Figure 5. Photomicrographs of the control and the treated buccal mucosal selections for light microscopy (LM) stained with H&E ($\times 40$): (a) control/untreated, (b) treated with 20 mg/mL TNF, (c) treated with 20 mg/mL ddI, (d) treated with 20 mg/mL TNF + 4% (w/v) PEG, (e) treated with 20 mg/mL ddI + 0.5% (w/v) PEG.

transcellular routes. Substances may use many several modes of transport for movement, including carrier-mediated transport across the epithelium lining; therefore, further investigations are needed to confirm how ddI, TNF and PEG move across the

mucosal barrier, including an investigation on possible endosomal transport mechanisms. Furthermore, the vacuolated appearance may not be considered deleterious and these findings are similar to Dhiman et al. (2009) who also found a vacuolated appearance

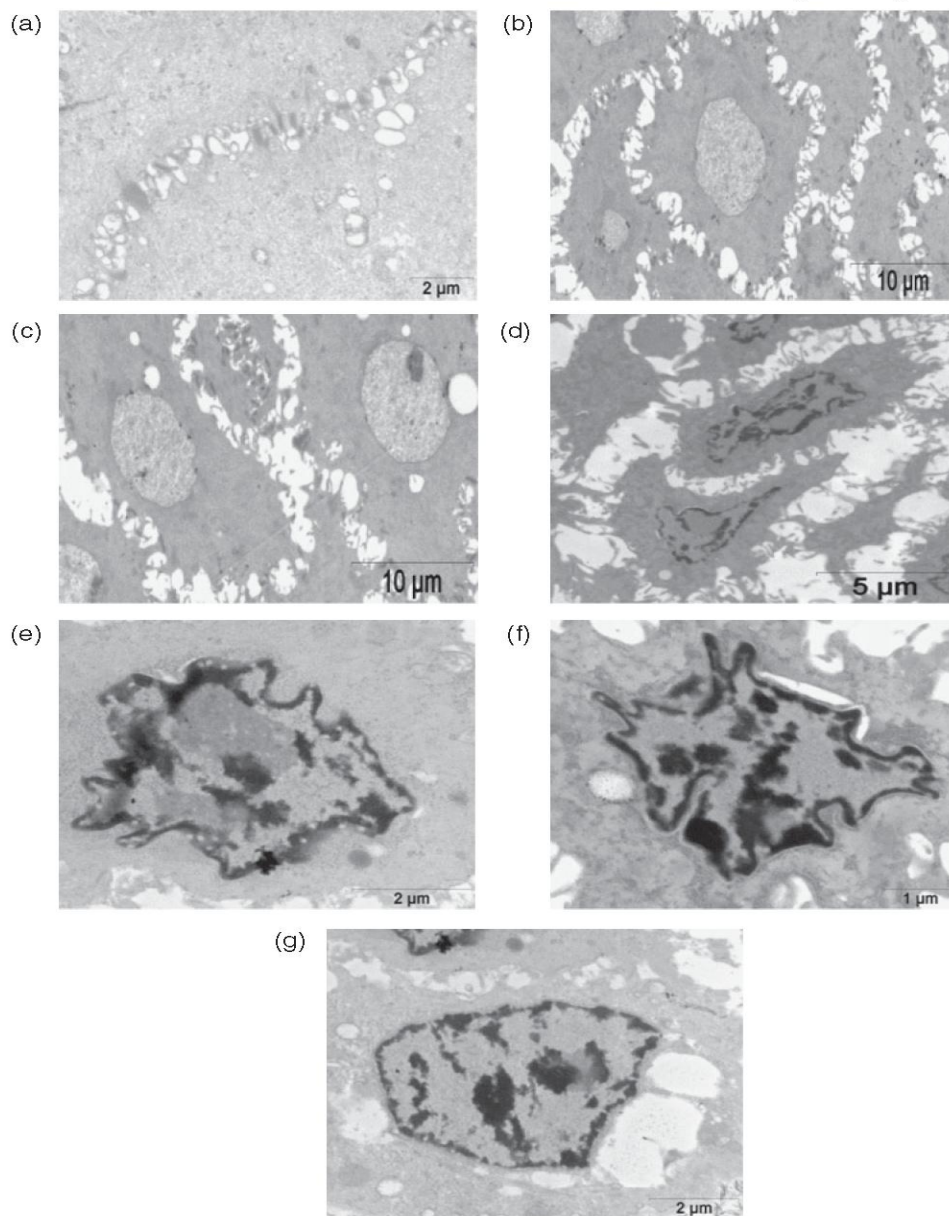


Figure 6. Electromicrographs of the control and the treated buccal mucosal sections for transmission electron microscopy (TEM): (a) control/untreated ($\times 10\,000$), (b) TNF 20 mg/mL ($\times 5\,000$), (c) ddi 20 mg/mL ($\times 5\,000$), (d) TNF + 4% PEG ($\times 5\,000$), (e) ddi + 0.5% PEG ($\times 5\,000$), (f) TNF + 0.5% PEG showing nuclear invagination ($\times 15\,000$) and (g) ddi 20 mg/mL showing intracellular vacuoles ($\times 15\,000$).

in the buccal mucosa after permeation experiments using sodium tauroglycocholate as an enhancer.

In addition, electron micrographs, compared to control (Figure 6a), also revealed an increase in the size of intercellular spaces when exposed to both drugs (Figure 6b and c respectively). The distinct expanded intercellular spaces observed for both drug treatments can be corroborated by the assertion that hydrophilic drugs could dissolve in the aqueous

intercellular space, and therefore favor the paracellular route of absorption^{26,34}.

Using LM we were unable to establish a loss in cellular integrity when PEG was coupled with both ddi and TNF at 0.5 and 4% w/v, respectively (Figure 5d and e). However, using TEM, larger intercellular spaces were observed with the addition of PEG to both drugs (Figure 6d and e, respectively). These larger intercellular spaces may be responsible for the increased

permeability observed with the addition of PEG³⁷. We also observed an increase in nuclear invaginations and changes in the nuclear envelopes (TNF + 4% w/v PEG electron micrographs) (Figure 6f). The mechanisms and functional significance of this is not really known but studies suggest the involvement of the cytoskeleton⁴². We speculate that, in this study, a combination of factors such as mechanical stress, ionic changes and increased cellular activity may have led to these effects in response to the permeation experiments. These changes observed by TEM for PEG with both drugs may not be considered deleterious.

Conclusions

This study investigated the buccal delivery potential of two ARV drugs, ddI and TNF. It further demonstrated the effects of polymeric excipients on the buccal permeation of ddI and TNF. Both TNF and ddI were able to permeate the buccal mucosa in a concentration-dependent manner at similar concentrations, with ddI having the greater permeability. The permeation of these drugs used alone can be attributed to passive diffusion via the paracellular route with transcellular route being an additional possibility for ddI. The addition of all polymeric excipients, i.e. PAA, SA, CMC and PEG, used in this study increased the permeability of TNF; however, only PEG was able to increase the permeability of ddI. The effect of these polymeric excipients and that of the drug appeared to be dependent on their ionic charge. Histological investigations revealed no significant loss in cellular integrity for mucosa treated with either TNF or ddI, alone or when coupled with PEG as an enhancer. The differences in histomorphological changes in response to TNF and ddI alone could support the greater permeation observed with ddI.

Selective polymeric excipients provide an effective means to increase the penetration of ddI and TNF. Their previously reported mucoadhesive and controlled release properties coupled with their permeation enhancing effects shown in this study highlight their potential use as multifunctional excipients for the design of buccal drug delivery systems.

Acknowledgements

The Biomedical Research Unit, Electron Microscope Unit and Miss Priyadeshni Naidoo at UKZN are acknowledged for their valuable technical assistance. Baynesfield Abattoir is acknowledged for kindly donating the buccal tissue.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Appendix A – Published Article

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Appendix B – Acceptance Letter

APPENDIX B – ARTICLE ACCEPTED FOR PUBLICATION

This is the acceptance letter for an original research article accepted for publication in an international ISI journal: Drug Development and Industrial Pharmacy, titled: “High-energy ball milling of Saquinavir increases permeability across the buccal mucosa.” Manuscript ID: LDDI-2013-0486.R1

Appendix B – Acceptance Letter

Drug Development and Industrial Pharmacy



High-energy ball milling of Saquinavir increases permeability across the buccal mucosa

Journal:	<i>Drug Development and Industrial Pharmacy</i>
Manuscript ID:	LDDI-2013-0486.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	01-Nov-2013
Complete List of Authors:	Rambharose, Sanjeev; University of KwaZulu natal, Pharmaceutical sciences Govender, Thirumala; University of KwaZulu Natal, Pharmaceutical Sciences
Keywords:	buccal permeability, saquinavir, ball milling, permeation enhancer, histology

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Appendix B – Acceptance Letter

From: maincent@pharma.uhp-nancy.fr

To: rambharoses@ukzn.ac.za

CC:

Subject: Drug Development and Industrial Pharmacy - Decision on Manuscript ID LDDI-2013-0486.R1

Body: Nancy, November 4, 2013

Dear Mr Rambharose:

Ref: High-energy ball milling of Saquinavir increases permeability across the buccal mucosa

Our referees have now considered your paper and have recommended publication in Drug Development and Industrial Pharmacy. We are pleased to accept your paper in its current form which will now be forwarded to the publisher for copy editing and typesetting. The reviewer comments are included at the bottom of this letter.

You will receive proofs for checking, and instructions for transfer of copyright in due course.

The publisher also requests that proofs are checked and returned within 48 hours of receipt.

Thank you for your contribution to Drug Development and Industrial Pharmacy and we look forward to receiving further submissions from you.

Sincerely,
Dr Maincent
Associate/Review Editor, Drug Development and Industrial Pharmacy
maincent@pharma.uhp-nancy.fr

Peer Reviewer(s)' Comments to Author:

None

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Date Sent: 04-Nov-2013