Intestinal Permeation Enhancers Safely Enable the Oral Delivery of Macromolecules

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Abstract

The unique physicochemical properties that make protein drugs powerful biologic therapeutics also prevent them from being delivered orally. They are structurally and chemically prone to degradation in the stomach, and their large size and hydrophilicity limit their absorption across the intestinal epithelium. Because oral drug delivery is much preferred by patients over parenteral delivery, herculean efforts have been made over the last several decades to improve the oral bioavailability of protein drugs. Permeation enhancers are chemicals that enhance the transport of molecules across the intestinal epithelium, and while they are widely studied, very few have made it into clinical formulations. One of the biggest concerns preventing the advancement of novel permeation enhancers is our poor understanding of how they function and how their chronic use will impact intestinal health. To address these concerns, this work used a combination of *in vitro* and *in vivo* models of the intestinal epithelium to assess the efficacy and toxicity profiles of phenylpiperazine and its derivatives as well as the bile salt sodium deoxycholate. For both 1-phenylpiperazine and sodium deoxycholate, this work is the first that assesses their ability to improve macromolecular absorption in the intestine. Additionally, this work extended the typical size range of macromolecular cargo studied up to 70 kDa to more accurately predict how these permeation enhancers may work with larger protein drugs. Finally, a month-long safety study was carried out in mice, leading to our ultimate conclusion that novel permeation enhancers are able to deliver cargos larger than previously published and can do so without irreparably damaging the intestinal epithelium.

Table of Contents

List of Tables

List of Figures and Illustrations

1 Introduction

Peptide and protein drugs are a critical sector of the pharmaceutical market that is expected to keep growing, with eight new peptide drugs approved in 2019, bringing the total number of approvals to 48.[1] Compared to small molecule drugs, peptide drugs have more specific interactions with their targets, and their biological activity is more inherently limited, reducing off target effects.[2] This limitation is not a weakness, however, as peptide drugs are capable of much more sophisticated pharmacological mechanisms than many small molecule drugs and are thus readily prescribed for diseases such as diabetes (insulin), osteoporosis (calcitonin), and growth disorders (human growth hormone, somatropin), not to mention the many antibody drugs used to treat rheumatoid arthritis to cancer. The most common types of peptide and protein drugs on the market are monoclonal antibodies, coagulation factors, and replacement enzymes, in that order.[3]

The benefits of peptide drugs are extensive, and it is likely that the number of peptide drugs available will continue to increase in the future; however, using peptide drugs as opposed to small molecule drugs comes with one major additional hurdle: delivery. The most convenient method of drug administration is oral drug delivery, and most patients can easily and accurately use drugs formulated as tablets, capsules, liquids, or suspensions. The minimal discomfort associated with oral drug delivery leads to higher levels of patient compliance compared to other routes of administration, particularly injection. Unfortunately, injection is how almost all protein drugs are delivered.[4,5]

Peptide drugs are not easily delivered as oral formulations because there are several physiological barriers that prohibitively limit their oral bioavailability. Engineering solutions to this problem of poor oral bioavailability of protein drugs stand to revolutionize the pharmaceutical industry as well

as drastically improve the current standards of patient care. The successful oral delivery of macromolecular drugs may also have a circular effect by which more effort is put into identifying and developing peptide and protein drug candidates because they will be easier to prescribe, distribute, and administer if they do not require injections and the pain and inconvenience that comes with them.

1.1 Physiological barriers to oral protein drug delivery

1.1.1 Proteins are susceptible to degradation in the GI tract

The first anatomical barrier to systemic delivery that protein drugs encounter after oral administration is the stomach. The stomach is very acidic, with a pH of 1-2, and contains digestive enzymes that cleave proteins as part of our natural digestion process. Protein drugs that are not engineered to withstand this environment are unlikely to survive the 30 min - 3 hour retention time in the stomach intact and remain functional.[6] Protein drugs are highly susceptible to acid-catalyzed denaturation in the low pH of the stomach, and their complex structures are readily digested by proteases and other enzymes in the GI tract.

1.1.2 Proteins are poorly absorbed leading to low bioavailability

Additionally, their large size (generally >1000 Da) means that, even if they make it to the highly absorptive small intestine intact, they are unlikely to cross the epithelium into systemic circulation. Most drugs need to reach the bloodstream to function, the major exception being enzyme replacement therapies that act locally in the GI tract, such as CREON®, a delayed-release capsule formulation of pancrelipase to aid digestion in patients with chronic pancreatitis.[7]

Transport across the intestinal epithelium can occur via the transcellular or paracellular route.[8,9] Transcellular transport is the process by which molecules cross the epithelium by moving through

the epithelial cells, whether by passive diffusion through the lipid membrane or active transport using one of the many transporter pathways that rely on membrane-bound transporter proteins.[10] Small and relatively hydrophobic compounds that partition into cell membranes can be transported transcellularly, and several proteins including IgG and lactoferrin have specific receptor-mediated endocytic pathways.[11]

The other general category of transepithelial passage is paracellular transport. This is defined as transport of ions or molecules through the spaces between the epithelial cells. The epithelial monolayer is maintained by a complex system of junctional and adhesive proteins that fill the paracellular space and serve to maintain the polarity and barrier integrity of the intestinal epithelium.[12] Paracellular transport is regulated by the tight junctions, which are multi-protein complexes that maintain barrier integrity through complex signaling cascades involving both surface receptors as well as intracellular mechanisms.[13] Tight junction regulation is not fully understood, but several key players in the system have been well-studied including claudins, occludins, junctional adhesion molecules (JAM), and zonula occludens (ZO).[14,15] See Figure 1.1.

The result of these physiological barriers is the poor oral bioavailability of macromolecular drugs, which is why almost all peptide and protein drugs are currently delivered parenterally. However, extensive research over the past several decades has created a rich subfield of drug delivery dedicated to the challenge of oral protein delivery and, as of June 2020, three FDA-approved oral formulations for peptide drugs, which will be discussed in more detail in Section 1.3.1. A selection of the technologies in marketed formulations and in development will be presented in the next section.

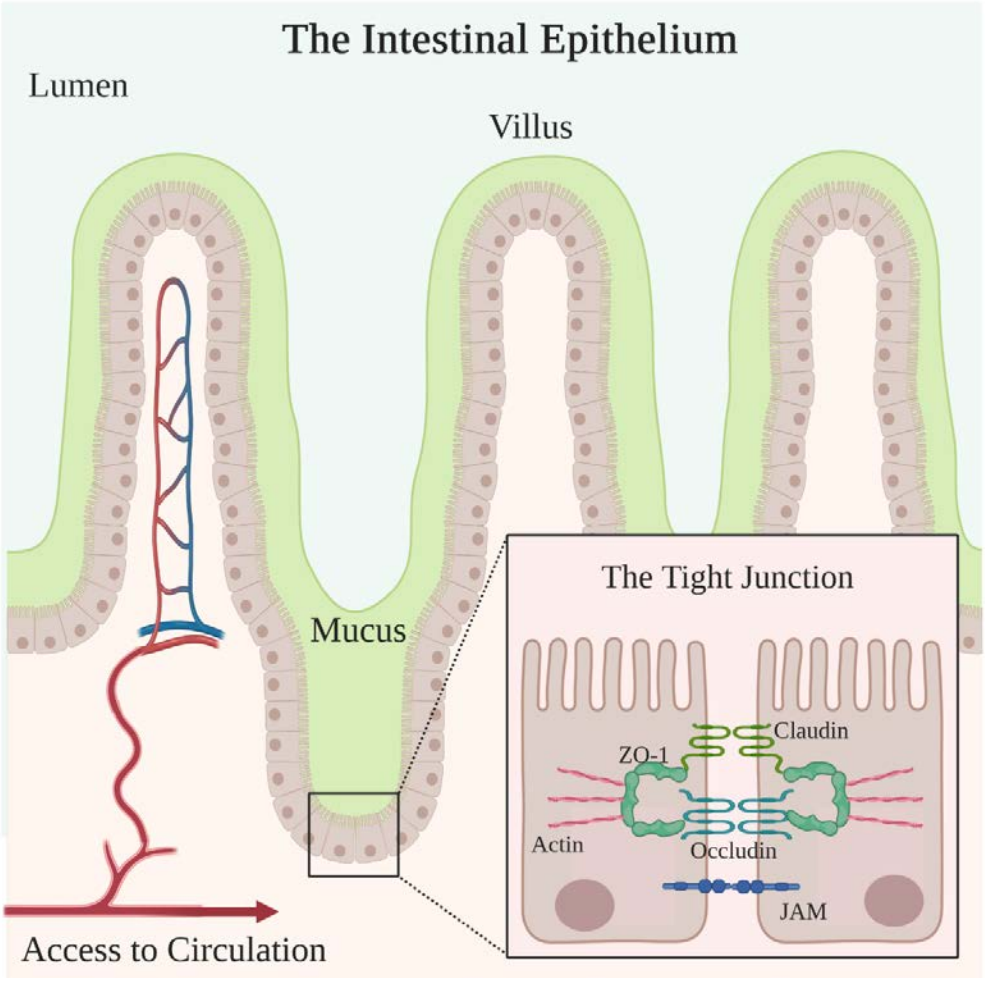

Figure 1.1: The intestinal epithelium is composed of a single layer of epithelial cells held together by protein complexes called tight junctions. (Created with BioRender.com)

1.2 Technologies for oral delivery of macromolecules

1.2.1 Strategies for peptide stabilization in the stomach

Peptide and protein drugs are at high risk of degradation in the acidic, enzyme-rich environment of the stomach. Strategies to improve protein stability generally fall into one of two categories: 1) molecular modifications that alter the structure of the peptide to increase its resistance to digestive processes, and 2) encapsulation methods that protect the drug cargo until it is selectively released in the absorptive region of the small intestine.[16]

The stability of peptides based on their chemical and structural characteristics is an active area of study, and detailed investigations of *in vitro* digestion suggest that the cleavage caused by proteases such as pepsin is more problematic than the low pH. Thus, structures and modifications that decrease the enzyme's access to the vulnerable peptide bonds are effective means of increasing gastric stability of peptide drugs.[17] Cyclosporin is a peptide drug that has outstanding stability in the GI tract because of its native cyclic structure. Cyclic peptides have exceptional stability in the GI tract and absorption in the intestine compared to polypeptides of similar size.[18] While cyclosporin is the only cyclic peptide currently on the market in an oral formulation, there are over forty cyclic peptide drugs available in parenteral formulations, with approximately one new cyclic peptide being approved annually.[19] Most of these are derived from natural sources, but the introduction of cyclic structures into peptides that are not naturally cyclic is a common tactic to improve stability.

Notably, a recent publication showed that phage display technology can be used to screen large libraries of peptides with genetically-introduced linkages for increased resistance to proteolysis by GI proteases. Kong, et al. used this novel method to develop a protease-resistant peptide that has potential to be an oral treatment for inflammatory disorders of the digestive system such as Crohn's disease.[20] Another type of molecular stabilization that protects peptide drugs from proteolysis is the addition of hydrocarbon linkages or "staples" between amino acids on alpha helices. Bird, et al. used this method to stabilize enfuvirtide, a 4.5 kDa peptide drug that can block HIV-1 entry in humans, but is not widely used because it lacks the oral bioavailability of other anti-HIV-1 therapies.[21]

Polymer conjugation is another molecular approach to increasing gastric stability of therapeutic peptides.[22] The addition of polymers, either by covalent conjugation or electrostatic attraction, has

been shown to increase stability in simulated gastric fluids as well as in animal models.[23–25] Many different polymer chemistries have been used, and they are extensively reviewed in the literature.[26]

A common concern with stabilization strategies like those described above that depend on altering the structure or chemistry of the peptide or protein drug is that these changes may affect how the drug is metabolized and excreted, which could be a negative consequence of a change that improves bioavailability. As an alternative to changing the drug structure, it is possible to increase protein stability on the formulation scale rather than the molecular scale.

Capsules coated with enteric polymers have been used successfully to selectively release peptide drugs once they are in the absorptive area of the intestine. Several peptide drugs that have reached clinical trials utilize enteric-coated capsules or tablets (see Table 1.1). The chemistry most commonly used is a system of acrylate/methacrylate and methacrylic acid copolymers. These polymers resist swelling and dissolution in the low pH environment of the stomach but release their cargo in the higher pH environment in the intestine because in pH 5-7 the carboxylic acid groups are transformed to carboxylate, causing the coating to dissolve.[27]

Eudragit is a widely used, commercially available brand of enteric polymers, with co-polymer formulations that serve several different purposes: Eudragit E is cationic and soluble below pH 5.5, making it useful for taste masking, and Eudragit L and S, which are soluble above pH 6 and 7 respectively, can be used to tailor release specifically to the small intestine or colon.[27] Eudragit E has been used to mask the taste of drugs and nutritional supplements such as iron encapsulated in microtablets for administration to infants and children.[28] Other enteric polymers have been used to coat microcapsules containing pancrelipase for oral delivery to patients with cystic fibrosis, who need pancreatic enzyme supplementation to allow nutrient absorption.[29] Enteric polymers are available in several different forms, including aqueous dispersions, organic solutions, dry powder,

or granules.[27] Recent advances in encapsulation technology include the development of capsules made out of intrinsically enteric polymers, thus eliminating the need for a coating step.[30]

In addition to enteric encapsulation, another common method of decreasing the loss of protein drugs to gastric digestion is the inclusion of protease inhibitors in the formulation. Given the plethora of proteases that attack specific amino acid sequences and cause cleavage of the peptide bonds, many different protease inhibitors with varied specificities, chemistries, and mechanisms have been investigated in oral protein drug delivery systems. These include small molecule and peptide/protein inhibitors that bind irreversibly to the protease and deactivate it. Examples include bacitracin, soybean trypsin inhibitor, and aprotinin, which have been found to improve the oral bioavailability of insulin in rodents.[31,32]

1.2.2 Overcoming poor peptide permeability in the intestine

In addition to degradation in the stomach, peptide drugs suffer poor oral bioavailability because they are not efficiently absorbed in the intestine. To reach systemic circulation, where most drugs need to be to have their pharmacological effect, drugs need to first cross the mucus layer and then be absorbed across the intestinal epithelium. Here, I present a survey of the techniques to overcome this barrier being used in clinical oral peptide formulations as well as some still in pre-clinical development.

The mucus layer is a major transport barrier to macromolecule absorption in the intestine, and it has been shown that steric hindrance and electrostatic attraction in the mucus mesh cause large molecules like proteins to be trapped and not reach the absorptive surfaces of the epithelial cells.[33,34] Many strategies have been employed to overcome the mucus barrier, including the use of mucolytic agents such as N-acetyl cysteine and polymeric carriers that alter the charge of the macromolecule and decrease its chances of getting stuck in the mucus due to electrostatic

attraction.[26,35] Physical strategies to improve absorption across the intestinal epithelium by increasing the residence time in the intestine or providing direct interaction between the drug and the epithelium include mucoadhesive devices, nanofabricated structures that interact with the cell surface, and hydrogels that localize drug within the intestine [36–39]

By far, the most common method of increasing drug transport across the intestinal epithelium is the use of permeation enhancers. In the broadest sense, these are chemicals that increase the permeability of the intestinal epithelium to allow passage of molecules that would not naturally be absorbed due to their large size or incompatible chemical characteristics.

1.3 Permeation enhancers

Permeation enhancers work by many different mechanisms, most of which are not fully understood. For our purposes, we can classify permeation enhancers into three broad categories based on general mechanism: enhancers that affect receptor-mediated uptake by epithelial cells, those that fluidize the lipid membrane of the epithelial cells, and those that affect the tight junctions between the cells. Permeation enhancers have been extensively reviewed in the literature; here, I limit discussion to clinically successful enhancers and novel enhancers in pre-clinical development.[40–42] A number of oral biopharmaceutical formulations containing permeation enhancers are in clinical trials, and the first oral peptide formulation including a permeation enhancer was FDA-approved in 2019, with two more added to the ranks by June 2020.[40]

In intestinal epithelial cells, there are several native mechanisms of transport from the apical side of the cell to the basolateral side that can be exploited for protein delivery. The neonatal Fc receptor pathway has been used for oral delivery in mice by fusing Fc fragments of IgG to nanoparticles loaded with insulin.[11] Another pathway that has potential for oral protein delivery is the lactoferrin

pathway. Lactoferrin is an iron-carrying protein, and there are receptors for its uptake on intestinal epithelial cells that could be used for a targeted method of uptake and transcytosis.[43,44] Oral peptide bioavailability can also be enhanced using surfactants and other molecules that interact physically with the lipid membranes of the epithelial cells, fluidizing their surface and allowing large molecules across the epithelial layer.

Many permeation enhancers act through a combination of mechanisms, meaning they have membrane fluidization activity in addition to altering tight junction structure. These chemicals fall into classes such as: ionic and nonionic surfactants, bile salts, fatty acids, toxins, and other small molecules like nitrogen-containing rings (see Section 1.3.1 for more information).[9,45,46] Sodium caprate (C_{10}) is perhaps the permeation enhancer with the most thoroughly investigated mechanism of action. Originally identified as a component of goat milk, C_{10} is a medium chain fatty acid that is an effective permeation enhancer in the small intestine with remarkably low toxicity compared to equally effective enhancers.[47] C_{10} is the primary permeation enhancer used in GIPET technology which was licensed by Novo Nordisk from Merrion Pharmaceuticals for use in an oral insulin formulation.[48] In addition to clinical trials for peptide oral delivery, C_{10} has also been investigated for the oral delivery of nucleotide-based drugs, increasing the available literature on its tolerability in humans.[49]

1.3.1 Permeation enhancers in clinical formulations

Currently, there are only three peptides that have FDA-approved oral formulations: cyclosporin, a systemic immunosuppressant used to prevent organ rejection in transplant patients; octreotide, a peptide hormone used to treat the growth disorder acromegaly; and semaglutide, a GLP-1 receptor agonist used to manage Type 2 diabetes (T2D). They each use a permeation enhancer or synergistic combination or permeation enhancers, and are available in liquid emulsion or encapsulated forms.

Cyclosporin is a small, cyclic peptide, and both of these native characteristics make it easier to deliver orally. Cyclization makes peptides more stable and resistant to acid-catalyzed hydrolysis and enzymatic degradation. Oral cyclosporin formulations are currently produced by Novartis (Neoral) and AbbVie Inc (Gengraf). Their formulations create spontaneous microemulsions from either liquid or capsule forms, and these emulsions promote absorption across the intestinal epithelium. Octreotide is the most recent addition to the group, with the approval of Mycapssa (Chiasma) occurring in June 2020. Mycapssa is the first drug approved that uses their Transient Permeation Enhancer (TPE) technology. Oral semaglutide is produced by Novo Nordisk (Rybelsus) and incorporates Eligen Technology (Emisphere Technologies, Inc.). Eligen Technology uses salcaprozate sodium (SNAC) to stabilize the fatty acid-acylated GLP-1 analog in its active, monomeric form and as a permeation enhancer that acts in the stomach.[50] Other oral peptide drugs in clinical development are summarized in Table 1.1.

The permeation enhancers that have made clinical progress have been limited mostly to GRAS substances and chemicals commonly used as food additives. For example, sodium caprate (C_{10}) is one of the most widely studied permeation enhancers, with many published *in vitro*, *in vivo*, and human studies.[47] A study by ISIS pharmaceuticals in 2007 found that oral solid dosage forms containing an antisense oligonucleotide drug and C_{10} were well-tolerated in human volunteers.[51] However, C_{10} is an anomaly in the field, as the majority of permeation enhancers have not been studied for the effects of repeat dosing in animals, much less tested in humans. Peptelligence Technology developed by Enteris Biopharma uses citric acid and acyl carnitines as a permeation enhancer in their delivery system which has been used in formulations of salmon calcitonin and leuprolide, both of which have completed multiple stages of clinical trials.[52]

Table 1.1: A selection of oral peptide and protein drugs that have reached clinical trials and/or FDA-approval.

Peptide	MW (Da)	Indication	Manufacturer	Drug Name	Oral Delivery Technology	Trial Status
Cyclosporin	1202.6	Immunosuppressant (systemic)	Novartis	Neoral (previous form: Sandimmune)	Cyclic peptide, spontaneous microemulsion	Marketed
			AbbVie Inc	Gengraf	Microemulsion capsules or solution	Marketed
Octreotide	1019.2	Acromegaly	Chiasma	Mycappsa (formerly Octreolin)	Transient Permeation Enhancer (TPE): lipophilic suspension of hydrophilic particles in hydrophobic medium	Marketed
Semaglutide	4113.6	GLP-1 receptor agonist for T2D	Novo Nordisk	Rybelsus	Eligen Tech (Emisphere Technologies, Inc.); SNAC as a permeation enhancer	Marketed
Exenatide	4186.6	Glucagon-like peptide-1 analogue for T2D	Oramed Pharmaceuticals, Inc.	ORMD 0901	Protein Oral Delivery (POD) technology: coated capsule, protease inhibitors, absorption enhancer	Preclinical/Investigational New Drug (Phase IB); pharmacokinetic study completed in T2D patients
Insulin	5808.0	T1/T2 diabetes	Oramed	ORMD-0801	POD technology	Phase 2 trials in progress for both T1D and T2D patients
			Novo Nordisk (licensed from Merrion Pharm.)	OI338GT	Gastro-Intestinal Permeation Enhancement Technology (GIPET): coated capsule with sodium caprate as a permeation enhancer	Phase 2 trials completed, but product development was discontinued due to high doses making it not commercially viable
			Oshadi Drug Administration, Ltd	Oshadi Icp	Silica nanoparticles with branched polysaccharide and suspension of insulin in a mixture of oils	Phase I and II completed
			Biocon	Tregopil (IN-105)	Insulin analogue with covalent PEG for stability and solubility in the GI tract	FDA Phase 1 trial paused in 2018; Phase 2 and 3 trials ongoing in India
			Diasome	Oral-HDV insulin	Hepatic delivery vesicles (HDV): phospholipid nanocarriers with surface-bound insulin and specific hepatocyte targeting molecules	Phase 2b trial in progress
Salmon calcitonin	3431.9	Osteoporosis	R-Pharm JSC	TBRIA	Peptelligence (Enteris Biopharm); acyl carnitines and citric acid	Phase 3 trial completed
			Nordic Biosciences	SMCC021	Eligen Technology from Emisphere Technologies, Inc.: 5-CNAC as a permeation enhancer	Phase 3 trial did not achieve primary endpoint of decreased incidence of bone fractures
Leuprolide	1209.4	Endometriosis, prostate cancer, premature puberty	Enteris Biopharma	Ovarest	Peptelligence (Enteris Biopharm) technology: surfactant permeation enhancer and citric acid	Phase 2 trial completed

1.3.2 Novel permeation enhancers in preclinical stage

The molecules, particles, and devices used to increase intestinal permeability in preclinical publications are numerous, with the simplest PubMed search for 'Intestinal Permeation Enhancers' yielding over 700 results from the last four decades. In this section, a small subset of novel permeation enhancers that have not yet reached clinical studies will be presented, including enterotoxin derivatives, bile salts, piperazines, ionic liquids, chitosan, and anionic nanoparticles. These enhancers represent a wide range of chemistries, mechanisms of action, and scale, but are by no means a comprehensive list of this extensive field.

Zonula occludens toxin (Zot) is a protein produced by *Vibrio cholerae*, the bacteria responsible for the disease cholera. It was originally discovered in attempts to eliminate the diarrhea-causing components of the pathogen to create a safe cholera vaccine.[46] It was found to affect tight junction arrangement in a reversible manner, and has since been studied extensively in preclinical studies such as the work by Fasano and Uzzau in which they produced Zot as a fusion protein that allowed the oral delivery of insulin in a diabetic rat model.[53]

Ionic liquids are a novel material consisting of loosely associating anions and cations that form liquids with unique properties that make them excellent biocompatible solvents and permeation enhancers. Many different compositions of ionic liquids with different drug delivery applications (oral, buccal, transdermal, etc.) have been published,[54] but of particular relevance to oral protein drug delivery is the work by Banerjee et al. showing that a system of choline and generate ions was able to significantly improve the oral bioavailability of insulin in rats.[55]

Chitosan is a cationic polysaccharide derived from the shells of crustaceans that has been widely studied for a vast array of biomedical applications. It has the delivery benefits of being a polycationic material that lacks much of the cytotoxicity associated with other polycations.[26] It is

easily modified with a wide variety of chemical modifications to change its transport or half-life and has been reported in many oral protein drug delivery systems with promising results delivering salmon calcitonin and insulin, among others, in rodents.[56,57] It has been shown to interact with the tight junctions of intestinal epithelial cells, thus improving oral bioavailability by increasing paracellular transport of protein cargos.[58]

Bile salts are naturally-occurring molecules synthesized by the liver to aid the absorption and clearance of lipids and cholesterol. They are ionic, amphiphilic molecules with a steroidal backbone, and function as permeation enhancers by fluidizing the cell membranes as well as by causing tight junction rearrangement. Sodium taurodeoxycholate was found to enhance the absorption of several compounds in the *in vitro* Caco-2 model and has also been used in a liposomal formulation to orally deliver salmon calcitonin to rats.[35,59] In this work, the bile salt sodium deoxycholate (SDC) was used as a permeation enhancer. SDC has been shown to increase permeation of several model drugs in Caco-2 cells as well as *ex vivo* intestinal tissue from rodents, but this work represents its first extensive testing in an *in vivo* model.[9,45,60,61]

In a study by Whitehead et al. testing 51 chemically diverse molecules for permeation enhancing ability, a family of small molecules called piperazines were found to have promise as potent permeation enhancers with minimal cytotoxicity. Piperazines contain six-membered, saturated rings with nitrogens ortho to each other at positions 1 and 4.[45] Two piperazine derivatives, 1-methylpiperazine and 1-phenylpiperazine (PPZ) emerged from this library as intestinal epithelial permeation enhancers with high potency and low toxicity.[45] The efficacy of the piperazine family was confirmed by a study of 14 piperazine derivatives with aliphatic (open carbon chain) substitutions that found all to be effective permeation enhancers.[62] The mechanism by which PPZ functions as a permeation enhancer has been investigated *in vitro* and in *ex vivo* rat tissue, and it

has been found to cause tight junction rearrangement that leads to increased paracellular transport.[63,64] In this work, we present further mechanistic investigations *in vitro*, as well as the first *in vivo* studies of PPZ.

1.4 Clinical translation of permeation enhancers suffers from our poor understanding of how they work

Despite a handful of successes, most permeation enhancers have generally failed to translate into the clinic. The blame for this stagnation largely falls on our poor understanding of how they work and how their repeated use in a drug regimen might impact intestinal health. Anecdotally, there is a pessimistic view of their potential, which is mainly driven by concerns about toxicity as well as catastrophic imaginings of how repeated permeation enhancement may cause permanent damage to the intestinal epithelium, resulting in formulation-induced colitis. In 2015, a study of repeated dosing of two common dietary emulsifiers in mice found evidence that they disturb the gut microbiota, resulting in increased inflammation and subsequent higher incidence rates of obesity and metabolic syndrome as well as colitis.[65] This study exemplifies the learnings that can be gained from repeat dosing experiments in animal models, but understandably, it triggered further concerns about the use of permeation enhancers.

Publications of novel permeation enhancers generally present efficacy and toxicity data from *in vitro* experiments using cell monolayers, e.g. the Caco-2 model, or from acute *ex vivo* or *in vivo* studies.[66] While these studies provide valuable information about the effects of these compounds on intestinal permeability and tissue damage on a single challenge, they do not answer the question of how chronic permeation enhancer use will affect the intestine. This critical knowledge gap hinders the development of formulations containing novel permeation enhancers.

1.5 The scope of this work

This work was performed to close some of the knowledge gaps discussed in Section 1.4. The phenylpiperazine family was explored to learn more about how paracellular permeation enhancers work and to identify possible structure-function relationships to predict the efficacy and toxicity of potential permeation enhancers (Chapter 2).

Phenylpiperazine and sodium deoxycholate were chosen for more in depth experiments to probe how the physical characteristics of the macromolecular cargo affects how permeation enhancers function. Specifically, FITC-dextrans of varying molecular weights were used to determine the maximum size of macromolecule that these two permeation enhancers are able to deliver. Generally, the molecules chosen to assess permeability are small molecules or peptides (< 4 kDa), and this limitation in the literature is reflected in the fact that the three peptides currently available in oral formulations are 4 kDa or less. For context, the oral delivery of insulin is considered one of the 'holy grails' of drug delivery and would have extraordinary effects on the quality of life of millions of diabetic patients; however, insulin, with a molecular weight of 5.7 kDa, lies outside the size range of the current understanding and ability of the field of oral drug delivery.

This work also presents the first *in vivo* results for phenylpiperazine as an intestinal permeation enhancer in mice (Chapter 3). Finally, this work investigates the effects of chronic exposure to permeation enhancers on the intestine (Chapter 4). Taken together, this body of work is intended to further the understanding of novel permeation enhancers in terms of their mechanism of action and their abilities to deliver macromolecular drugs in a wider range of therapeutically relevant sizes.

2 Structure-function analysis of phenylpiperazine derivatives as intestinal permeation enhancers

2.1 Introduction

Because biological membranes are often sensitive to small changes in molecular structure, it was hypothesized that further exploration of the piperazine family, specifically, derivatives of 1-phenylpiperazine, could identify increasingly efficacious compounds. To do so, we assembled a small library containing 13 phenylpiperazine derivatives and tested it for paracellular permeation enhancing ability and cytotoxicity. In addition to identifying novel enhancers, we anticipate that improving our understanding of structure-function relationships within this promising molecular family will facilitate the discovery of next-generation piperazine-derived enhancers in the future.

2.2 Materials and Methods

2.2.1 Materials

Thirteen derivatives of phenylpiperazine were studied. 1-phenylpiperazine (A) and 1-(4-methylphenyl)piperazine (J) were purchased from Sigma-Aldrich (St. Louis, MO). 2-phenylpiperazine (B), 1-(4-hydroxyphenyl)piperazine (D), 1-(3-hydroxyphenyl)piperazine (M), and 1-(4-aminophenyl)piperazine (E) were obtained from Alfa Aesar (Ward Hill, MA). 4-phenylpiperidine (I), 1-(3-methylphenyl)piperazine (K), and 1-(2-methylphenyl)piperazine (L) were purchased from Acros Organics (Morris Plains, NJ). 1-phenylpiperidine (H) was purchased from CombiBlocks (San Diego, CA). 4-(4-methylpiperazin-1-yl)aniline (G) was purchased from Enamine (Monmouth Jct., NJ). 1-benzylpiperazine (C) was obtained from Matrix Scientific (Columbia, SC). 1-methyl-4-phenylpiperazine (F) was purchased from Ontario Chemicals (Guelph, ON).

Dulbecco's modified Eagles medium (DMEM), Falcon 225 cm^2 tissue culture flasks, Corning BioCoat™ HTS 1.0 µm porous support Transwell plates, Falcon 24-well plates, Corning CellBIND 96-well plates, sodium butyrate, amphotericin B, and MITO+ serum extender were procured from VWR (Radnor, PA). Penicillin/streptomycin, trypsin-EDTA, phosphate buffered saline (PBS), and fetal bovine serum (FBS) were purchased from Life Technologies (Thermo Fisher subsidiary, Carlsbad, CA). Caco-2 cells and methyl thiazole tetrazolium (MTT) assay kits were obtained from American Type Culture Collection (Manassas, VA). CellTiter-Fluor™ Cell Viability assay kits were purchased from Promega (Madison, WI). Alexa-Fluor 594 anti-ZO-1, Alexa-Fluor 488 phalloidin, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes (Thermo Fisher subsidiary, Eugene, OR). Calcein was purchased from Thermo Fisher Scientific (Waltham, MA).

2.2.2 Cell Culture

Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 10 IU/mL of penicillin, 0.1 mg/mL of streptomycin, and 1 µg/mL amphotericin B. Cultures were incubated in a 5% CO_2 environment at 37°C. The cells were subcultured using 0.25% trypsin-EDTA, with cells at passage numbers 25-55 used for experiments. For transepithelial electrical resistance (TEER) experiments, cells were seeded on BioCoat™ HTS membranes at a density of 2×10^5 cells per well (cell growth area of 0.3 cm^2) and incubated in basal seeding medium (BSM) for two days. BSM consisted of DMEM supplemented with MITO+ serum extender according to the manufacturer's instructions and 1 µg/mL amphotericin B. Then the media was changed to enterocyte differentiation medium (EDM) and incubated for two more days. EDM comprised DMEM supplemented with MITO+ serum extender, 1 µg/mL amphotericin B, and 2 mM sodium butyrate. Monolayer formation was

confirmed by measuring the TEER values and only wells that had developed monolayers with TEER values of 200 Ω-cm^2 or higher were used in experiments.

2.2.3 TEER Experiments

The Caco-2 monolayers grown on HTS membranes were transferred to 24-well plates with 1 mL of EDM per well and incubated at 37°C with 5% CO_2 for 15 minutes to allow the system to equilibrate. Initial TEER values were measured using a Millicell voltohmmeter. The phenylpiperazine derivatives were dissolved in EDM and applied to the apical side of the monolayers. TEER readings were taken after 30 and 60 min. Negative control wells receiving only EDM were included in each TEER experiment.

2.2.4 Permeability Experiments

The paracellular diffusion marker calcein was dissolved in EDM at a concentration of 0.5 mM along with the phenylpiperazine derivative treatments at 1, 3, 10, or 30 mM and applied to the apical side of the Caco-2 monolayers. The negative control wells received EDM with only calcein. After 60 min, 120 min, and 180 min incubation at 37°C with 5% CO_2, samples of media were taken from the basolateral side of the monolayers. The fluorescence of these samples at 495/515 nm was measured using a BioTek Synergy2 plate reader. Calibration curves were used to convert fluorescence measurements into the mass of calcein transferred across the monolayer, which was used to calculate the apparent permeability of calcein across the monolayer:

$$P_{app} = \frac{\Delta M}{C_a A \Delta t} \qquad (1.1)$$

where P_{app} is the apparent permeability, ΔM is the mass of calcein transported into the basolateral compartment, C_a is the concentration of calcein in the apical well, A is the area of the monolayer, and Δt is the length of time the calcein was applied to the monolayer. Normalization to obtain the

fold-increase in permeability was done by dividing P_{app} of treated monolayers by the P_{app} of untreated monolayers on the same 24-well plate.

2.2.5 MTT Experiments

Cells were seeded in 96-well plates at a density of 1×10^5 cells per well in DMEM and incubated overnight. The phenylpiperazine derivatives dissolved in media at concentrations of 1, 3, 10, 30, or 100 mM were applied to the cells for 1 hour. Then 10 µL/well of the MTT reagent was added, and the plate was incubated for 3 hours at 35°C. 100 µL of detergent was added to each well, and the plate was incubated in the dark at room temperature overnight. Absorbance values at 570 nm were measured using a BioTek Synergy2 plate reader. Absorbance data were normalized to those of control wells that contained no cells but the same quantities of phenylpiperazine treatments, MTT reagent, and detergent.

2.2.6 CellTiter-Fluor™ Experiments

Cells were seeded in 96-well plates at a density of 1×10^4 cells per well in DMEM without phenol red and incubated overnight. Phenylpiperazine derivatives D, E, G, and M were dissolved in clear DMEM at concentrations of 1, 3, or 10 mM and applied to the cells for 1 hour. Then the CellTiter-Fluor™ reagent was prepared according to the manufacturer's instructions by mixing the GF-AFC substrate with the provided assay buffer. 100 µL was added to each well, and the plate was incubated at 37°C for 45 minutes. Fluorescence values at 400/505 nm were read using a BioTek Synergy2 plate reader. Wells containing treatments and the toxicity reagent but no cells were included to control for background fluorescence of the phenylpiperazine derivatives.

2.2.7 Confocal Imaging

Four sets of Caco-2 monolayers were cultured as for TEER experiments and treated with phenylpiperazine derivatives A, E, or F at 10 mM or PBS (untreated control) for 1 hour. The monolayers were washed 3 times with PBS then fixed with 4% w/v paraformaldehyde in PBS for 10 minutes at room temperature. The monolayers were then washed four times with PBS, permeabilized with 0.2% v/v Triton-X for 10 minutes at room temperature, and washed three more times with PBS. The monolayers were blocked with 0.2% BSA in PBS for 30 minutes at room temperature to reduce non-specific labelling. The monolayers were stained with Alexa-Fluor 594 anti-ZO-1, Alexa-Fluor 488 phalloidin, and 4',6-diamidino-2-phenylindole (DAPI) by incubation at room temperature for one hour. The monolayers were rinsed once with PBS. Then the monolayers were cut from the well inserts and mounted on glass slides with ClearMount. Confocal microscopy was performed on a Zeiss LSM 700 using Zen 2012 software (Carl Zeiss, Inc.) with a 555 nm filter (AF-594-anti-ZO-1), 488 nm (AF488-phalloidin), and 405 nm (DAPI).

2.2.8 Statistical Analysis

TEER and permeability experiments were performed in triplicate. MTT and CellTiter-Fluor™ experiments were performed in quadruplicate. Error is displayed in all plots as the standard error of the mean and accounts for propagated error from all normalization calculations.

2.3 Results

2.3.1 Enhancement Potential of Phenylpiperazine Derivatives

The 13 compounds used in this study are shown in Figure 2.1. Previous studies have shown 1-phenylpiperazine to be an effective paracellular permeation enhancer on Caco-2 monolayers at the relatively low concentration of 0.1% w/v, which corresponds to approximately 6 mM.[45,63] Therefore, for this study of chemically similar compounds, concentrations of 1, 3, and 10 mM were used for an initial screen of efficacy. Higher concentrations (30 and 100 mM) were tested only for compounds that induced minimal cytotoxicity at the lowest concentration of 10 mM. All studies were conducted using Caco-2 cells, which, when differentiated into monolayers, represent the most widely-accepted model of the intestinal epithelium.[67,68]

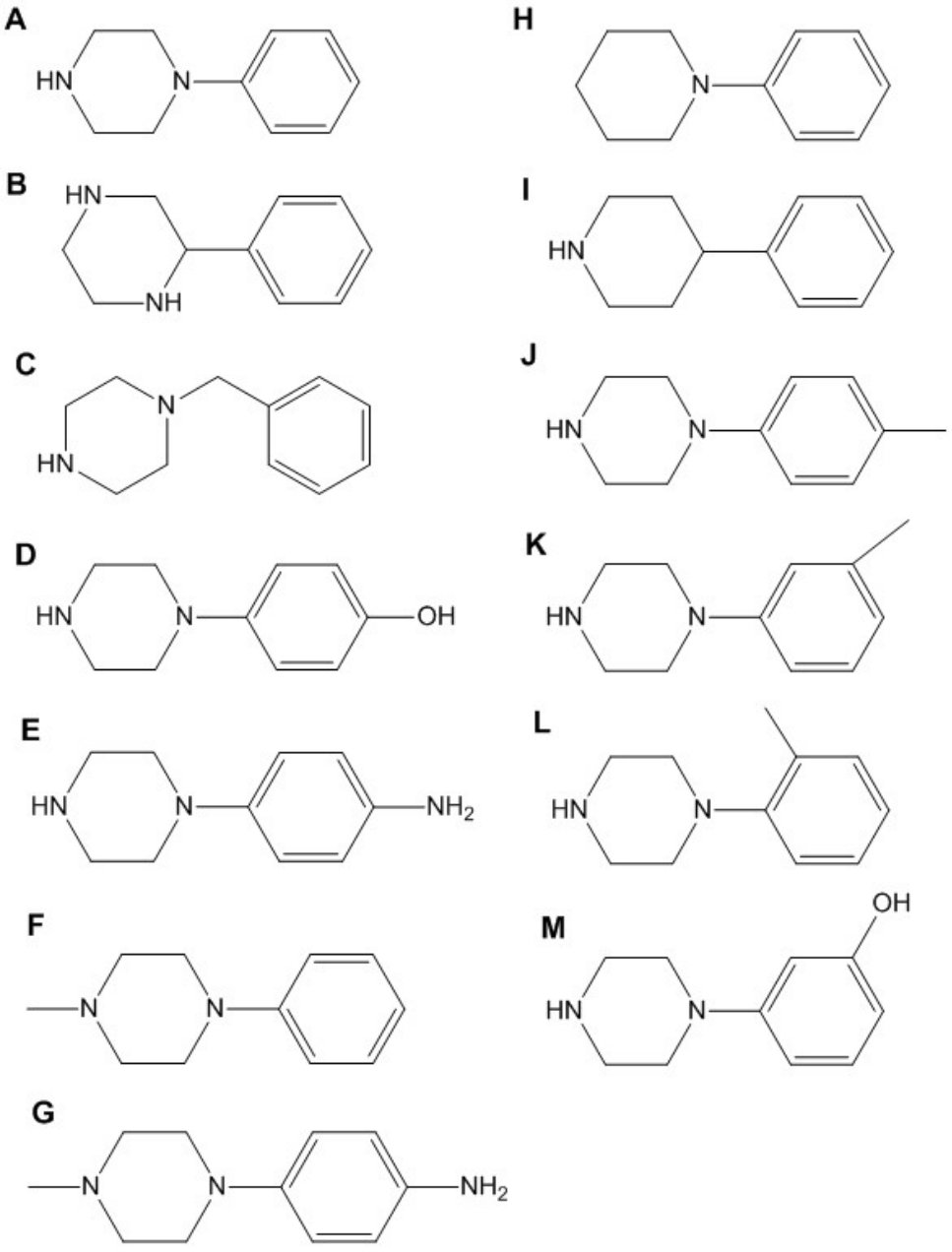

Figure 2.1: A library of 13 piperazine compounds was studied for intestinal permeation enhancing ability. These compounds included 1-phenylpiperazine (A) and 12 of its derivatives: B, 2-phenylpiperazine; C, 1-benzylpiperazine; D, 1-(4-hydroxyphenyl)piperazine; E, 1-(4-aminophenyl)piperazine; F, 1-methyl-4-phenylpiperazine; G, 4-(4-methylpiperazin-1-yl)aniline; H, 1-phenylpiperidine; I, 4-phenylpiperidine; J, 1-(4-methylphenyl)piperazine; K, 1-(3-methylphenyl)piperazine; L, 1-(2-methylphenyl)piperazine; M, 1-(3-hydroxyphenyl)piperazine.

The ability of phenylpiperazine derivatives to increase the permeability of Caco-2 monolayers was initially screened by measuring trans-epithelial electrical resistance (TEER). TEER, which indicates the ability of ions to cross the monolayer via the paracellular pathway,[69] has been shown to have a strong inverse relationship with paracellular flux of diffusion markers such as calcein.[45,62]

Following the application of the three concentrations of each phenylpiperazine derivative to Caco-2 monolayers, a normalized parameter called the enhancement potential (EP) was calculated for each treatment to represent efficacy:

$$EP = 1 - \frac{(TEER_P)/(TEER_{P,i})}{(TEER_C)/(TEER_{C,i})} \quad (1.2)$$

where $TEER_P$ is the average electrical resistance of the monolayer after 1 hour of treatment with a phenylpiperazine derivative, $TEER_C$ is the average electrical resistance of untreated monolayers at 1 hour, and the subscript 'i' indicates the average initial TEER value for the respective monolayers. EP values range from 0 to 1, with 0 indicating that a compound has no permeation enhancing ability and 1 indicating maximum permeation enhancement ability (i.e. complete disruption of barrier function).

EP values for the 13 phenylpiperazine derivatives of this study covered a wide range of possible values from 0 to 0.9, with a general dependence on concentration. The median EP value for 1 mM treatments was 0, with only one compound, 1-(4-methylphenyl)piperazine (J), possessing a non-zero EP ($p < 0.1$). At 3 mM, the median EP value was 0.15, and at 10 mM it increased to 0.68. Most compounds showed an increasing EP with increasing concentration, with one notable exception being compound G that was ineffective (EP = 0) at all concentrations. The full set of EP values can be found in Table S1.

2.3.2 Toxicity Potential of Phenylpiperazine Derivatives

When considering therapeutic potential of chemical permeation enhancers, efficacy alone should not be used to identify promising compounds for future study. Because permeation enhancement can occur by reversible mechanisms such as tight junction rearrangement, or by irreversible mechanisms such as cell death, cytotoxicity assays were performed to identify compounds that are both potent and safe. For compounds A, B, C, F, H, I, J, K, and L, an MTT assay was used to calculate the toxicity potential at each concentration tested:

$$TP = 1 - \frac{A_P - A_{P,b}}{A_C - A_{C,b}} \quad (1.3)$$

where A_P is the average absorbance value of reduced MTT reagent by cells treated with phenylpiperazine derivatives, A_C is the average absorbance value of reduced MTT reagent by untreated cells, and the subscript 'b' indicates the absorbance reading from wells containing treatments and MTT reagent but no cells. TP values ranged from 0 to 1, with 0 indicating negligible toxicity and 1 indicating maximum toxicity.

Interestingly, the cytotoxicity of compounds D, E, G, and M could not be evaluated using the MTT assay, which relies on cellular machinery to reduce the MTT reagent into a purple product. This was because compounds D, E, G, and M reduced the MTT reagent in the absence of cells. As such, an alternative assay (the CellTiter-Fluor™ Cell Viability Assay) was used for these phenylpiperazines. The CellTiter-Fluor™ Cell Viability Assay measures the activity of a protease that only functions in living cells via a substrate that fluoresces when cleaved by an active protease. A similar toxicity potential was calculated for these compounds:

$$TP = 1 - \frac{F_P - F_{P,b}}{F_C - F_{C,b}} \quad (1.4)$$

where F_P is the average fluorescence of cleaved assay substrate by cells treated with phenylpiperazine derivatives, F_c is the average fluorescence of cleaved GF-AFC substrate by untreated cells, and the subscript ‘b’ indicates the fluorescence from wells containing treatments and GF-AFC substrate but no cells.

As expected, toxicity potential increased with concentration for most of the compounds tested. The TP values of all formulations can be found in Table S1. The median values were low for 1 mM and 3 mM treatments, at 0.05 and 0.06 respectively, indicating that many of the phenylpiperazine derivatives were nontoxic at these low concentrations. However, there was a large increase in TP for the 10 mM treatments, with a median value of 0.72.

2.3.3 Relationship between Enhancement Potential and Toxicity Potential

To assess the relationship between efficacy and toxicity for each of the phenylpiperazine treatments tested, EP was plotted versus TP for all compounds at each concentration. When considering the quadrants shown in Fig. 2.2a, effective and non-cytotoxic materials appear in the top left quadrant, while non-efficacious, toxic materials appear in the bottom right quadrant. Concentration played an important role when considering the utility of each treatment. None of the 1 mM treatments are located in the upper half of the graph, and they are fairly evenly distributed throughout the range of TP values, indicating low efficacy and wide variation in toxicity at low concentrations. Many of the 3 mM treatments appear on the left side of the graph, indicating low toxicity, with several possessing the desirable characteristics of low toxicity and high efficacy. Because toxicity increased for several of the 10 mM treatments, their points are distributed throughout all four of the quadrants.

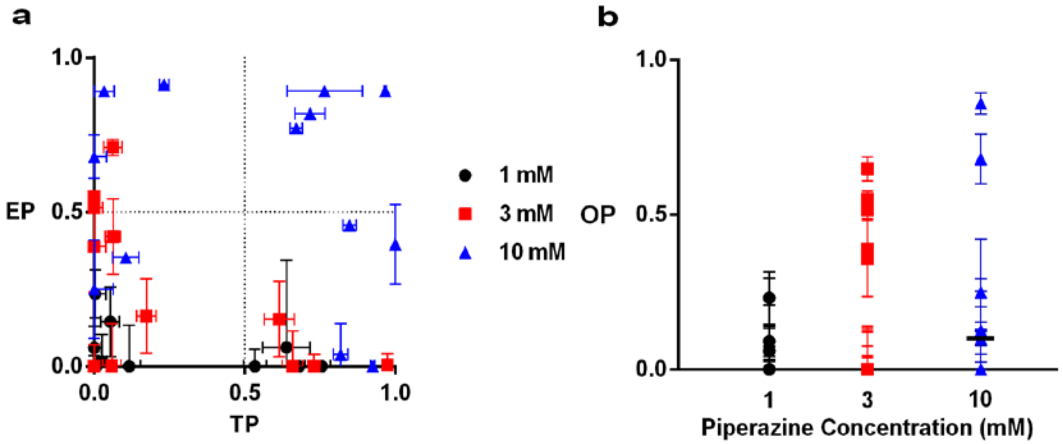

Figure 2.2: Phenylpiperazine derivatives have a wide range of efficacy and toxicity potentials that tends to increase with concentration. (a) Most compounds were ineffective at 1 mM, with increased efficacy (and sometimes toxicity) at higher concentrations. (b) Overall potential is shown for each compound. Multiple points overlapping at an OP value of 0 cause the median OP values for 1 mM and 3 mM treatments to be 0, with the median value for 10 mM treatments shown as a horizontal black bar at 0.1. Error bars represent SEM (n = 3 - 4).

To further examine the relationship between efficacy and toxicity, an overall potential (OP) parameter was calculated as the difference between EP and TP:

$$\mathrm{OP} = \begin{cases} \mathrm{EP} - \mathrm{TP}, & \text{if } \mathrm{EP} - \mathrm{TP} > 0 \\ 0, & \text{if } \mathrm{EP} - \mathrm{TP} \leq 0 \end{cases} \tag{1.5}$$

While it is necessary to consider EP and TP individually to assess a chemical permeation enhancer, OP is a useful parameter for looking at broad trends, as higher OP values typically indicate a more promising enhancer. Fig. 2.2b shows that, in general, OP increases with concentration. While many treatments resulted in OP values of 0, there were several phenylpiperazine derivatives at each concentration that showed more relative efficacy than toxicity.

2.3.4 Therapeutic Windows for Phenylpiperazine Derivatives

To develop a more thorough understanding of the relationship between efficacy and toxicity and the resulting potential of a phenylpiperazine derivative as a therapeutic permeation enhancer, EP

and TP were plotted versus concentration on the same graph to show therapeutic windows. There were three general profiles that appeared upon examination of these graphs. Figure 2.3 shows representative examples, and plots for all derivatives are in the supplemental information (Figure S2.1).

The first type of profile (Fig. 2.3a) is seen for compounds A, B, F, I, J, K, and L, and contains at least a small range of concentration for which EP is significantly higher than TP. This gap between EP and TP as a function of concentration can be considered a therapeutic window – the bigger the window, the more potential therapeutic utility. Compounds that displayed therapeutic windows included 1-phenylpiperazine and several of the compounds most chemically similar, including 2-phenylpiperazine (B) and 4-phenylpiperidine (I). All of the derivatives that deviated from the structure of 1-phenylpiperazine with the addition of a single methyl group, regardless of position, also possessed therapeutic windows (compounds F, J, K, and L). Compound B produced the broadest window, which spanned two orders of magnitude, with TP and EP converging at 100 mM. The plot for compound B has a logarithmic x-axis, so it does not appear to be the largest based simply on shape.

A second type of profile (Fig. 2.3b) exists for compounds D, E, G, and M, and shows that for each concentration tested, the TP was significantly higher than the EP. These four compounds containing amine and hydroxyl group substitutions were toxic at even the lowest concentration tested. The final type of profile (Fig. 2.3c) observed in plots of EP and TP versus concentration is seen for compounds C and H. In these plots, the EP and TP values fell almost on top of each other for every concentration, with no significant differences at any concentration. Phenylpiperazine derivatives resulting in the second or third type of profile (as seen in Fig. 2.3b and c) are not viable permeation enhancers.

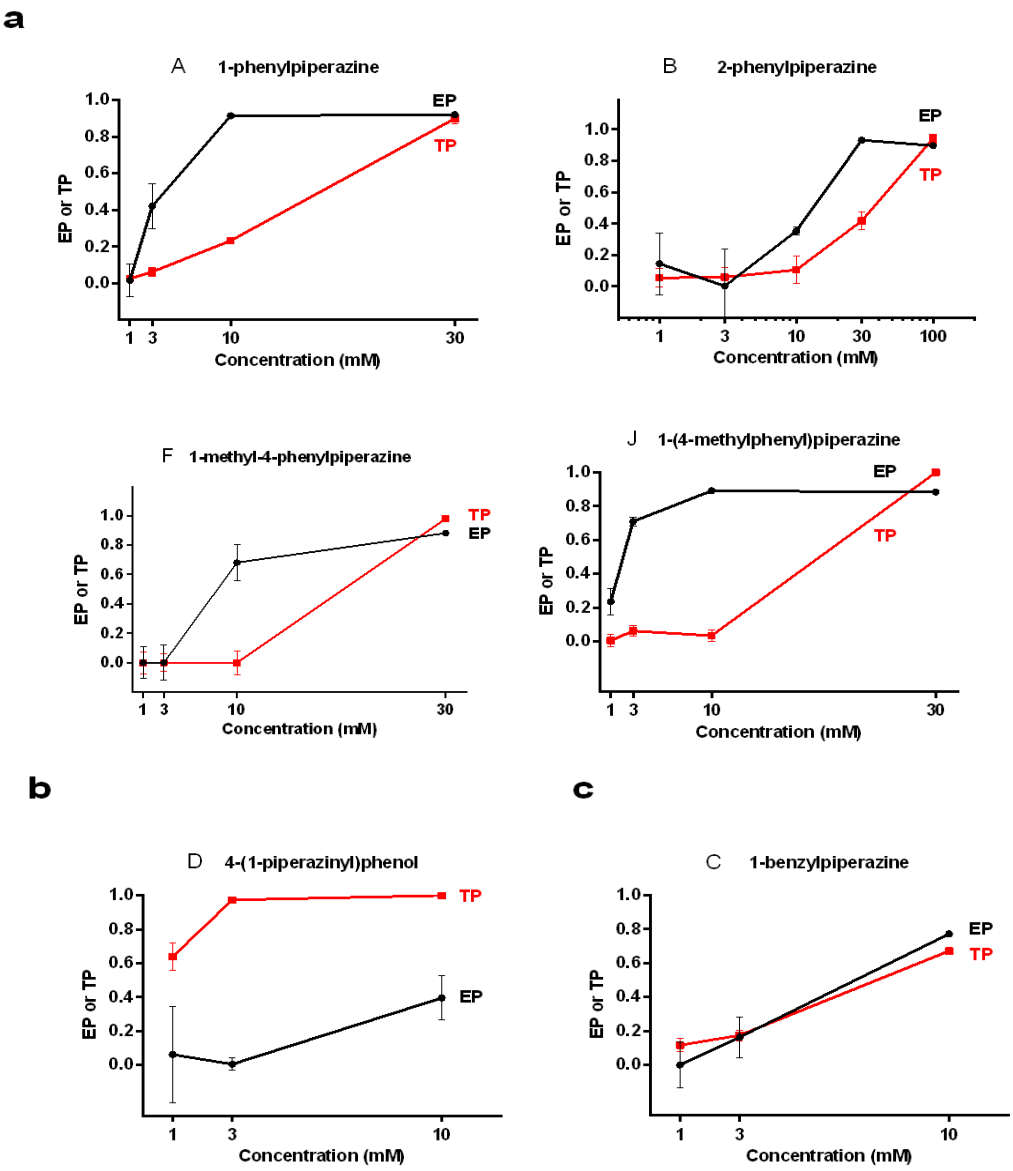

Figure 2.3: Enhancement potential (EP) and toxicity potential (TP) plotted as a function of concentration reveal enhancer "therapeutic windows." (a) Compounds A, B, F, and J had profiles where EP was greater than TP for some range of concentrations, indicating potential utility as permeation enhancers. (b) Some compounds, such as D, resulted in profile in which TP was greater than EP for all concentrations studied. (c) Compound C represents a third type of profile in which EP and TP were overlaid. Error bars represent SEM (n = 3 - 4).

2.3.5 Phenylpiperazine Derivatives Affect the Permeability of Caco-2 Monolayers

Until this point, TEER measurements was used as a surrogate measure for paracellular permeability. To further assess the effects of phenylpiperazine derivatives on the permeability of Caco-2 monolayers, experiments were conducted in which the fluorescent marker molecule, calcein, was applied to the apical side of Caco-2 monolayers in the presence of enhancer treatments. Basolateral calcein concentrations were measured as a function of time. These measurements were converted to P_{app} using equation (1.1), and the relative calcein permeability was determined for each derivative by normalizing the P_{app} of treated wells to their pre-treatment baseline permeability values and the permeability of the untreated control (Figure 2.4a).

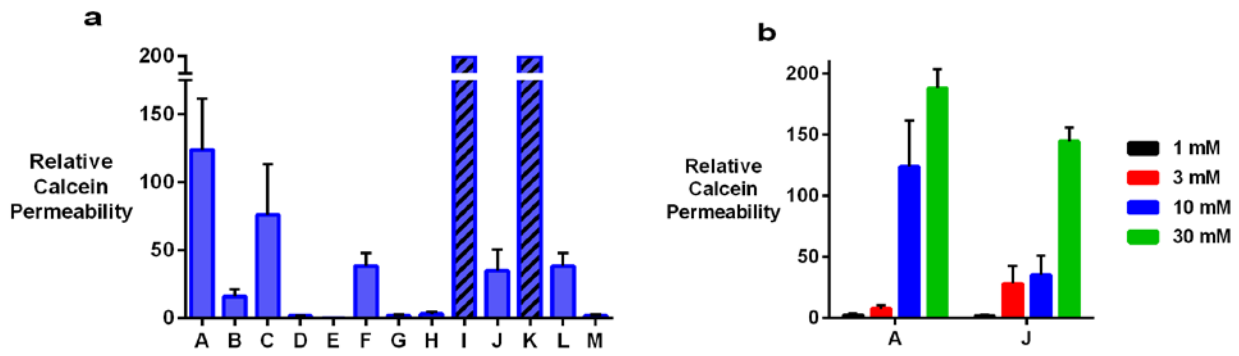

Figure 2.4: 1-phenylpiperazine and several derivatives increase the permeability of Caco-2 monolayers to the fluorescent marker calcein. (a) Relative calcein permeability across Caco-2 monolayers with 10 mM treatments was determined over the course of three hours. Derivatives I and K increased permeability to the maximum detectable fluorescence levels. Values shown are fold-increases compared to untreated monolayers. (b) Relative calcein permeability was concentration dependent for efficacious derivatives. Compounds A and J show representative increasing trends. Error bars represent SEM (n = 3).

Eight of the thirteen phenylpiperazine derivatives tested were able to increase the permeability of the Caco-2 monolayers to the paracellular flux marker calcein. Derivatives D, E, G, H, and M caused less than three-fold increases in permeability as compared to the untreated control monolayers even at the highest concentration tested (10 mM). The rest of the derivatives caused

significant increases in permeability, with several treatments achieving over 100-fold increases in calcein permeability. The highest increases in permeability were caused by 1-phenylpiperazine (A), 4-phenylpiperidine (I), and 1-(3-methylphenyl)piperazine (K) at 10 mM. Compounds I and K increased calcein permeability to the maximum detectable level, and A caused a relative permeability ~120-fold over control values.

The relative calcein permeability was concentration dependent for the derivatives that increased permeability (Figure 2.4b). This finding is consistent with the concentration dependence in EP as calculated by changes in TEER. Previously, TEER and diffusion marker permeability through Caco-2 monolayers have been shown to be strongly correlated. [45,62]

2.3.6 Confocal Microscopy of Caco-2 Monolayers Treated with Phenylpiperazines

To assess potential mechanisms of action for phenylpiperazine permeation enhancers, confocal microscopy experiments were conducted on Caco-2 monolayers. In these experiments, nuclei, F-actin, and the tight junction protein zonula occludin-1 (ZO-1) were stained. Untreated samples showed continuous ZO-1 expression with a ruffled appearance in the paracellular spaces between cells as well as semi-continuous F-actin expression that was more concentrated at the cell periphery (Figure 2.5a). In contrast, monolayers incubated with 10 mM 1-phenylpiperazine (A) for 1 hour showed extensive ZO-1 rearrangement, indicating a significant reduction of tight junction integrity (Figure 2.5b). This finding is consistent with the high EP measured for this treatment, 0.913, as measured by TEER. F-actin expression also appears to have been dramatically affected by A, with much less F-actin visible in Figure 2.5b as compared to the untreated control in Figure 2.5a.

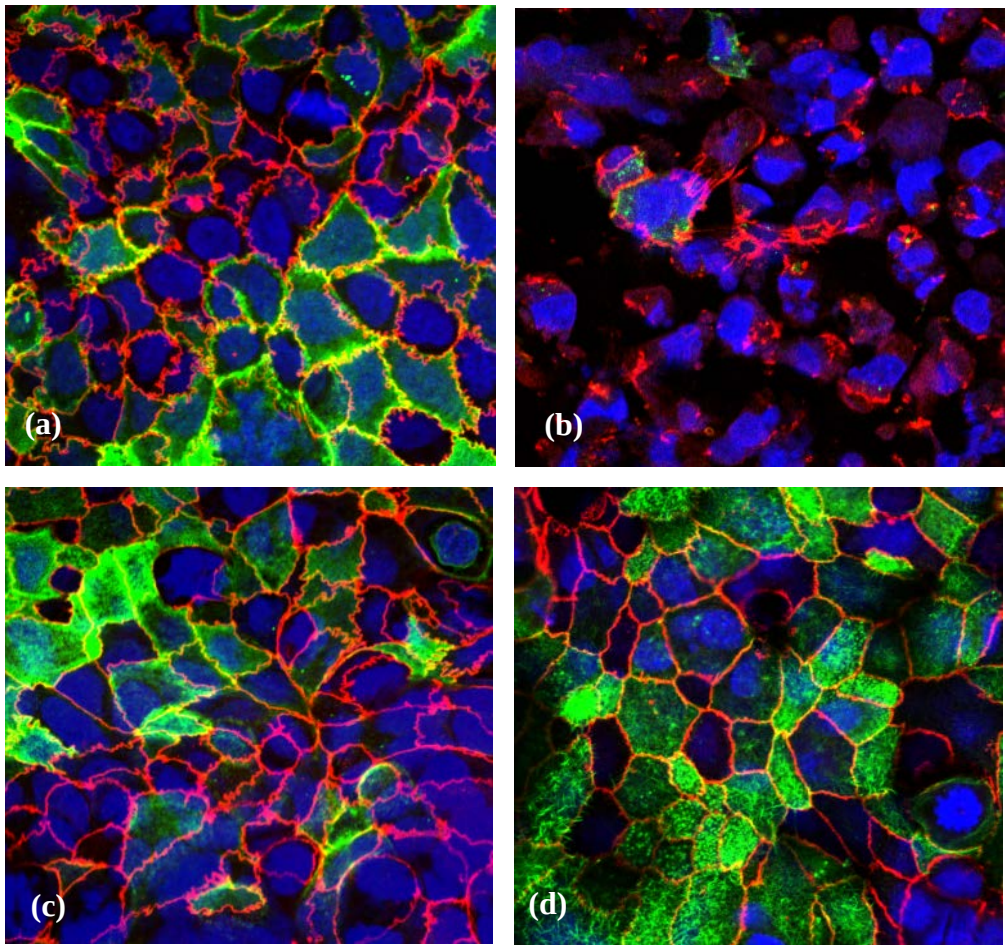

Figure 2.5: Confocal images of phenylpiperazine derivative-treated Caco-2 monolayers show rearrangement of tight junction protein ZO-1 and the cytoskeletal protein actin. Caco-2 monolayers were incubated with phenylpiperazine derivatives and stained with DAPI (blue – nuclei), AF488-phalloidin (green – F-actin), and AF594-anti-ZO-1 (red – Zonula occludin-1). (a) Untreated Caco-2 monolayers show continuous ZO-1 expression typical of intact tight junctions. (b) Treatment with10 mM 1-phenylpiperazine (compound A) resulted in ZO-1 rearrangement and a loss of typical actin expression. (c) Treatment with 10 mM compound E, which possessed minimal permeation enhancing ability, resulted in little to no ZO-1 or actin rearrangement compared to control cells. (d) 10 mM Compound F caused some ZO-1 rearrangement with smoothing around the paracellular spaces and changes in actin arrangement.

Figure 2.5c shows an image of Caco-2 monolayers incubated with 10 mM derivative E for 1 hour. 10 mM E was found to be ineffective based on TEER (EP = 0.039), which is mirrored in the similarity of the ZO-1 and F-actin expression between Figure 2.5c and the untreated control, Figure 2.5a. Caco-2 monolayers treated with 10 mM F, a phenylpiperazine derivative with moderate

efficacy (EP = 0.679) and low toxicity (TP = 0), showed nearly continuous ZO-1 expression in the paracellular spaces (Fig. 2.5d). However, the tight junctions had a smoother appearance as compared to the ruffled outline seen in the untreated control. Some F-actin rearrangement appears to have occurred as well, with more feathery, discrete areas of F-actin that appear throughout the cell (not just at the periphery). These images suggest that phenylpiperazine derivatives capable of permeation enhancement alter tight junction protein expression and induce rearrangements of F-actin when applied to Caco-2 monolayers.

2.4 Discussion

When considering challenges in the development of oral delivery systems for macromolecules, chemical permeation enhancers are a potential solution to the problem of poor intestinal absorption. While many promising candidates have been identified, concerns of toxicity remain a major barrier to the approval of drug formulations containing chemical permeation enhancers. To develop a better understanding of efficacy and cytotoxicity structure-function relationships for a promising class of permeation enhancers, 13 derivatives of 1-phenylpiperazine were examined in Caco-2 cells. Caco-2 cells cultured to mimic the intestinal epithelium are more sensitive to chemical permeation enhancers than other models such as isolated rat intestinal mucosae. As such, false positives are possible when assessing cytotoxicity, which would lead to the exclusion of piperazine-derived enhancers from further study that may actually be nontoxic *in vivo*. Similarly, false positives are also possible when determining efficacy by permeation measurements, which would lead us to recommend materials for *in vivo* study that may not be sufficiently potent. Despite these caveats, Caco-2 models are useful in that they provide an efficient means for the screening of larger arrays of compounds. Eight of the 13 derivatives were found to be effective permeation enhancers at the concentrations studied, as determined by their effect on the TEER and calcein

permeability of Caco-2 monolayers. Three were less cytotoxic than and similarly effective as 1-phenylpiperazine at 10 mM.

From this small library, 1-phenylpiperazine once again emerged as one of the best enhancers when looking at permeability increases alone; however, it has a relatively low toxicity potential of 0.23 ± 0.015 at 10 mM. In contrast, two other derivatives stood out from the group when both permeability increases and toxicity were considered. Derivatives F and J both caused significant improvements in calcein permeability, 38- and 35-fold respectively, but notably, they have toxicity potentials much lower than that of 1-phenylpiperazine at 10 mM (TP = 0 ± 0.040, 0.03 ± 0.033 respectively). This characteristic of improved efficacy with low toxicity is also seen in their therapeutic windows, which are large compared to other derivatives, and indicate that these compounds warrant further study as permeation enhancers.

The phenylpiperazine derivatives in this library were generally more effective at lower concentrations compared to the piperazine derivatives with aliphatic substitutions examined by Lamson, et al.[62] In that study, only one piperazine derivative, 1-isopropylpiperazine, had an OP greater than 0.5 (0.72 ± 0.16) at the lowest concentration tested of 10 mM. At the same concentration, compounds A, F, and J from this study had OP values greater than 0.5, and at a lower concentration of 3 mM, compounds J, K, and L had OP values greater than 0.5. The aliphatic piperazines library reached their maximum OP at 30 mM, with several derivatives having overall potentials of 1.0 at 30 mM, whereas for the phenylpiperazine derivatives here, the maximum OP occurred at 10 mM. This difference can be primarily attributed to the higher cytotoxicity induced by phenylpiperazine derivatives at higher concentrations. Together, these libraries showcase the wide range of permeation enhancing activity possible in the piperazine family.

Structure-function relationships can powerfully inform drug formulation, and while a study of such a small library is unlikely to yield predictive relationships, the close comparison of such chemically similar compounds can identify trends that should be examined further. In particular, this cohort of phenylpiperazine derivatives indicated that there may be a correlation between the degree of amine substitution and the toxicity of a chemical permeation enhancer. The two phenylpiperazine derivatives containing primary amines, E and G, were among the most toxic of the compounds studied, with TP > 0.8 at 10 mM. The median toxicity potential for derivatives containing at least one secondary amine at 10 mM was 0.74, whereas the two derivatives with no primary or secondary amines (F and H) were the only ones to have a TP of 0 at 10 mM. Of these two compounds, 1-methyl-4-phenylpiperazine was much more effective (EP = 0.679 ± 0.071) than 1-phenylpiperidine (EP = 0.250 ± 0.159). Substitution at both the 1 and 4 positions of the piperazine ring was previously found to correspond with improved tolerance by Caco-2 cells in a study of piperazines with simple hydrocarbon substitutions. [62]

Another indication of the importance of amine substitution is seen in the comparison of 1-phenylpiperazine and two of its closest non-piperazine derivatives. At 10 mM, neither compound H nor I were effective permeation enhancers (OP = 0.250 ± 0.172, 0 ± 0.004, respectively). Both are piperidines, having only one nitrogen in their non-aromatic ring. 1-phenylpiperidine (H) was both ineffective and non-toxic at 10 mM. In contrast, 4-phenylpiperidine (I) had both a high EP and a high TP, which suggests that perhaps its permeation enhancing effect comes not from modulating tight junctions but from reducing monolayer integrity by inducing cell death. Comparing these structures and efficacies to 1-phenylpiperazine (OP = 0.681 ± 0.015), which is highly effective and less toxic at 10 mM, suggests that the piperazine ring with its two amines may be necessary for permeation enhancement resulting from a mechanism other than cell death.

Seven derivatives in this library contained substitutions on the aromatic ring, and four of these had substitutions other than methyl groups. Some clear differences emerged between the toxicities associated with these different substitutions. Compound D had a hydroxyl group at the 4 position, and compounds E and G contained primary amines at the 4 position. These three derivatives were among the most toxic of the derivatives studied, each having TP > 0.8 at 10 mM, whereas J, which had a methyl group at the 4 position had an insignificant toxicity potential of 0.033 ± 0.034 at 10 mM. An interesting difference also appeared between derivatives J, K, and L, which had the structure of 1-phenylpiperazine with a methyl group added to the aromatic ring at the 4, 3, or 2 position, respectively. All three had at least one concentration at which EP was significantly greater than TP; however, K and L had large increases in TP from 3 mM to 10 mM, while J did not. The dramatic cytotoxicity differences between very similar phenylpiperazine derivatives underscore the general sensitivity of biological membranes to small changes in molecular structure, and suggest that the search for safe and effective permeation enhancers could benefit from more thorough examinations of molecular families with enhancement potential.

Finally, it is important to note that piperazines are a pharmacologically active class of molecules,[70] and cytotoxicity as measured in Caco-2 cells does not ensure safe use *in vivo*. However, low toxicity *in vitro* suggests that phenylpiperazines may be safe their effect is limited to intestinal epithelial tissue. Thus, when considering therapeutic applications, we envision that piperazine-derived permeation enhancers would be incorporated into a localized drug delivery device, such as intestinal patches,[37] to limit systemic exposure.

2.5 Conclusion

The oral delivery of macromolecular drugs like proteins has the potential to improve compliance and medical outcomes for the many patients currently prescribed injectable protein therapeutics.

To achieve oral protein delivery, the two major barriers of drug degradation in the upper gastrointestinal tract and poor absorption across the intestinal barrier need to be addressed. The use of chemical permeation enhancers is a promising approach to improve the transport of macromolecules across the intestinal epithelium. The balance of efficacy and toxicity is paramount, and the trends identified here will inform further study of 1-phenylpiperazine and its derivatives as intestinal permeation enhancers for their potential incorporation into a drug delivery vehicle capable of systemic protein delivery. The addition of methyl groups to the 1-position in the piperazine ring or the 4-position in the aromatic ring was found to decrease toxicity while maintaining impressive levels of permeation enhancement as indicated by TEER and calcein permeability of Caco-2 monolayers. Confocal imaging indicated that phenylpiperazine derivatives capable of enhancing permeability do so by acting on tight junction and cytoskeletal proteins. Finally, the large differences in enhancement and toxicity potential between chemically similar compounds indicate that the intestinal epithelium is a typically sensitive biological membrane. Attempts to modulate its permeability in a therapeutic manner will benefit from detailed examinations of molecular families in which intestinal permeation enhancers have already been identified.

3 Intestinal permeation enhancers enable oral delivery of macromolecules up to 70 kDa in size

3.1 Introduction

Permeation enhancers have been studied for decades in the grand attempt to deliver protein drugs orally, but only recently have any made it past regulatory hurdles to become FDA-approved.[40] These approved permeation enhancers are very limited in terms of their chemical class, mostly consisting of surfactants that form spontaneous emulsions or medium chain fatty acid derivatives such as salcaprozate sodium (SNAC).[71] The first protein drugs that have been incorporated into formulations with permeation enhancers are fairly small peptides like octreotide (1 kDa) and semaglutide (4.1 kDa).[50,72] It remains to be seen how permeation enhancers will fair with larger molecules including insulin (5.7 kDa) and the many antibodies used to treat diseases ranging from cancer to rheumatoid arthritis (~150 kDa).

In this study, FITC-dextrans were used as model macromolecules to study how two permeation enhancers previously identified as good enhancers of small molecule permeability *in vitro* and *ex vivo*, 1-phenylpiperazine (PPZ) and sodium deoxycholate (SDC), would fare as enhancers for larger molecules.[45,60,63,64,73] While FITC-dextrans do not perfectly emulate proteins, they are good model molecules because they are relatively inexpensive, available in a variety of macromolecular weights, and non-digestible. The latter property ensures that the environment of the gut, which includes degradative enzymes and denaturing acidic conditions, will not impact the molecule's stability during the study. PPZ and SDC were evaluated for their ability to improve the absorption of FITC-dextrans from 4-70 kDa in three different models of oral drug delivery: Caco-2 monolayers (*in vitro),* intestinal injections, and oral gavage (*in vivo*-mice). These experiments

provide guidance on the upper bound of drug size that can benefit from these two permeation enhancers.

3.2 Materials and Methods

3.2.1 Materials

FITC-Dextrans (4, 10, 40, 70, 150 kDa), sodium deoxycholate, 1-phenylpiperazine, sodium bicarbonate, and N-acetylcysteine were purchased from Millipore Sigma (Burlington, MA). Insulin syringes (29 G), needles, serum collection tubes, Dulbecco's modified Eagles medium (DMEM), Hank's Balanced Salt Solution (HBSS), Falcon 225 cm^2 tissue culture flasks, Corning BioCoat^{TM} HTS 1.0 µm porous support Transwell plates, Falcon 24-well plates, sodium butyrate, Amphotericin B, and MITO+ serum extender were purchased from VWR (Radnor, PA). Phosphate buffered saline, penicillin/streptomycin, trypsin-EDTA, and fetal bovine serum (FBS) were purchased from Thermo Fisher (Waltham, MA). Isoflurane was purchased from Henry Schein (Melville, NY). Flexible plastic oral gavage needles were purchased from Instech Laboratories, Inc. (Plymouth Meeting, PA). Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA).

3.2.2 Caco-2 Cell Culture

Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 10 IU/mL of penicillin, 0.1 mg/mL of streptomycin, and 1 µg/mL Amphotericin B. Cultures were incubated in a 5% CO_2 environment at 37°C. The cells were subcultured using 0.25% trypsin-EDTA, with cells at passage numbers 25-55 used for experiments. For transepithelial electrical resistance (TEER) experiments, cells were seeded on BioCoat^{TM} HTS membranes at a density of 2×10^5 cells per well and incubated in basal seeding medium (BSM) for two days. BSM consisted of DMEM supplemented

with MITO+ serum extender according to the manufacturer's instructions and 1 µg/mL Amphotericin B. Then the media was changed to enterocyte differentiation medium (EDM) and incubated for two more days. EDM comprised DMEM supplemented with MITO+ serum extender, 1 µg/mL Amphotericin B, and 2 mM sodium butyrate. Monolayer formation was confirmed by measuring the trans-epithelial electrical resistance (TEER), and only wells that had developed monolayers with TEER values of 200 Ω cm^2 or higher were used in experiments.

3.2.3 *In vitro* Permeability Experiments

Monolayers were transferred into transport buffer (HBSS with 12.5 mM glucose and 25 mM HEPES) and allowed to equilibrate at 37°C for one hour before the permeability experiments began. The FITC-dextrans were dissolved in transport buffer at a concentration of 10 mg/mL, and PPZ or SDC was added for the treatment groups, and the solutions were applied to the apical side of the Caco-2 monolayers. At 1 and 2 hours after treatment began, samples were taken from the basolateral side of the monolayers. The fluorescence of these samples at 490/520 nm was measured using a BioTek Synergy2 plate reader. Individual calibration curves were made for each combination of dextran size and treatment and used to convert fluorescence measurements into the amount of FITC-dextran transferred across the monolayer, which was used to calculate the apparent permeability of calcein across the monolayer:

$$P_{app} = \frac{\Delta M}{C_a A \Delta t} \qquad \text{(Eq. 3.1)}$$

where P_{app} is the apparent permeability, ΔM is the mass of FITC-dextran transported into the basolateral compartment, C_a is the concentration of FITC-dextran in the apical well, A is the area of the monolayer, and Δt is the length of time the treatment was applied to the monolayer.

3.2.4 Animal Care and Use

Animal protocols were approved by the Institutional Care and Use Committee at Carnegie Mellon University (Pittsburgh, PA), and all experiments were performed in accordance with protocol PROTO201600017 as well as all institutional, local, and federal regulations. Male C57/BL6 mice were obtained from an institutionally managed breeding colony. Mice were housed in standard cages with a 12 hour light/dark cycle and free access to water and food. Mice were 14-30 weeks old for all studies to ensure the minimum size necessary for procedures involving anesthesia and the collection of multiple blood samples (>30 g).

3.2.5 Intestinal Injections of FITC-Dextrans and Permeation Enhancers

Mice were fasted for 12 hours, after which an initial blood sample was collected from the submandibular vein. Then mice were anesthetized using vaporized isoflurane, and their intestines were surgically exposed. Solutions containing PBS, 3.2% (v/v) PPZ, or 10% (w/v) SDC and one of the five different-sized FITC-dextrans were injected into the duodenum using a 29 G syringe with a dose of 2 µL/g. FITC-dextran solutions were a constant molar concentration of 1.25 mM (equivalent doses expressed in mg/kg: 10 mg/kg FD4, 25 mg/kg FD10, 100 mg/kg FD40, 175 mg/kg FD70). The surgical incisions were closed and secured with tissue adhesive. They were kept under anesthesia for 3 hours after injection using a heating pad to maintain their body temperature. Blood was collected from the submandibular vein 30 min and 90 min after injection. The mice were kept anesthetized until they were sacrificed by cervical dislocation after 3 hours. The final blood sample was collected by cardiac puncture. Blood was collected in serum tubes and centrifuged at 13,000 RPM for 10 minutes to isolate the serum. 10 µL of serum was diluted 1:10 with PBS in black 96-well plates, and fluorescence was measured on a BioTEK Synergy H1 plate reader at 490 nm excitation and 520 nm emission wavelengths. Separate calibration curves were

prepared for each size of FITC-dextran in PBS, PPZ, and SDC to account for any possible influence of the permeation enhancers or dextran size on their fluorescence spectra. The blank fluorescence value of serum from the initial blood sample was subtracted from each measurement to account for autofluorescence of biological materials at these wavelengths.

3.2.6 Oral Gavage of FITC-Dextrans and Permeation Enhancers

Mice were fasted for 12 hours, after which an initial blood sample was collected from the submandibular vein. Flexible plastic oral gavage needles were used to administer all oral gavages in these experiments. Mice were gavaged with 10 µL/g of solutions containing PBS, 0.6% (v/v) PPZ, or 2% (w/v) SDC and one of the five different-sized FITC-dextrans. In the oral gavage experiments, the dose of FITC-dextran was kept constant at 200 mg/kg (20 mg/mL). Blood was collected 3 hours after dosing. Blood was collected and analyzed as described in the previous section. For the experiments containing additional excipients, mice receiving sodium bicarbonate as part of the treatment received 100 µL of 10% sodium bicarbonate solution 15 minutes prior to the FITC-dextran/permeation enhancer solution, and mice receiving N-acetylcysteine (NAC) had 5% (w/v) added to the solutions of FITC-dextran and permeation enhancer.

3.2.7 Statistics

All data are presented as the mean with error bars representing the standard error of mean (SEM). Statistical significance was determined by two-tailed, unpaired Student’s t-tests or one-way ANOVA performed in GraphPad Prism 8.

3.3 Results

3.3.1 PPZ and SDC increase the permeability of macromolecules *in vitro*

Caco-2 monolayers were used to assess how PPZ and SDC affect the permeability of FITC-dextrans in a range of sizes (4-70 kDa). There was no relationship between apparent permeability and molecular weight for the untreated control, but trends with molecular size appeared in the groups treated with 0.1% PPZ or 0.05% SDC. As seen in Figure 3.1, PPZ caused a nearly 10-fold increase in P_{app} for FD4 and a 2-fold increase in P_{app} for FD10. However, it did not significantly increase the permeability of the larger FITC-dextrans (40 and 70 kDa). The effect of SDC was also limited to the two smaller sizes, with a nearly 100-fold increase in the permeability of FD4 and a 10-fold increase for FD10, but no changes for FD40 or FD70.

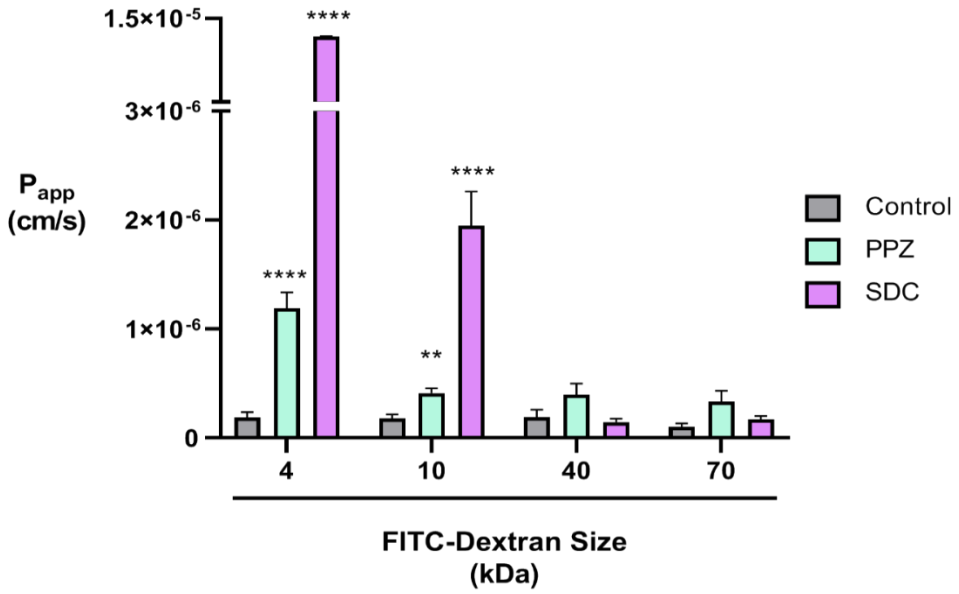

Figure 3.1: PPZ and SDC increase the Caco-2 intestinal cell monolayer permeability of FITC-dextrans in a size-dependent fashion. PPZ significantly increased the permeability of FD4 ($p < 0.0001$) and FD10 ($p < 0.01$) but did not significantly increase that of FD40 or FD70. Likewise, SDC significantly increased the permeability of FD4 ($p < 0.0001$) and FD10 ($p < 0.0001$) but did not affect the permeability of FD40 and FD70. Significance determined by unpaired, two-tailed Student's t-test. n = 3-12, error bars represent SEM.

3.3.2 PPZ and SDC increase the intestinal permeability of macromolecules after direct injection into the small intestine

Following from the promising results *in vitro*, PPZ and SDC were tested *in vivo* to determine if they function as macromolecular permeation enhancers. Mice were anesthetized, and their intestines were surgically exposed. Solutions of PPZ or SDC mixed with one of the sizes of FITC-dextran were injected into the duodenum, and blood samples were taken over the next two hours. Corresponding sizes of FITC-dextran dissolved in PBS alone served as the untreated controls.

Because SDC is a bile salt that is naturally found in the intestine, acute toxicity was not a major concern, and a high dose of 200 mg/kg (corresponding to a concentration of 10% (w/v)) was chosen for these experiments. In the human intestine, bile salt concentration varies widely between individuals and between fed and fasted states for a single person, with fed-state values falling between 0.74-86.14 mM in one study of human volunteers.[74] The 10% concentration used here is equivalent to ~240 mM, but this is for a small volume bolus, which would be diluted in the larger volume of the intestine after administration. According to the MSDS, the oral LD50 for SDC in mice is over 1 g/kg, which is 5-fold higher than the dose used in this experiment. For PPZ, concentration was more of a concern, as the oral LD_{50} in rats is published as 210 mg/kg, giving a much smaller window in which to operate. Thus, a preliminary experiment using only FD4 and two different concentrations of PPZ was performed. As seen in Figure 3.2a, 13 mg/kg PPZ (0.5% (v/v)) was not effective at increasing the amount of FD4 absorbed into the bloodstream after intestinal injection, but 65 mg/kg PPZ (3% (v/v)) caused a statistically significant 10-fold increase.

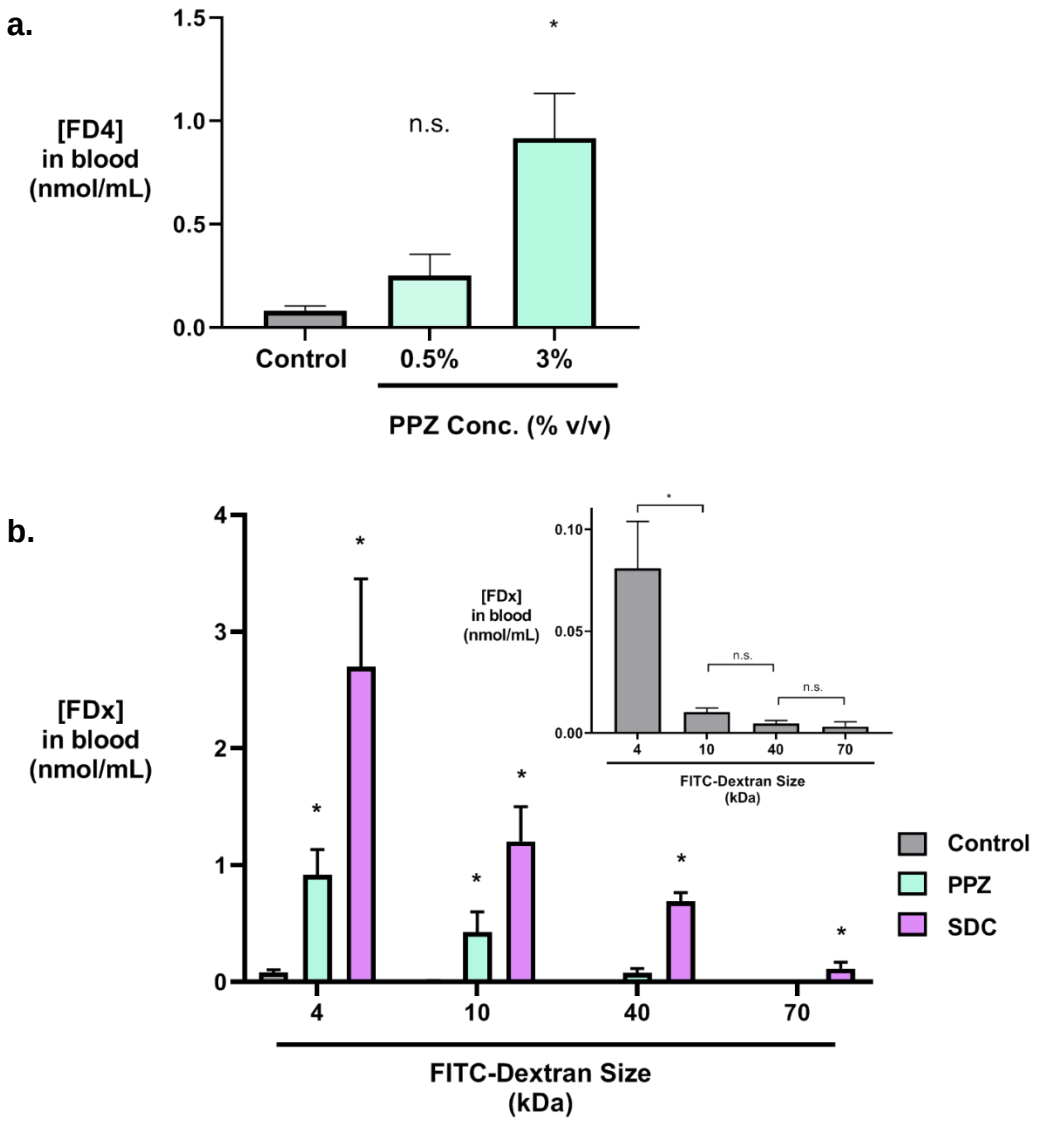

Figure 3.2: PPZ and SDC enhance the permeability of different sized FITC-dextrans in mice when delivered by intestinal injection. (a) PPZ causes dose-dependent increases in the permeability of FD4 when delivered via intestinal injection. 0.5% PPZ does not produce a statistically significant increase in the concentration of FD4 in the blood 2 hours after injection, but increasing the dose of PPZ to 3% does cause a significant increase compared to the control of FD4 injected with just PBS. *$p < 0.05$ by unpaired, two-tailed Student's t-test. n = 3-5, error bars represent SEM. **(b)** PPZ causes statistically significant increases in the blood concentration of FD4 and FD10 (both have $p < 0.05$ compared to control). SDC increases the permeability of all four sizes of FITC-dextrans tested (all p values less than 0.05 compared to untreated control). Significance determined by unpaired, two-tailed Student's t-tests. n = 3-6, error bars represent SEM. **(inset)** Inset shows untreated control data on a different y-axis scale so all bars are visible.

After the concentrations were chosen, intestinal injections were performed using four different sizes of FITC-dextrans (4, 10, 40, and 70 kDa). See Figure 3.2b. Similarly to the *in vitro* experiments (Figure 3.1), PPZ caused a statistically significant increase in the absorption of FD4 and FD10, but was ineffective for the two larger sizes of FITC-dextran that were tested. In contrast, SDC significantly improved the intestinal permeability of all four sizes of FITC-dextran. Interestingly, a trend in permeability versus dextran molecular weight emerged from the untreated control in the intestinal injection experiments, unlike in the Caco-2 monolayers (see inset of Figure 3.2b).

3.3.3 PPZ requires an additional excipient to function as a permeation enhancer when delivered by oral gavage

After finding that PPZ and SDC both enhance the intestinal permeability of macromolecules after direct injection into the small intestine, we performed a set of experiments in which we evaluated the same compounds but changed the delivery method to oral gavage. Oral gavage better approximates the actual physiology of oral delivery, as it includes passage through the stomach unlike surgical injection into the intestine. For these experiments, the concentrations of PPZ and SDC in the solutions were 5-fold less than those used for the intestinal injections. However, because the delivered volume was increased from 2 µL/g to 10 µL/g, the doses were equivalent.

After oral gavage of solutions containing FITC-dextrans with PPZ or SDC, only SDC caused significant increases in intestinal permeability (Figure 3.3a). Similarly to the intestinal injection experiments, mice treated with SDC had significantly higher concentrations of FITC-dextrans in their blood three hours after receiving the oral gavage. SDC was effective at increasing the absorption of all four tested sizes of FITC-dextran. In contrast, PPZ did not improve the absorption of any of the FITC-dextrans.

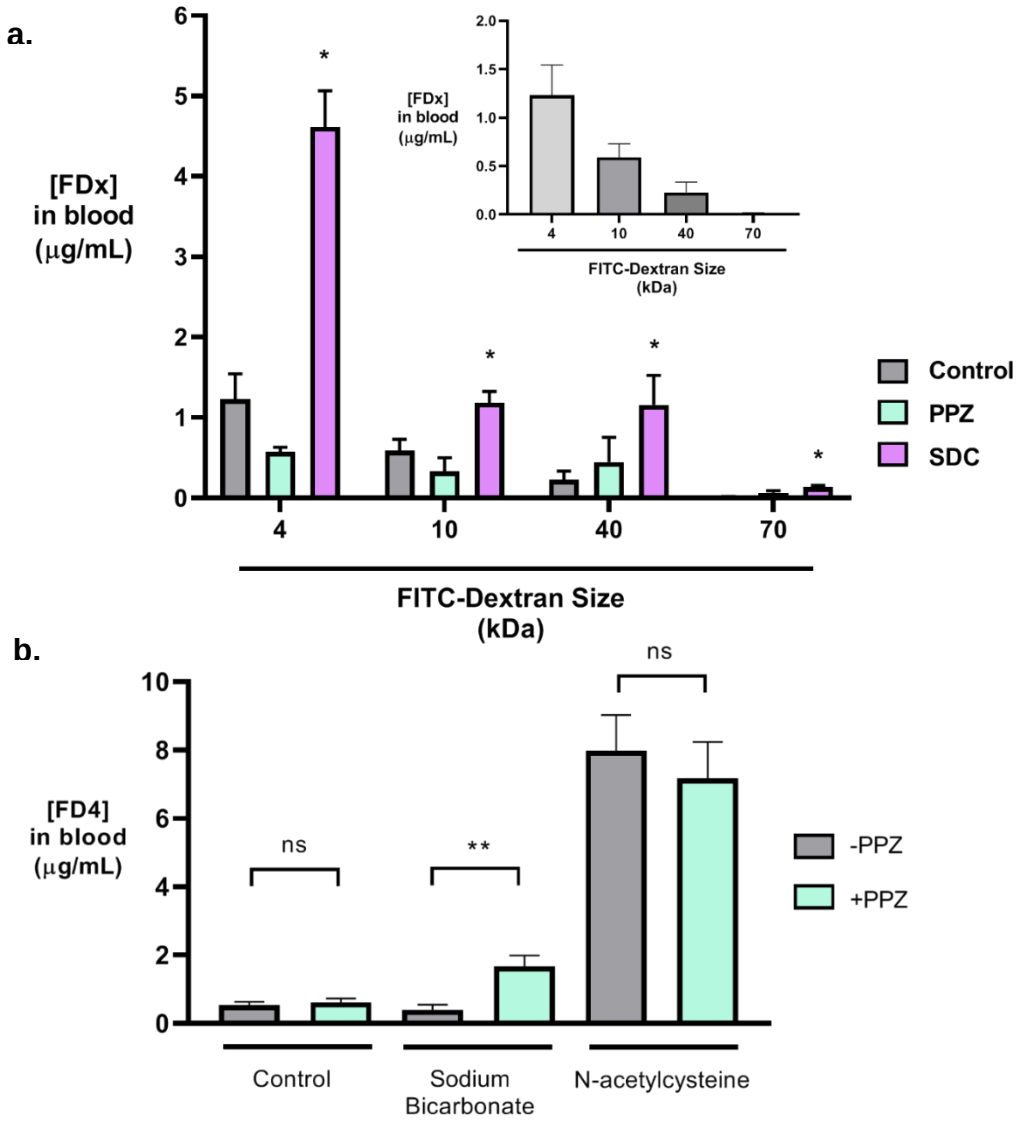

Figure 3.3: PPZ is an effective permeation enhancer by oral gavage when mice are pretreated with an oral gavage of sodium bicarbonate solution. (a) SDC produces significant increases in the concentration of FD4, FD10, FD40, and FD70 after oral gavage (*$p < 0.05$ by unpaired, two-tailed Student's t-test). PPZ does not cause any significant changes to the absorption of FITC-dextran when administered orally. **(inset)** Inset shows the data from the untreated control group on a modified y-axis so that all bars are visible. n = 4-5, error bars represent SEM. **(b)** PPZ alone does not increase FD4 absorption after oral gavage. Pre-treatment with sodium bicarbonate solution makes PPZ an effective permeation enhancer. The mice treated with PPZ and sodium bicarbonate had a significantly higher blood concentration of FD4 than those receiving just sodium bicarbonate and FD4 (**$p < 0.01$ by two-tailed, unpaired Student's t-test. N-acetylcysteine co-delivered with FD4 causes an increase in FD4 absorption compared to the untreated control but does not enhance the action of PPZ. n = 5, error bars represent SEM.

There are several potential reasons for the loss in PPZ efficacy after oral gavage compared to direct intestinal injection. It is possible that PPZ is affected by the low pH of the stomach or unable to penetrate the intestinal mucus layer when it is not injected in a single large bolus in the intestine. To account for these possibilities, additional excipients were added to the oral gavage procedure in a follow-up experiment with FD4. One group of mice received an oral gavage of 10% sodium bicarbonate solution 15 minutes before the oral gavage of FD4 and PPZ. A control group of mice receiving the sodium bicarbonate followed by FD4 with no PPZ was included. Sodium bicarbonate would increase the pH of the stomach and possibly negate any effect of the stomach acid on PPZ. Another group of mice received a solution of FD4 and PPZ with N-acetylcysteine (NAC) added to it. NAC is a mucolytic compound that is known to loosen the mucus layer in the intestinal epithelium.[35]

As seen in Figure 3.3b, the pre-treatment with sodium bicarbonate significantly improved the permeation enhancing action of PPZ. Mice receiving sodium bicarbonate followed by PPZ had a 4-fold higher concentration than mice receiving just sodium bicarbonate or no treatment at all. Interestingly, NAC dramatically increased the absorption of FD4 regardless of whether PPZ was included or not. This compound clearly deserves further exploration as a permeation enhancer but is not an effective excipient to improve the action of PPZ.

3.4 Discussion

Both PPZ and SDC were found to be effective permeation enhancers for macromolecules *in vitro*. They both caused significant increases in P_{app} of FD4 and FD10 in Caco-2 monolayers but did not impact the permeability of FD40 and FD70. When transitioning to *in vivo* models of the intestine, we began with direct injection of the working solutions into surgically exposed intestines of anesthetized mice. This eliminates the extra variable of how the permeation enhancer and dextran

solutions travel through the stomach by delivering them in one bolus directly into the duodenum. In this model, both PPZ and SDC were once again effective permeation enhancers. In this *in vivo* model, there was a difference in their performance, in that SDC was able to significantly increase the absorption of all four tested dextran sizes (up to 70 kDa), but PPZ was once again only effective for FD4 and FD10. This difference may be attributed to the different mechanisms by which these two permeation enhancers act.

PPZ is known to be a paracellular permeation enhancer, meaning that it increases intestinal permeability by affecting the tight junction protein complexes that hold the epithelial cells together.[9,63,73,75] In contrast, SDC is able to fluidize the lipid membrane of the epithelial cells so that cargo can cross through the epithelial cells themselves, making it a transcellular permeation enhancer.[9,60,61] In the case of a paracellular permeation enhancer, there is an additional constraint to the permeation process in that while the tight junctions may loosen when PPZ is added, the size of the paracellular space, or the gaps between the epithelial cells, may not increase. The dimensions of the paracellular space are reported as being on the order of 5-10 Å in diameter for the pores formed by tight junction proteins and 100 Å in diameter for the entire space between the epithelial cells.[76] The hydrodynamic diameters of FITC-dextrans increase with molecular weight, from FD4 with a reported diameter of 28 Å up to 120 Å for FD70. It is plausible that tight junction loosening may not be an effective mechanism for delivering very large molecular cargos.[77] SDC may benefit from the fact that fluidization of the lipid membrane may not have such strict constraints on the sizes of molecules that are transported. The possible size limitations of paracellular permeation enhancers do not inherently limit their potential for oral protein drug delivery because for some protein drugs that may be very sensitive to chemical degradation, it might be beneficial that they could be transported through the tight junctions and avoid the enzyme-rich cytosol of the epithelial

cells. Please see Section 7.3 for supplementary data from a small pilot study comparing the intestinal permeability of FITC-dextrans versus fluorescently-labeled proteins. Further experiments will be necessary to elucidate these relationships between macromolecular properties and permeation enhancement, and it is possible that choosing a permeation enhancer for a drug formulation may end up being a process that must be investigated and optimized for each protein individually.

Another interesting finding of this work was that PPZ on its own was completely ineffective when delivered via oral gavage. We hypothesized that this was due to one of the physiological barriers that it encounters after oral gavage but did not have to overcome when it was directly injected into the intestine. The acidity of the stomach and the mucus barrier in the intestine were two such barriers for which common excipients for oral formulations could be added to compensate and hopefully improve the performance of PPZ. The pH of the stomach is 1-2, meaning that it is very acidic, which could cause PPZ with a pKa of 8.71 to be protonated as it passes through, possibly rendering it ineffective. To address this, mice were orally gavaged with a 10% (w/v) solution of sodium bicarbonate 15 minutes prior to the oral gavage of FD4 and PPZ. Pre-treatment with sodium bicarbonate is commonly used in mouse models of infectious disease when bacteria challenges need to be delivered orally without losing viability in the acidic environment of the stomach.[78,79] It temporarily increases the pH of the stomach, and in the case of PPZ, it significantly improves the permeation enhancement of FD4.

The second possible issue preventing the action of PPZ after oral gavage was the intestinal mucus layer, which we attempted to address by adding the well-known mucolytic, N-acetylcysteine (NAC) to the solution of FD4 and PPZ.[35,80,81] Interestingly, NAC was able to significantly increase the permeability of FD4 even without PPZ present, suggesting that it is itself a permeation

enhancer. It did not augment the performance of PPZ, but future investigations into the efficacy of NAC may be warranted.

A final interesting finding from these experiments is that macromolecular size impacted permeability in the untreated control groups differently in the three models used. In Caco-2 monolayers, there was no apparent relationship between molecular weight and P_{app}, but in both the intestinal injection and oral gavage data, the larger sizes of FITC-dextran were significantly less permeable than the smaller sizes. The discrepancy between the *in vitro* and *in vivo* models used in this study may be attributed to the fact that it is well-known that Caco-2 monolayers (125-250 Ω-cm^2) are much less permeable than actual intestinal tissue (60-115 Ω-cm^2 for rabbit small intestine).[14] Using trans-epithelial electrical resistance (TEER) as a surrogate for permeability, comparing published values for Caco-2 monolayers to those for small intestine, it becomes clear that Caco-2 cells are not an ideal model for studies investigating size dependence. However, they are a convenient model to screen potential permeation enhancers before moving to *in vivo* experiments. Other cell lines have been developed that have TEER values that are more physiologically relevant, including IPEC-J2 cells (derived from neonatal pig jejunal tissue).[66,82] These may be more predictive, but they are not yet standard in the literature.

3.5 Conclusion

This work represents the first *in vivo* evaluation of PPZ as an intestinal permeation enhancer, and the first study of how macromolecular size impacts permeation enhancer efficacy. PPZ and SDC were used as examples of a paracellular permeation enhancer and a transcellular permeation enhancer, respectively. It was found that both enhancers increased the transport of FITC-dextrans of multiple sizes (4-70 kDa) in Caco-2 monolayers as well as after injection into the small intestine. SDC significantly improved the permeability of dextrans up to 70 kDa after oral gavage. In

contrast, PPZ required the addition of a sodium bicarbonate pre-treatment to neutralize the stomach acid to be effective as an oral permeation enhancer. Encapsulation in enteric-coated capsules may be able to protect both PPZ and the macromolecular drug cargo from the harsh environment of the stomach, and this strategy would allow the replacement of the FITC-dextrans with actual protein drugs to better simulate the complex problem of oral protein drug delivery.

4 Long-term daily oral administration of intestinal permeation enhancers is safe and effective in mice

4.1 Introduction

In this study, we set out to interrogate the effects of long-term permeation enhancer use with a longitudinal study in mice using three chemical permeation enhancers delivered via oral gavage every day for thirty days. We chose to use phenylpiperazine (PPZ), a novel permeation enhancer studied previously by our group, which we and others have found to improve paracellular permeation by tight junction rearrangement. We also used sodium deoxycholate (SDC), which is a bile salt that acts by a combination of membrane fluidization and tight junction rearrangement, but has not made the clinical progress of other bile salts. We also included C_{10} to provide a point of comparison for the other two permeation enhancers, as there is extensive literature on its safety and efficacy, including studies in humans. The goal of this work is to determine if repeated oral dosing of permeation enhancers has permanent effects on the intestine and if the mechanism of the enhancer affects the efficacy and safety profiles *in vivo*.

4.2 Methods

4.2.1 Materials

Phosphate buffered saline (PBS), AlexaFluor-antibody conjugates, DAPI, Fluoromount-G™ and primers were purchased from ThermoFisher (Waltham, MA). IL-6, TNF-α, and zonulin ELISA kits were purchased from Abcam (Cambridge, UK). Hemoccult Guaiac Fecal Occult Blood Test slides, serum collection tubes were purchased from VWR (Radnor, PA). FITC-Dextrans, PPZ, SDC, and C_{10} were purchased from Millipore Sigma (Burlington, MA). Plastic oral gavage needles were purchased from Instech Laboratories, Inc. (Plymouth Meeting, PA).

4.2.2 Animal Care and Use

Animal protocols were approved by the Institutional Care and Use Committee at Carnegie Mellon University (Pittsburgh, PA) and all experiments were performed in accordance with protocol PROTO201600017 as well as all institutional, local, and federal regulations. Female C57/BL6 mice aged 10 weeks were purchased from Charles River Laboratories and acclimated to facility conditions for four weeks before the study began. Mice were housed in standard cages with a 12 hour light/dark cycle and free access to water and food.

4.2.3 One Day Time Point Permeability Study

Mice were fasted for 12 hours prior to the start of the experiment in cages with fasting grates and no food or bedding but free access to water. Negative control mice were orally gavaged with 600 mg/kg 4 kDa FITC-Dextran (FD4) dissolved in PBS. Treated mice received either 60 mg/kg 1-phenylpiperazine (PPZ), 200 mg/kg sodium deoxycholate (SDC), or 390 mg/kg sodium caprate (C_{10}) dissolved in PBS in addition to 600 mg/kg 4 kDa FD4. Blood samples were taken from the submandibular vein at 0, 0.5, 1, 1.5, 2, and 3 hours post-gavage. Blood was collected in serum tubes (VWR, Radnor, PA) and centrifuged at 13,000 RPM for 10 minutes to isolate the serum. 10 µL of serum was diluted 1:10 with PBS in black 96-well plates, and fluorescence was measured on a BioTEK Synergy H1 plate reader at 490 nm excitation and 520 nm emission wavelengths. A calibration curve of FD4 was prepared for each experiment to calculate the concentration of FD4 in the blood. A blank fluorescence value of serum from an untreated mouse (not one of the subjects of this study) was subtracted from each measurement to account for autofluorescence of biological materials at these wavelengths. After 3 hours, mice were sacrificed, and the small intestine and colon were dissected, and tissue samples were collected for RNA extraction (see section on qRT-PCR).

4.2.4 Baseline Permeability Measurement

One week before the study began, baseline intestinal permeability was measured as follows. Mice were fasted for 12 hours prior in cages with fasting grates and no food or bedding but free access to water. Then, mice were orally gavaged with 600 mg/kg FD4. After three hours, blood was taken from the submandibular vein and mice were returned to standard cages and fasting was ended. FD4 concentrations in the blood were determined as described above.

4.2.5 Safety Study Design

The experiment began when mice were 14 weeks old. Mice were weighed daily and their condition was monitored. Conditions requiring sacrifice for humane considerations were defined as mice losing 20% or more of their body weight. Mice were randomly assigned to treatment groups prior to the start of the study.

On day 1 of the study, mice were weighed and received 600 mg/kg FD4 dissolved in PBS with no treatment, 60 mg/kg PPZ, 200 mg/kg SDC, or 390 mg/kg C_{10} by oral gavage. After three hours, blood was taken from the submandibular vein, and mice were returned to standard cages and fasting was ended. Blood concentrations of FD4 were evaluated as described above. This procedure to measure intestinal permeability was repeated weekly for four weeks, on days 8, 15, 22, and 30.

On all other days (2-29), mice were weighed and received PBS, 60 mg/kg PPZ, 200 mg/kg SDC, or 390 mg/kg C_{10} via oral gavage. On day 30, the protocol for treatment and intestinal permeability measurement was carried out as previously done, and then the treatment groups were randomly subdivided into two groups each. One subgroup (chronic exposure group) was sacrificed on

day 30. Blood was collected by cardiac puncture, and the small intestine and colon were removed and sections were collected for RNA extraction and histology.

The other subgroup (washout group) was returned to normal cages for a one-week recovery period during which mice received no treatment. Mice were weighed daily. On day 37, mice were weighed and orally gavaged with 600 mg/kg FD4 in PBS with no added permeation enhancers. After three hours, blood was taken to assess FD4 serum concentrations as described above. Then the mice were sacrificed, and blood and tissue samples were taken as described above.

4.2.6 Fecal Scoring

Mouse feces were collected on each of the permeability measurement days and assigned a score from 0-3 based on solidity, presence of mucus, and presence of blood as determined using Hemoccult Guaiac Fecal Occult Blood Test slides.

4.2.7 Histology

After dissection, small intestine and colon samples were immediately put into 4% formaldehyde for 24 hours. Then samples were rinsed with PBS and transferred to 30% sucrose and stored at 4°C. Samples were embedded in Sakura Tissue-Tek Optimal Cutting Temperature Compound (VWR) and stored at -80°C. Samples were sectioned to 10 µm thickness on a Shandon Cryotome FSE and then stained as follows.

4.2.8 Immunofluorescence and Confocal Microscopy

Samples were rinsed in PBS to remove the residual OCT and blocked with 10% (w/v) bovine serum albumin in 0.1% (v/v) TritonX-100 for two hours at room temperature. Samples for tight junction staining were incubated with a primary antibody against Claudin 3 overnight at 4°C. Then the samples were rinsed and incubated with anti-rabbit Alexa-Fluor 488 for secondary staining of

Claudin 3 and anti-ZO1 AlexaFluor 594 for two hours. The samples were rinsed again, and Hoechst was added for 25 minutes to stain the nuclei. Samples were rinsed a final time, mounted in Fluoromount Gold, and coverslips were added. Samples for actin staining were blocked in 10% BSA in TritonX overnight at 4°C. Then the samples were rinsed and Phalloidin-Alexa-Fluor 594 was added for 45 minutes. Samples were rinsed again and then incubated with Hoechst for 25 minutes. Samples were rinsed a final time, mounted in Fluoromount Gold, and coverslips were added. Microscopy was performed using a Keyence BZ-x810 fluorescence microscope with 405, 488, and 555 nm filters.

4.2.9 RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

After dissection, tissue samples from the small intestine and colon were immediately placed into RNA*later* (ThermoFisher) and stored at -20°C until they were processed. Tissue samples were placed in 250 µL Trizol reagent (ThermoFisher) and homogenized with the BeadBug Microtube Homogenizer. 150 µL chloroform was added, and samples were centrifuged for 15 minutes at 12000 rpm. The aqueous layer was removed to a fresh 1.5 mL RNAse-free tube, and an equal volume of ethanol was added. The sample was briefly mixed and then transferred to a spin column from the QIAGEN RNeasy Mini Kit. Further washing steps were performed according to the manufacturer's instructions and using buffers provided in the kit. cDNA was synthesized from 2000 ng of each RNA sample using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. qRT-PCR was performed on a Viia 7 Real-Time PCR System (Applied Biosystems) using SYBR Select Master Mix (Applied Biosystems). Primer sequences can be found in Table 4.1.

Table 4.1: PCR primer sequences.

Gene	Forward	Reverse
β-Actin	CACTGTCGAGTCGCGTCC	TCATCCATGGCGAACTGGTG
Claudin 2	GAAAGGACGGCTCCGTTTTC	CAGTGTCTCTGGCAAGCTGA
Claudin 3	GTACAAGACGAGACGGCCAA	GGGCACCAACGGGTTATAGA
ZO-1	CTCTTCAAAGGGAAAACCCGA	GTACTGTGAGGGCAACGGAG
JAM-A	TCCCGAGAACGAGTCCATCA	GAACTTCCACTCCACTCGGG

4.2.10 Serum Zonulin Quantification by ELISA

After the mice were sacrificed, blood was collected by cardiac puncture and centrifuged to isolate the serum. The serum concentration of Zonulin was measured using an enzyme-linked immunosorbent assay (ELISA) kit purchased from Abcam. For Zonulin, a 1:1000 dilution factor was used to assess all samples according to the manufacturer's instructions.

4.2.11 Statistics

For the time point study, the group size was n = 6. For the longitudinal experiment, mice were randomly assigned to treatment groups such that the initial group size was n = 12. All data are presented as the mean with error bars representing the standard error of mean (SEM). Statistical significance was determined by two-tailed, unpaired Student's t-tests performed in GraphPad Prism 8.

4.3 Results

4.3.1 PPZ, SDC, and C_{10} enhance intestinal permeation of 4 kDa FITC-Dextran

Before beginning the month-long safety study, we conducted a one day experiment to confirm that our chosen permeation enhancers, 1-phenylpiperazine (PPZ), sodium deoxycholate (SDC), and sodium caprate (C_{10}), produced significant increases in permeability and to establish the time profiles of each enhancer's activity. To assess the efficacy of the selected permeation enhancers, we measured the oral absorption of 4 kDa FITC-Dextran (FD4) co-delivered in solution with PBS (untreated control), 60 mg/kg PPZ, 200 mg/kg SDC, or 390 mg/kg C_{10}. Blood samples were taken 30, 60, 90, and 180 minutes after administration to determine FD4 blood serum concentrations (Figure 5.1a). When no permeation enhancer was administered, FD4 serum concentrations were low, and the resultant area under the curve (AUC) was 5.67 ± 0.64 μg/mL*hr (Figure 5.1b). In contrast, mice that received PPZ, SDC, or C_{10} absorbed significantly more FD4, although with unique concentration profiles over time.

SDC caused the largest increase in permeation, with the blood concentration reaching its maximum by 30 minutes and remaining elevated compared to the untreated control out to three hours after administration. C_{10} also caused a rapid increase in FD4 blood concentration reaching its maximum value by 30 minutes, but the increase in permeation caused by C_{10} did not persist as long as that caused by SDC, with values dropping back to near those of the untreated control by the 90 minute time point. PPZ also significantly enhanced permeation, remaining above the untreated control values for all time points in the study. All three permeation enhancers caused statistically significant increases in AUC compared to the untreated control, producing fold increases of 1.7, 5.0, and 2.3 for PPZ, SDC, and C_{10}, respectively. Because these doses of the permeation enhancers significantly increased the absorption of FD4 after oral gavage, we decided to carry them through

the rest of the study, as they represent amounts of the enhancer that could have a therapeutic effect in a hypothetical oral drug formulation.

4.3.2 Permeation enhancers alter mRNA expression of tight junction proteins

To assess how one dose of a permeation enhancer affects the structure of the intestine on a molecular level, we used quantitative real-time polymerase chain reaction (qRT-PCR) to measure the mRNA expression of tight junction proteins. Small intestine and colon were harvested from mice sacrificed after one three-hour treatment with permeation enhancers. These tissues were

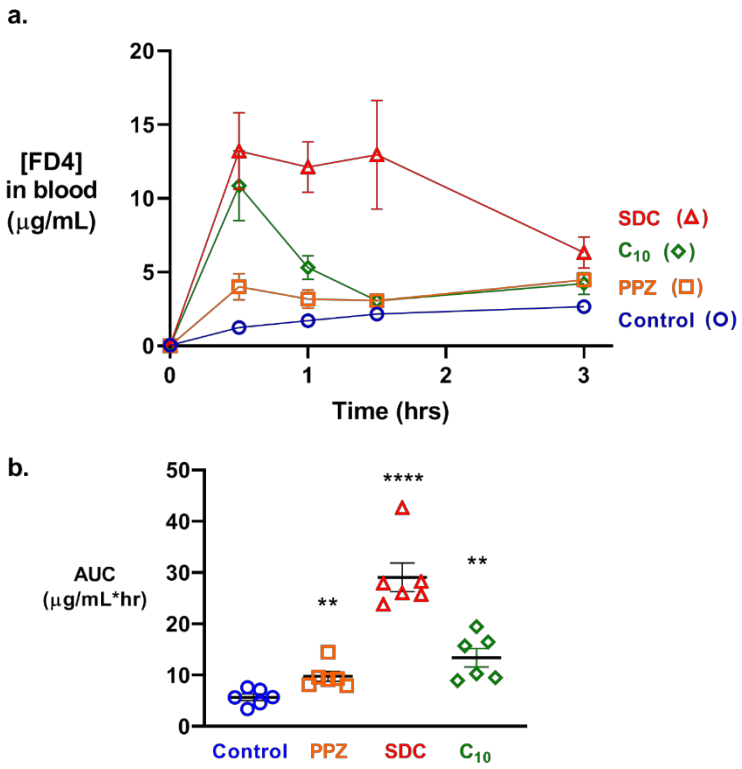

Figure 4.1: The permeation enhancers 1-phenylpiperazine (PPZ), sodium deoxycholate (SDC), and sodium caprate (C_{10}) increase oral macromolecular absorption. FITC-dextran 4 kDa (FD4) was co-delivered with either PBS (Control), PPZ, SDC, or C_{10} by oral gavage. a) All enhancers increased FD4 blood concentrations compared to control over three hours. b) Each enhancer increased the area under the curve (AUC) of FD4 over three hours. n = 6, error bars represent SEM **p < 0.01, ****p < 0.0001 compared to untreated control by unpaired, two-tailed Student's t-test.

processed to extract the RNA, and qRT-PCR was used to assess the relative expression levels of Claudin 2, Claudin 3, ZO-1, and JAM-A.

After one dose of permeation enhancer, C_{10} caused a five-fold increase in the expression of Claudin 2 in the small intestine compared to the untreated group and an almost two-fold increase in the expression of Claudin 3 (Figure 5.2a). C_{10} also caused a two-fold increase in the expression of JAM-A in the small intestine after one dose, but no other permeation enhancers affected small intestine tight junction gene expression after one dose. In the colon, SDC induced a five-fold decrease in the expression of Claudin 2, while PPZ caused a two-fold decrease (not statistically significant) (Figure 5.2b). Claudin 3 expression in the colon was more variable in all groups compared to the other genes studied, and PPZ and SDC both caused statistically significant decreases in the expression of Claudin 3. No changes were seen in the expression of ZO-1 in the small intestine or colon compared to the untreated group after just one dose of PPZ, SDC, or C_{10} (Figure 5.2a and b).

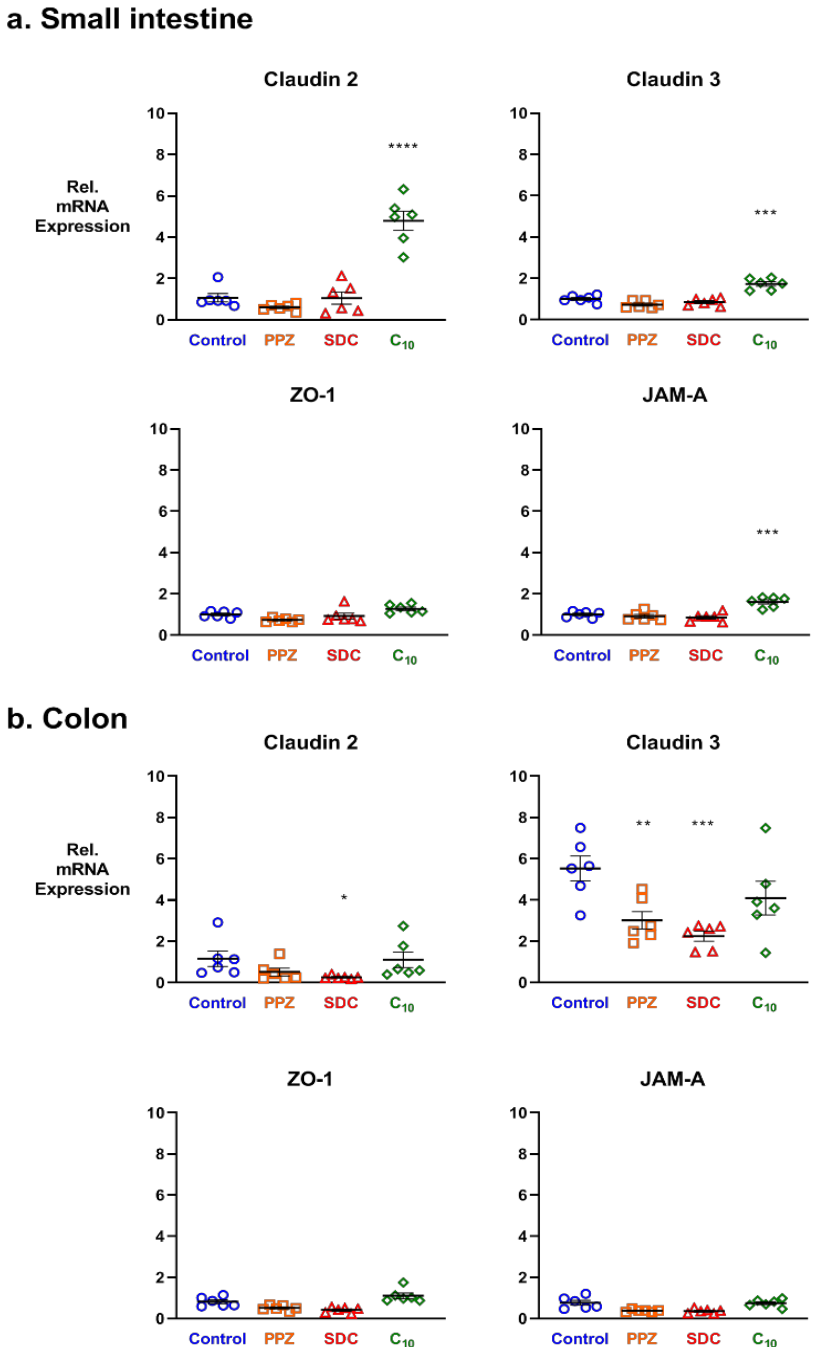

Figure 4.2: The permeation enhancers PPZ, SDC, and C_{10} affect gene expression of tight junction proteins. Mice received a single dose of PBS (Control) or one of the three enhancers. After three hours, small intestine and colon tissue samples were harvested, and mRNA expression was determined by qRT-PCR for four tight junction proteins: Claudin 2, Claudin 3, Zonula occludens-1 (ZO-1), and Junctional adhesion molecule-A (JAM-A). (a) In the small intestine, only C_{10} induced significant gene expression changes compared to the control. (b) In the colon, PPZ and SDC altered gene expression of the claudins compared to the control. No differences in ZO-1 or JAM-A expression were found. n = 6, error bars represent SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by unpaired, two-tailed Student's t-test.

4.3.3 Repeat dosing of permeation enhancers does not permanently impair intestinal barrier function

To evaluate the effect of long-term, daily oral administration of permeation enhancers on intestinal barrier function, we designed a month-long experiment during which mice received daily oral gavages of PBS, PPZ, SDC, or C_{10} at the concentrations found to be effective in the previous experiment. One week before the experiment began, each animal's baseline intestinal permeability was measured by delivering FD4 with no enhancer to mice by oral gavage and measuring the concentration of FD4 in the serum three hours after administration. On day 1 of the study, mice received FD4 in solution with one of the permeation enhancers or PBS for the untreated control, and permeability was again measured three hours after administration. For ethical reasons regarding the frequency of blood draws, permeability was only measured once per week on days 8, 15, 22, and 30, with mice receiving oral gavages of just the permeation enhancer treatments on all other days. On day 30, half of the mice in each group were sacrificed, and tissues were collected for further analysis. The remaining half of the mice were given a one week washout period, then permeability was assessed by once more measuring FD4 permeability without permeation enhancer treatment.

The critical indicator of safety in this study was whether or not the permeability of the intestine increased upon repeat dosing of the permeation enhancers. The concentrations of FD4 measured in the blood on the pre-treatment day, treated days 1, 8, 15, 22, and 30, and after the one week washout period are compared in Figure 5.3 (a-d). All values shown as light grey open squares are from measurements made on days where no permeation enhancer treatment was given. All values shown as colored shapes are from measurements made on days where mice were treated with their respective permeation enhancer.

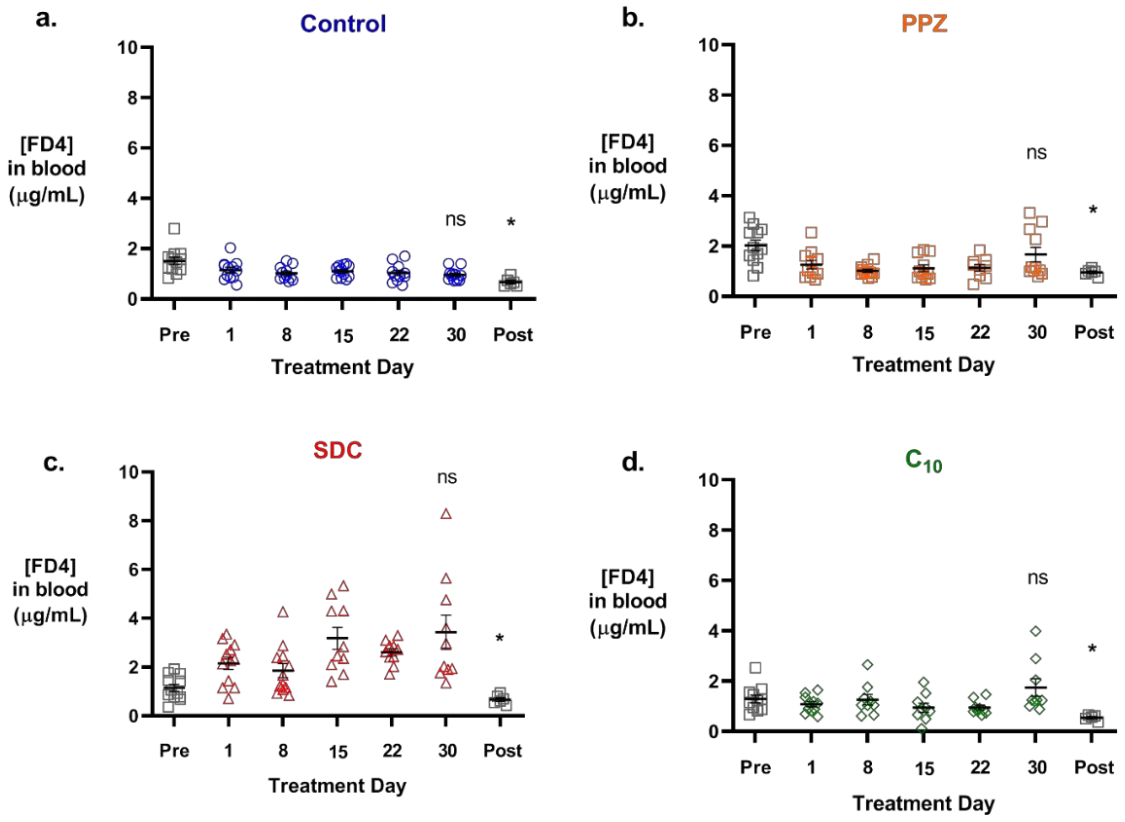

Figure 4.3: Enhancers do not permanently increase intestinal permeability after four weeks of daily oral administration. Baseline untreated intestinal permeability was measured one week before treatment began (grey squares-Pre). Mice were dosed with PBS (a), PPZ (b), SDC (c), or C_{10} (d) every day for 30 days, and the permeability of FD4 was measured five times throughout the 30 day period. Half of the mice were sacrificed on day 30, and half had a one week washout period before their untreated permeability was measured again (grey squares-Post). None of the groups had significant differences between the concentrations measured on Treatment Day 1 and Treatment Day 30. All groups had significantly lower blood concentrations of FD4 after the recovery period compared to before the experiment started. n = 6-12, error bars represent s.e.m., *$p < 0.05$ by unpaired, two-tailed Student's t-test.

The key comparisons that we were interested in were between treated days 1 and 30, and between the pre-treatment and post-treatment permeability. Comparing the FD4 concentrations on treated days 1 and 30 assesses whether the permeation enhancers have the same effect when used for the first time in enhancer-naïve mice and after sustained exposure. In all groups, when the FD4 concentrations on treatment day 30 were compared to those on treatment day 1, there was no statistically significant difference as evaluated by an unpaired two-tailed Student's t-test. The

treated groups did show some increase in permeability and the variability of FD4 blood concentration over the four weeks of treatment, but these effects were not significant, nor did they persist after the recovery week. For all groups, the post-recovery period values were significantly different from the pre-treatment values, but, surprisingly the average values were lower after the recovery period compared to the pre-treatment. This is in direct contrast to the general expectation that repeated use of permeation enhancers would cause lasting increases in intestinal permeability reflected in the literature, which will be discussed further in the discussion.

4.3.4 Chronic permeation enhancer exposure does not negatively affect GI health indicators

aIn addition to intestinal permeability, we were also interested in several other indicators of GI and general health in mice receiving long-term permeation enhancer treatment. Mice were weighed daily, as well as assessed for general behavioral signs of healthy versus stressed states, including barbering, hunching, and fecal abnormalities including mucus in the stool and diarrhea. Mice in all groups gained weight (Figure 5.4), with the untreated group gaining an average of 5.2 g over the four weeks of the study.

The SDC and C_{10} groups gained averages of 4.0 and 3.9 g, respectively. The PPZ group gained an average of 3.4 g in the four week period, and while this value was found to be statistically significantly different from the untreated control, we believe that this is a result more of the reduced variability in the weights in the PPZ group affecting the statistical analysis, rather than a biological effect of orally administered PPZ.

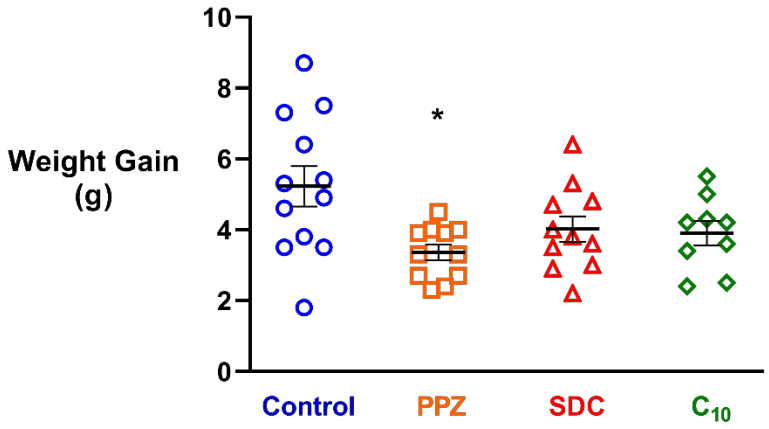

Figure 4.4: Daily permeation enhancer treatment does not cause weight loss. Mice were weighed daily during the study. Shown here is the difference between the starting weight measured one week before the study began and the weight on treatment day 29. Both values were non-fasted weights to remove the variable of weight loss associated with the 12-hour fasting period before each permeability measurement day. While all groups showed weight gain, the PPZ group did gain significantly less weight than the control group, with an average gain around 3.5 g as opposed to the untreated mice which gained an average of 5 g in 4 weeks. n = 9-12, error bars represent s.e.m. * $p < 0.05$ by a two-tailed, unpaired Student's t-test.

No mice were sacrificed over the course of the study for reaching pre-determined indicators of poor health. These included loss of ≥ 20% of their body weight, excessive grooming or barbering, multiple consecutive days of diarrhea or stool containing mucus or blood, and hunching that did not resolve within a few minutes after handling. The only observed instances of barbering were resolved by separating a dominant mouse from one of the cages and housing it separately for the rest of the study. Over the course of the thirty-day treatment period, three mice in the C_{10} group and one in the SDC group were sacrificed due to procedural complications resulting from the oral gavage, and one mouse in the PPZ group was sacrificed due to complications from a blood draw.

4.3.5 Fecal analysis

It is well known that damage to the intestinal epithelium can result in changes to stool quality, thus, fecal quality was used as another marker of intestinal health during the long-term study. Fecal samples were collected on permeation measurement days, and visually inspected for the presence

of mucus and for the solidity. Hemoccult testing was used to identify blood in the stool samples. The qualitative characteristics of solidity, presence of mucus, and presence of blood were aggregated into a quantitative fecal score on a scale of 0-3, as shown in the table in Figure 5.5.

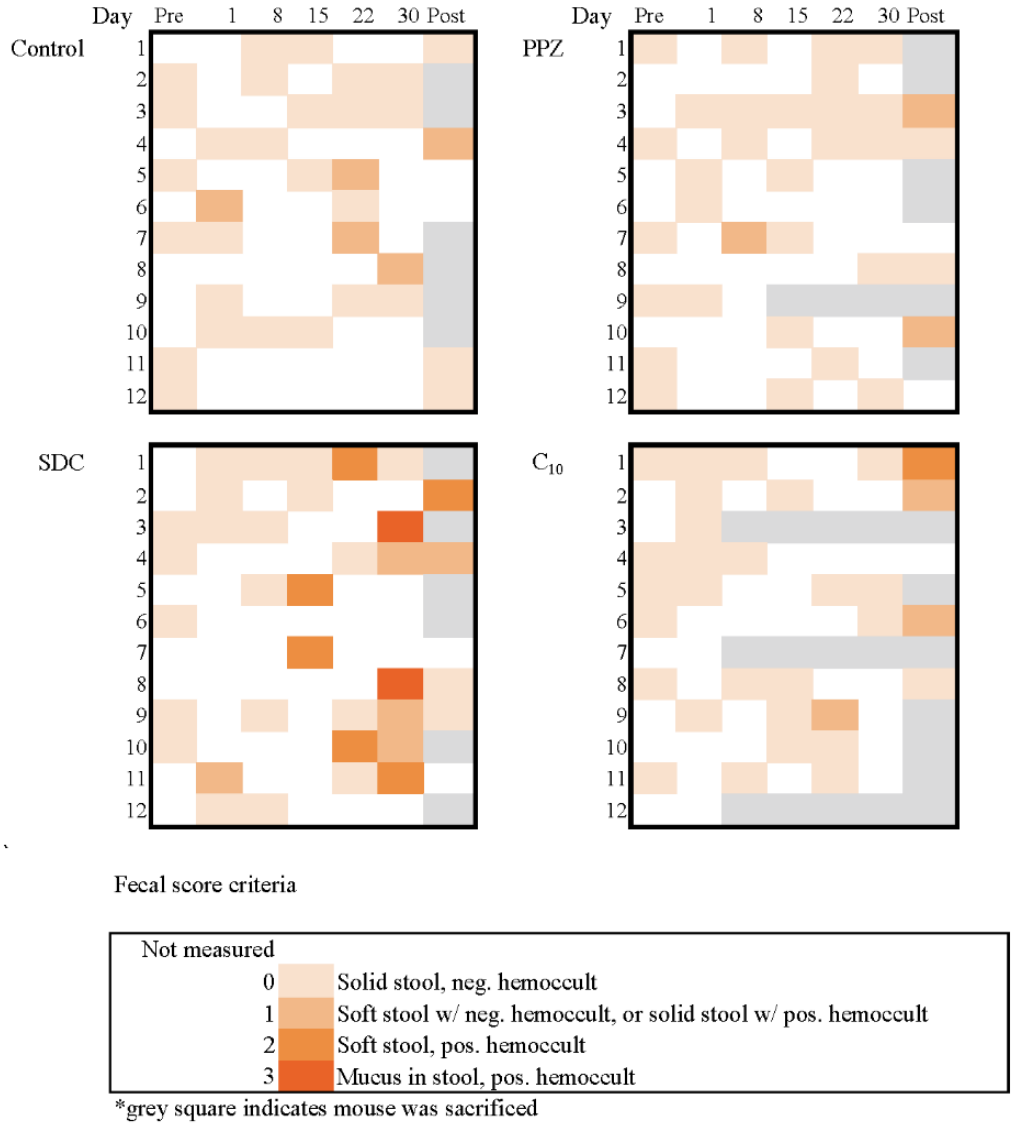

Figure 4.5: SDC has the strongest effect on the quality of mouse stool. Daily SDC administration caused an increase in fecal score over time, but fecal indicators of altered intestinal health decreased after a one week washout period. The scoring system used here assesses stool solidity, mucus in the stool, and blood in the stool (pos. hemoccult).

Variability in scores from 0-1 were seen even in the untreated group, suggesting that minor changes to solidity or the presence of blood in the stool without changes to solidity are normal fluctuations seen even in healthy mice. The PPZ and C_{10} groups showed no clear differences in fecal score over the course of the study, but mice in the SDC group showed increases in fecal score over time. On the last treatment day, all but one of the fecal samples had scores of 1 or higher, with two samples having the highest score of 3. After the recovery period, the fecal scores in the SDC group showed a general decrease, suggesting that the changes SDC was causing to fecal quality were temporary.

4.3.6 Serum zonulin concentrations

The final measure of GI health that we assessed was the amount of the protein zonulin found in the blood, which has been shown to correlate with increased intestinal permeability.[83,84] The concentration of zonulin in the blood of mice sacrificed on treatment day 30 and after the washout period was measured by ELISA (Fig. 5.6). On treatment day 30, mice from the SDC group had an average serum zonulin concentration of 4 ng/mL, which was higher than that of the untreated mice, with an average of approximately 2.5 ng/mL. Mice in the PPZ and C_{10} groups showed no difference from the untreated control. After the one week recovery period, the concentrations of zonulin in blood samples from the three treated groups were indistinguishable from those of the untreated group, suggesting that the observed differences are not permanent once permeation enhancer treatment ends.

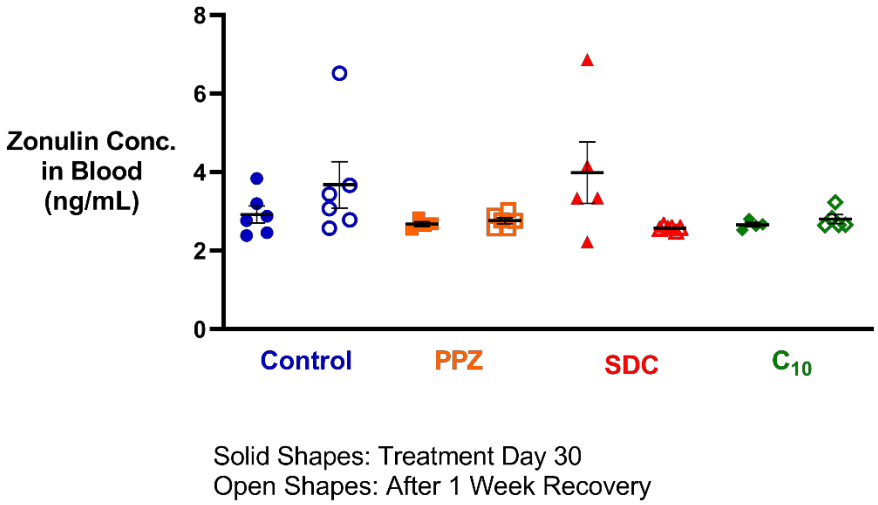

Figure 4.6: Elevated serum zonulin levels do not persist after permeation enhancer treatment ends. The serum concentration of zonulin was measured by ELISA. In blood samples collected on the final day of treatment, mice treated with SDC had serum zonulin concentrations noticeably higher than those of the control group, which received PBS. After the one week washout period, the serum zonulin concentrations of SDC-treated mice were no longer elevated. Mice treated with PPZ and C_{10} did not show differences from the untreated control at either time point. n = 4-6, error bars represent SEM.

4.3.7 Repeated dosing of permeation enhancers affects mRNA expression of tight junction proteins

Based on our previous observation that just one dose of permeation enhancer causes changes to the gene expression of several tight junction proteins, we were interested in the effects of chronic exposure to these enhancers. Tissue samples were collected from the small intestines and colons of mice sacrificed either on treatment day 30 or after the washout period, and the mRNA expression of Claudin 2, Claudin 3, ZO-1, and JAM-A were measured by qRT-PCR. After 30 days of permeation enhancer exposure, SDC and C_{10} both caused highly variable changes to Claudin 2 expression (Figure 5.7a). In several animals, expression was between 4- and 10-fold higher than that measured in the untreated group, but the extreme variability among the SDC- and C_{10}-treated animals precluded statistical significance. There was a moderate amount of variability observed

within and among groups for Claudin 3 and ZO-1 expression in the small intestine after 30 days of treatment, but none of the permeation enhancers tested caused significant differences in the expression of either of these tight junction proteins.

After the one week washout period, the expression of Claudin 2 was no longer elevated relative to the untreated control for C_{10}. For the mice treated with SDC, we were surprised to see that they all had significantly reduced levels of expression of Claudin 2 in the small intestine. This was the only notable difference observed in any gene analyzed for all small intestine samples that were collected after the washout period (Figure 5.7b).

Similarly to the changes observed in the expression of Claudin 2 in the small intestine after chronic exposure, C_{10} caused significant and highly variable changes to Claudin 2 expression in the colon (Figure 5.7c). PPZ also caused a smaller, but still statistically significant two-fold increase in Claudin 2 expression in the colon and an almost three-fold increase in the expression of JAM-A in the colon compared to the untreated control.

We were surprised to see that Claudin 2 expression in the colons of mice in the washout group was still highly elevated in the C_{10} group and that Claudin 2 expression in the SDC group was higher and more variable than it was before the recovery period. The increase in JAM-A expression observed in the colons of the PPZ group did not persist after the recovery period. C_{10} also affected ZO-1 expression in the colon, causing a two-fold increase in expression that was not apparent upon chronic exposure but appeared only after the washout period.

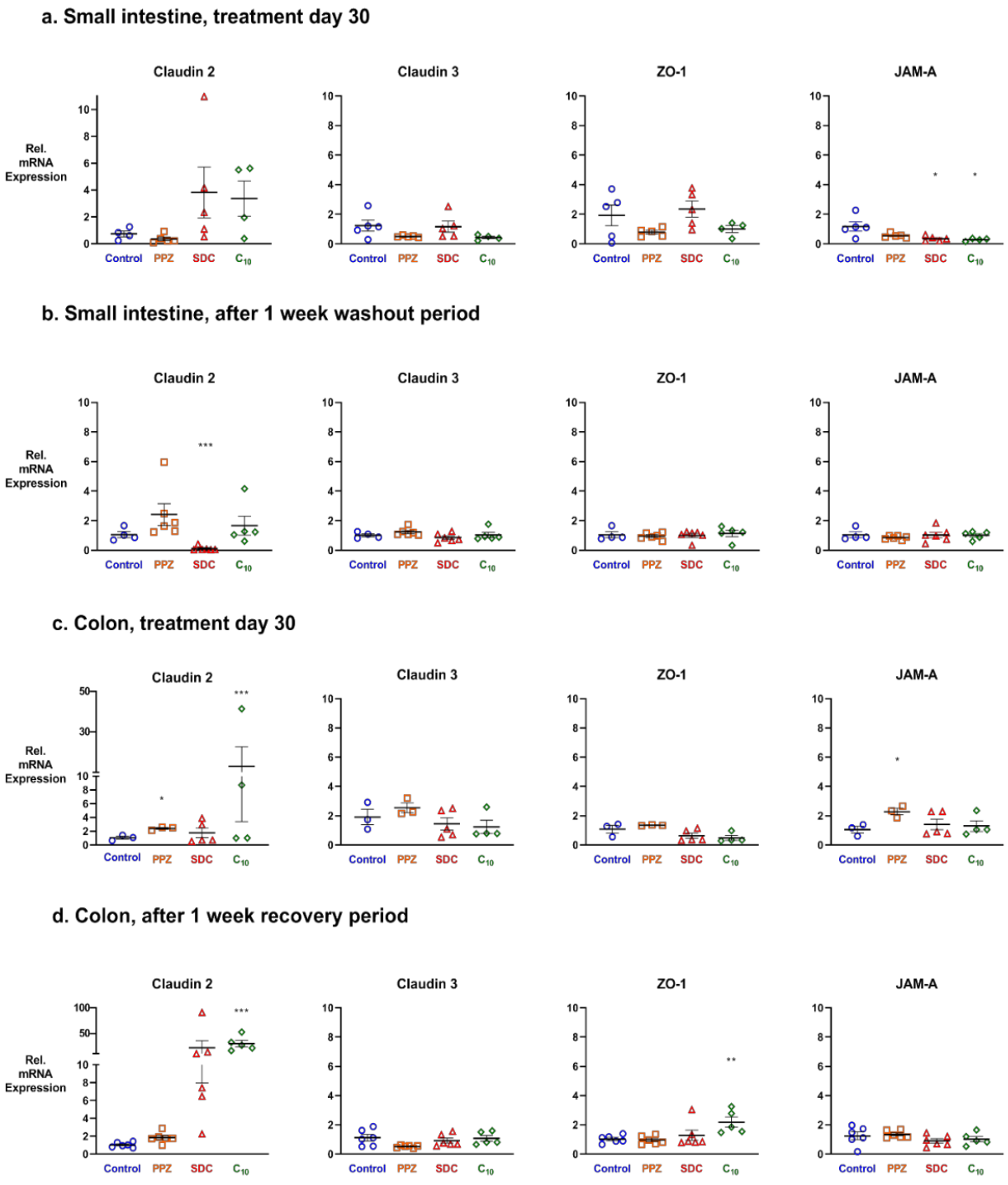

Figure 4.7: Chronic exposure to permeation enhancers affects gene expression of tight junction proteins. (a) On day 30, mice that received SDC and C_{10} had increased variability in claudin 2 expression in the small intestine but no statistically significant increases. SDC and C_{10} both caused significant decreases in JAM-A expression in the small intestine.

Figure 4.7, continued: (b) After one week of recovery, there were no differences in claudin 3, ZO-1, or JAM-A expression in the small intestine for any of the groups. For claudin 2, PPZ and C_{10} expression were similar to the control group, but the SDC group had significantly decreased expression. (c) In the colon, C_{10} caused a dramatic increase in the average expression of claudin 2, although the variability was much higher than for the other groups. PPZ caused a two-fold increase in the expression of claudin 2 as well as a small but statistically significant increase in JAM-A expression. (d) After the recovery period, mice treated with C_{10} had significantly increased levels of claudin 2 and ZO-1 expression in the colon, and SDC showed increased but not statistically significant expression in claudin 2 expression. Claudin 3 and JAM-A were unaffected. n = 6, error bars represent SEM, *p < 0.05, **p < 0.01, ***p < 0.001 by unpaired, two-tailed Student's t-test.

4.3.8 Intestinal architecture shows no damage by permeation enhancers as assessed by immunofluorescence microscopy

The dramatic changes to the gene expression of tight junction proteins in permeation enhancer-treated mice prompted the question of whether the effects of these molecular changes could be seen on a larger scale in the architecture of the intestine. Therefore, we used immunofluorescence staining and microscopy to assess the architecture and tight junction arrangement of samples of small intestine tissue collected from mice sacrificed on treatment day 30. Sections from each treatment group were stained with Hoechst to visualize the nuclei, anti-Claudin 3 antibody with an AlexaFluor 488-tagged secondary antibody, and anti-ZO1 tagged with AlexaFluor 594.

Representative images are shown in Figure 5.8a. In all samples, we were able to see intact villi with no cell sloughing at the tips. Claudin 3 and ZO-1 were localized at the junctions between the epithelial cells, with clear outlines of the nuclei visible in each sample. If there was permeation enhancer-associated tight junction rearrangement occurring, we would expect to see differences between the localization patterns of these proteins between the untreated control and the treated groups. Sections from each treatment group were stained with Hoechst to visualize the nuclei and phalloidin-AlexaFluor 594 to visualize F-actin (Figure 5.8b). Again, the villi are intact, and F-actin can be seen surrounding the luminal side of each villus as well as surrounding each epithelial cell.

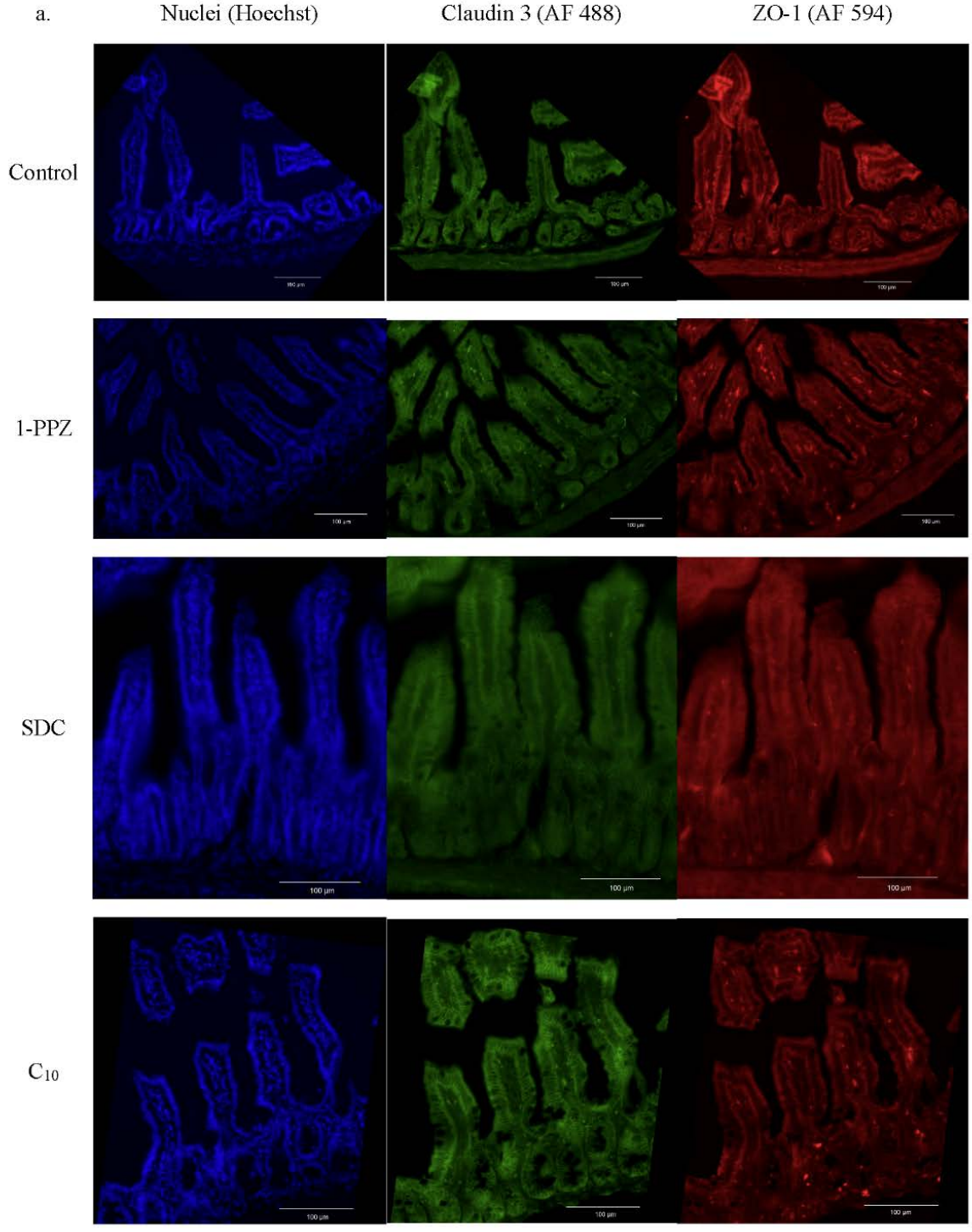

Figure 4.8: Intestinal architecture and tight junction protein localization are not affected by four weeks of treatment with permeation enhancers. (a) Sections of small intestine from mice receiving PBS, PPZ, SDC, or C_{10} were stained for nuclei (blue, Hoescht), the barrier-forming claudin 3 (green, AF 488), and the tight junction protein ZO-1 (red, AF 594). All scale bars are 100 µm.

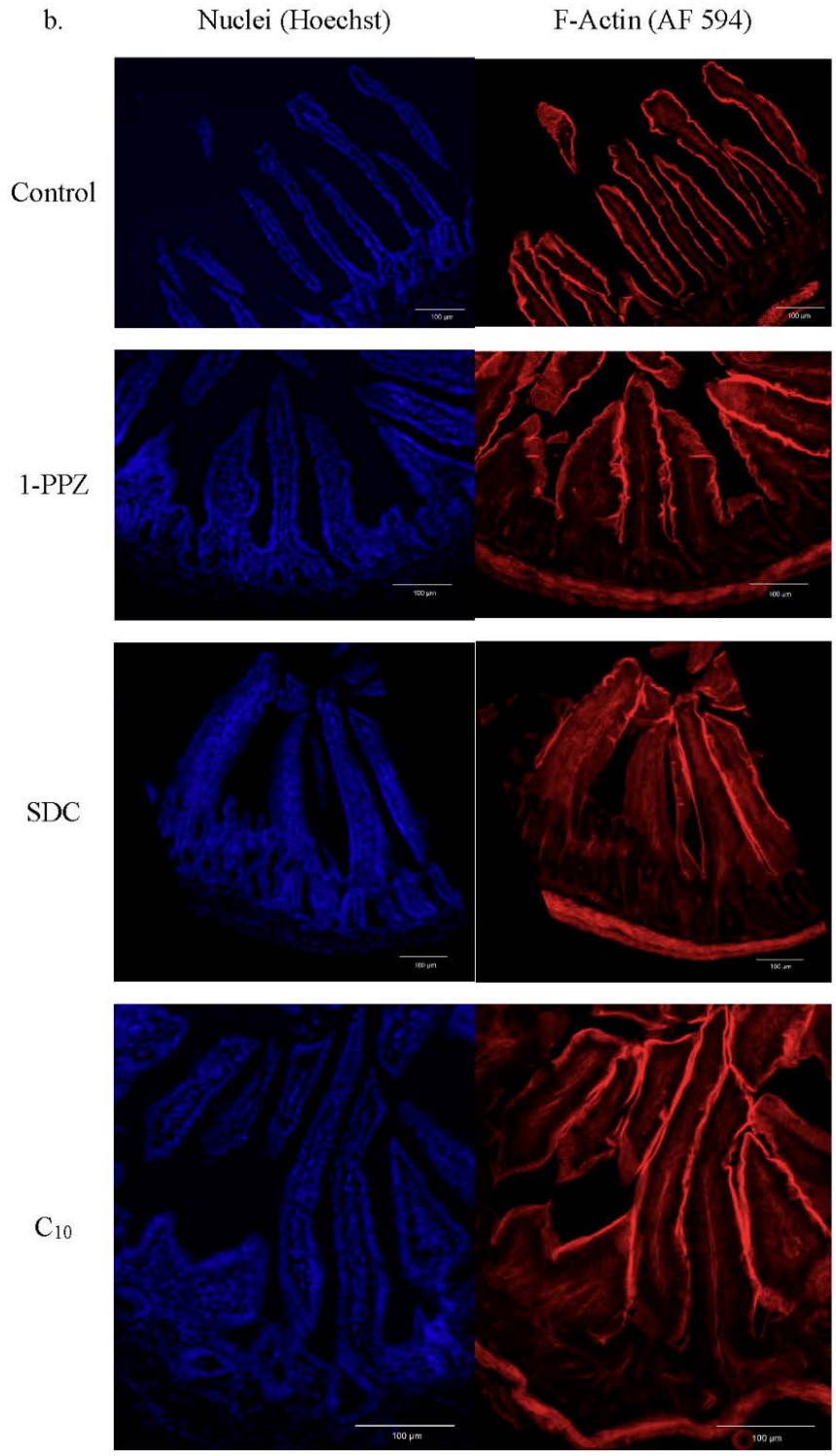

Figure 4.8: Intestinal architecture and tight junction protein localization are not affected by four weeks of treatment with permeation enhancers. (b) Sections of small intestine from mice receiving PBS, PPZ, SDC, or C_{10} were stained for nuclei (blue, Hoescht) and F-actin (red, AF 594). All scale bars are 100 µm.

4.4 Discussion

The clinical translation of permeation enhancers has been limited by our incomplete understanding of their mechanisms and impact on long-term intestinal health. Anecdotally, we have encountered consistent and significant skepticism that permeation enhancers can act without toxicity. The most commonly cited concerns include 1) that increased intestinal permeability will result in the absorption of toxic molecules from the intestinal lumen and 2) that prolonged use will permanently permeabilize the intestinal epithelium.[85]

Given our lab's interest in the clinical translation of permeation enhancer technology, we were motivated to design a study in response to the most common concerns we've encountered while presenting our work. Specifically, we sought to characterize the short- and long-term effects of daily oral permeation enhancer administration in mice. We included three permeation enhancers with distinct mechanisms of action. To our knowledge, the paracellular enhancer, 1-phenylpiperazine (PPZ), and the transcellular enhancer, sodium deoxycholate (SDC), have not been studied for safety or efficacy *in vivo*.[60] These enhancers were compared to sodium caprate (C_{10}) which acts both by fluidizing the lipid membrane of the epithelial cells and by rearranging tight junctions and is included in FDA-approved oral formulations.[47]

The major finding of this study is that there are no permanent increases in intestinal permeability induced by any of the three permeation enhancers that we tested. For each group, the FD4 concentration in the blood measured on treatment day 30 was not significantly different than that measured on treatment day 1. Previous studies of repeat oral administration of C_{10} in rodent and dog models found similar results in which the repeated use of the permeation enhancer did not change the efficacy as determined by measuring several pharmacokinetic profiles.[49] We were pleased to see that SDC and PPZ also showed similar efficacy each week and did not cause

continual increases in permeability, which would have indicated damage to the epithelium. Studies of PPZ and SDC have been limited to the Caco-2 model and *ex vivo* studies using tissue from rats; however, even in these models that lack many of the repair mechanisms of the *in vivo* intestine, recovery of intestinal barrier integrity was observed after a single dose of the permeation enhancer at effective concentrations.[45,61,63,64,73] There was a noticeable increase in the variability of FD4 serum concentration in the SDC group over the course of treatment, but it is worth noting that the same mice within the SDC group had the highest FD4 serum concentrations each week. This observation that individual responses to permeation enhancement can be very different is not surprising based on literature over the last few decades showing that intestinal absorption is highly variable between individuals.[86] Additionally, daily handling of animals and their associated stress responses could contribute to increased variability.

Zonulin is an endogenous protein that regulates intestinal permeability by contributing to the disassembly and rearrangement of tight junction proteins. Its pathogenic counterpart, zonula occludens toxin, is produced by Vibrio cholerae and has been studied extensively as a permeation enhancer.[46,53,87] While there are fewer studies on zonulin, several publications show that serum zonulin levels are elevated in humans and rodents with increased intestinal permeability associated with autoimmune diseases such as type 1 diabetes and celiac disease.[83,84] We measured the concentration of zonulin in serum samples taken from mice on treatment day 30 as well as after the recovery period. Only mice receiving SDC had elevated levels of serum zonulin on treatment day 30, although the difference was not statistically significant (Figure 5.6). This fits with our overall observation that mice in the SDC group had moderate increases in intestinal permeability as the treatment period progressed, as well as the fact that they were the only group with noticeable changes to fecal quality that would suggest increased intestinal permeability, including the

presence of mucus and blood in the stool (Figure 5). We were delighted to observe that the elevated levels of zonulin did not persist after the one week recovery period, suggesting that any increases to intestinal permeability and tight junction integrity caused by repeated administration of SDC are not permanent.

While we chose permeability over time as the main parameter to observe the effects of repeated permeation enhancer use, we were also curious about changes to both the molecular and gross architecture of the intestinal epithelium. We used qRT-PCR to measure the relative gene expression of four different tight junction proteins after one day of treatment (Figure 5.2), on the 30th day of chronic exposure, and after a one week washout period (Figure 5.7). The proteins of interest were claudin 2, a pore-forming tight junction protein, claudin 3, a barrier-forming tight junction protein, JAM-A, and ZO-1, which was of particular interest due to the fact that it has contact with most other tight junction proteins as well as the actin cytoskeleton. The most dramatic expression changes were seen in claudin 2 and claudin 3, with claudin 2 having the highest increases in expression among the four genes, and the only one with dramatic increases that persisted after the week of recovery. Many permeation enhancers have been reported to interact with members of the claudin family of tight junction proteins, but to our knowledge, the effects of chronic permeation enhancer exposure on tight junction expression is poorly understood.[12,14]

Despite the observed differences in gene expression, imaging of intestinal tissue samples did not reveal any gross morphological damage in any group. Previous *in vitro* investigations suggest that PPZ disrupts pathways that affect actin conformation to increase paracellular permeability.[63] We were particularly pleased to see that intestinal tissue from PPZ-treated mice did not show changes in actin arrangement that would suggest damage was done to the cytoskeleton. The architectural characteristics of small intestine were preserved in all samples, including intact villus tips and no

shortening of the villus-crypt axis. These types of changes to the villi structure have been reported in studies of intestinal damage.[88]

Recent successes in the field of oral protein drug delivery, such as the FDA approval of an oral octreotide formulation, Mycapssa® (Chiasma), are promising signs that repeated use of fatty acid and surfactant-based permeation enhancers is safe. Our results showing that novel permeation enhancers that rearrange tight junction proteins are also safe and effective with repeated use in a mouse model add further encouragement for more exploration into novel permeation enhancers in preclinical and clinical studies.

4.5 Conclusion

To our knowledge, this is the first study to look at repeated dosing of a paracellular permeation enhancer. Most publications that identify novel permeation enhancers are based on acute studies, whether *in vivo, ex vivo,* or *in vitro.*[45,89,90] While we are fairly confident that epithelial cell turnover, mucus shedding, and other native recovery mechanisms in the intestine are capable of repairing damage inflicted by permeation enhancers that fluidize the lipid membrane, the effects of repeated tight junction rearrangement caused by paracellular enhancers are unknown.[91] Interestingly, PPZ affected tight junction protein expression less than C_{10} did, but in terms of larger scale changes to epithelial architecture or FD4 permeability, there were no discernible differences between mice that received C_{10} or PPZ. While many questions remain about the long-term use of permeation enhancers in the formulation of oral protein drugs, we show here that repeated oral use of a paracellular permeation enhancer *in vivo* does not have permanent negative effects on the integrity of the intestinal barrier. This study shows the value of including longitudinal *in vivo* studies in reports of novel permeation enhancer development and hopefully dispels some of the pessimism that precludes the advancement of oral protein drug delivery research.

5 Conclusions and Future Perspectives

In the midst of 2020, a year of uncertainty and stalled projects for many in the scientific community, there was a brief moment of triumph when the third oral formulation of a protein drug was approved by the FDA (Mycapssa). This formulation includes a permeation enhancer to aid the delivery of octreotide and represents a growing momentum in the field of drug deliver towards a future where more and more life-saving drugs are available in patient-friendly oral forms. However, with the current state of the literature, there are several critical gaps in understanding that will impede this progress.

First, the mechanism by which intestinal permeation enhancers function is understood only for a handful of chemicals, most of which operate by the relatively simple mechanism of cell membrane fluidization. There is incomplete, if any understanding of how paracellular permeation enhancers open tight junctions and permit macromolecules to pass through the spaces between the intestinal epithelial cells. Second, the drugs that have made it into successful oral formulations are peptides that max out at a size of 4 kDa. Most publications of novel permeation enhancers in the literature use small molecule markers such as mannitol or peptide-sized markers like FITC-dextran, with insulin (5.7 kDa) being the largest macromolecule cargo seen in the average permeation enhancer paper. This leaves behind a massive array of protein drugs, which reach upwards of 150 kDa in size for antibody therapeutics. Finally, despite the successes of a few formulations containing permeation enhancers, there are many in the field of drug delivery who doubt that any oral formulation containing a permeation enhancer could be used regularly without causing damage to the intestinal epithelium, perhaps with grave consequences. For drugs like insulin, which is dosed multiple times a day in many diabetic patients, the worst imagined scenario is the permeation

enhancer formulation causing widespread increases to intestinal permeability with painful and dangerous symptoms like those of ulcerative colitis emerging over time.

This work addresses some of these issues, in the hope that permeation enhancer research will soon enable the oral delivery of all protein drugs. In Chapter 2, a small library of phenylpiperazine derivatives is assessed for their permeation enhancing potential as well as any associated cytotoxicity. This set of *in vitro* experiments highlights that very small changes in chemical structure can have outsized effects on the biological function of the molecule. This may inform future work in this area by encouraging the exploration of chemical variants of promising permeation enhancers, with the possibility of safer and more effective candidates emerging from the screening process.

Chapter 3 presents the first *in vivo* examination of two permeation enhancers, 1-phenylpiperazine and sodium deoxycholate, that have been previously identified as potent enhancers in *in vitro* and *ex vivo* models of the intestinal epithelium. These experiments also go beyond the typical assessment of permeation enhancement with a small molecule or peptide-sized tracker and assess the efficacy of these two compounds with FITC-dextrans ranging from 4-70 kDa. Both enhancers are able to improve the permeability of these larger macromolecules upon surgical injection into the small intestine, but interestingly, only sodium deoxycholate is functional when delivered via oral gavage. The addition of a pre-treatment with sodium bicarbonate to neutralize the acidic environment of the stomach restores the permeation enhancing function of 1-phenylpiperazine. With regards to both the sizes of molecules that enhancers can deliver and potential excipients that aid permeation enhancement, this work shows that novel permeation enhancers and larger and more complex macromolecular drugs may require extensive formulation optimization.

The safety study presented in Chapter 4 is an answer to the pessimism towards permeation enhancers that I have frequently encountered in the oral delivery community. The permeation enhancers studied earlier in this thesis, 1-phenylpiperazine and sodium deoxycholate, were orally delivered to mice every day for a month, and several metrics of intestinal health were assessed. One of the gold standard enhancers in the field of drug delivery, sodium caprate, was included in the experiment to provide clinical context, as it is included in several formulations that have performed well in human trials. The results of this study were in many ways a pleasant surprise, in that none of the three enhancers caused irreversible increases in intestinal permeability, even though sodium deoxycholate in particular is a very potent enhancer. Additional analysis of mRNA expression and staining of several different tight junction proteins shows that while chronic permeation enhancer use does affect the expression of certain genes, it does not have damaging effects on the structure of the epithelium.

The successful oral delivery of protein drugs hinges on our ability to overcome the mechanisms that the human body has in place to digest proteins for food and prevent the absorption of harmful pathogens. Innovative encapsulation methods using pH-sensitive polymers have largely made the issue of digestion in the stomach a problem of the past, and the remaining issue at hand is overcoming the low bioavailability caused by poor absorption in the intestine. Permeation enhancers have been studied for decades, and as they begin to succeed in the clinic, researchers must drive that success by filling the critical knowledge gaps of mechanistic understanding, the specific formulation needs of large protein drugs, and the paramount issue of safety for patients who rely on protein drugs every day.

6 References

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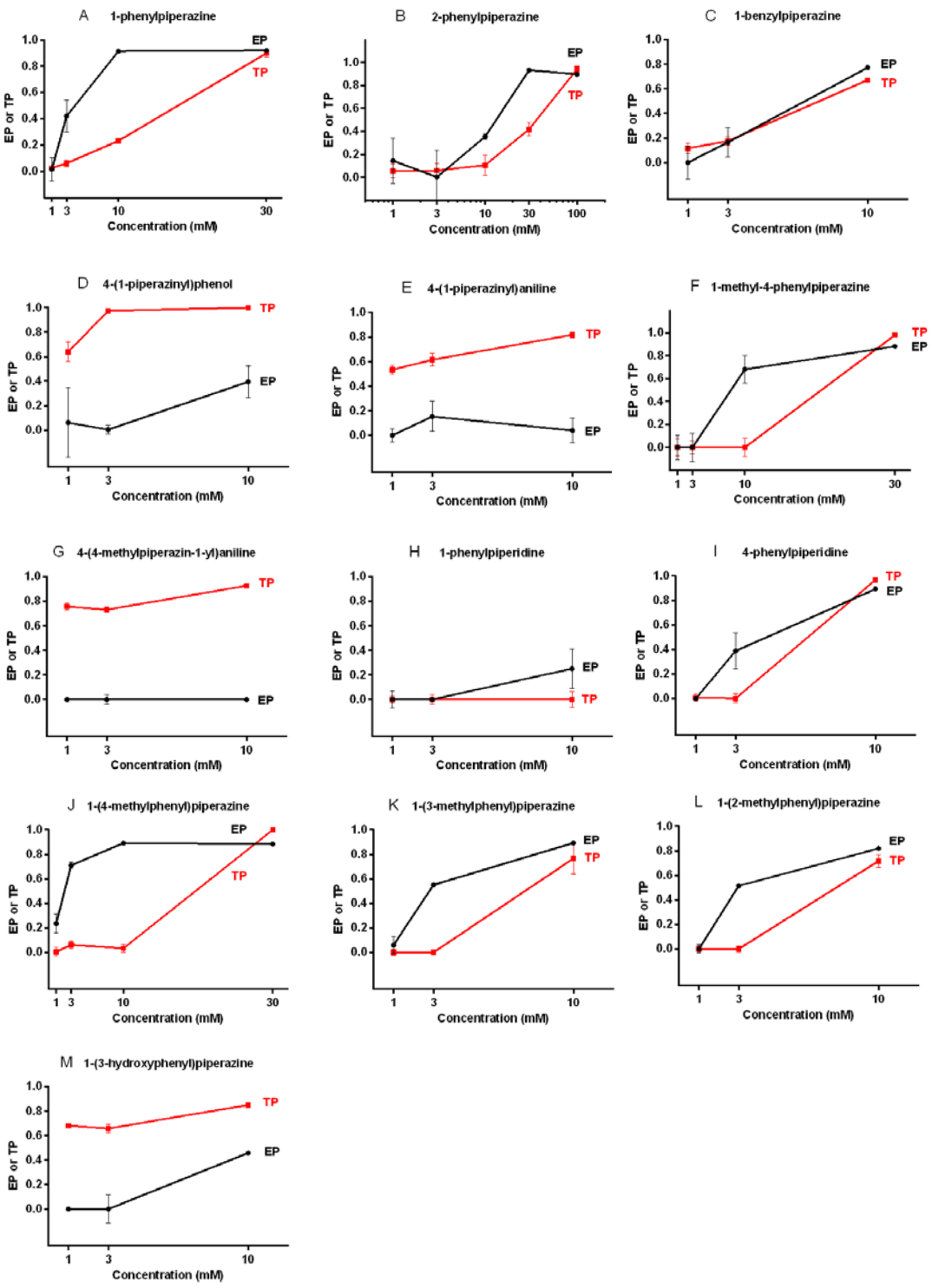
7 Appendices

7.1 Table S1: Tabulated Potential Values for Phenylpiperazine Derivatives

Compound		Concentration (mM)	pH	1 hr EP	SEM	TP	SEM	1 hr OP	SEM
A	1-phenylpiperazine	1	8.1	0.016	0.087	0.025	0.015	0	0.089
		3	8.7	0.421	0.122	0.063	0.025	0.358	0.124
		10	8.89	0.913	0.002	0.232	0.015	0.681	0.015
		30	9.17	0.920	0.005	0.898	0.029	0.021	0.029
B	2-phenylpiperazine	1	8.55	0.145	0.113	0.054	0.030	0.091	0.117
		3	8.72	0.002	0.137	0.059	0.031	0	0.140
		10	9.06	0.353	0.013	0.105	0.043	0.248	0.045
		30	9.16	0.931	0.004	0.417	0.029	0.514	0.029
		100	9.72	0.896	0.006	0.940	0.015	0	0.016
C	1-benzylpiperazine	1	8.53	0	0.134	0.116	0.039	0	0.140
		3	8.73	0.163	0.120	0.174	0.032	0	0.124
		10	9.02	0.772	0.011	0.671	0.020	0.100	0.023
D	1-(4-hydroxyphenyl)piperazine	1	8.09	0.061	0.283	0.639	0.080	0	0.294
		3	8.32	0.004	0.037	0.973	0.010	0	0.039
		10	8.63	0.395	0.130	1.000	0.001	0	0.130
E	1-(4-aminophenyl)piperazine	1	7.76	0	0.055	0.534	0.038	0	0.067
		3	7.5	0.153	0.123	0.615	0.050	0	0.133
		10	6.9	0.039	0.101	0.819	0.024	0	0.104
F	1-methyl-4-phenylpiperazine	1	8	0	0.063	0	0.037	0	0.073
		3	8.01	0	0.069	0	0.030	0	0.075
		10	8.02	0.679	0.071	0	0.040	0.679	0.081
		30	7.9	0.880	0.001	0.978	0.010	0	0.010
G	4-(4-methylpiperazin-1-yl)aniline	1	8.14	0	0.018	0.757	0.029	0	0.034
		3	8.26	0	0.040	0.731	0.021	0	0.045
		10	8.5	0	0.012	0.926	0.006	0	0.014
H	1-phenylpiperidine	1	7.73	0.001	0.069	0	0.028	0.001	0.075
		3	7.71	0	0.016	0	0.041	0	0.044
		10	7.75	0.250	0.159	0	0.063	0.250	0.172
I	4-phenylpiperidine	1	7.9	0	0.002	0.008	0.028	0	0.028
		3	8.28	0.388	0.148	0.000	0.038	0.388	0.153
		10	8.97	0.892	0.001	0.967	0.004	0	0.004
J	1-(4-methylphenyl)piperazine	1	7.72	0.235	0.078	0.004	0.035	0.231	0.085
		3	7.95	0.709	0.026	0.062	0.030	0.648	0.040
		10	8.4	0.891	0.005	0.033	0.034	0.859	0.034
		30	8.56	0.883	0.003	1.000	0.012	0	0.013
K	1-(3-methylphenyl)piperazine	1	7.82	0.060	0.070	0	0.025	0.060	0.074
		3	8.14	0.550	0.016	0	0.019	0.550	0.025
		10	8.66	0.892	0.012	0.766	0.125	0.126	0.126
L	1-(2-methylphenyl)piperazine	1	8.08	0	0.034	0	0.026	0	0.043
		3	8.31	0.514	0.006	0	0.028	0.514	0.029
		10	8.79	0.818	0.007	0.717	0.051	0.101	0.051
M	1-(3-hydroxyphenyl)piperazine	1	8.12	0	0.019	0.681	0.018	0	0.026
		3	8.38	0	0.115	0.658	0.035	0	0.120
		10	8.64	0.457	0.004	0.849	0.023	0	0.023

7.2 Figure S2.1: Therapeutic Windows for Piperazine Derivatives



7.3 Figure S3.1

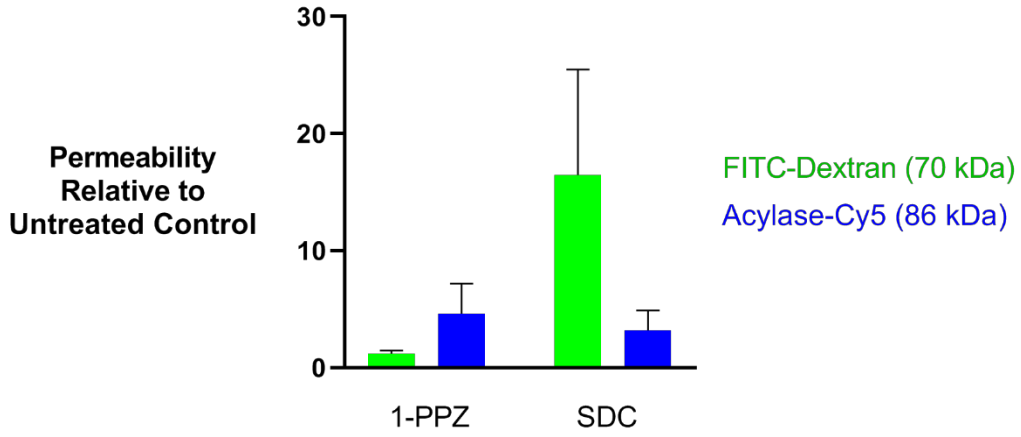


Figure S3.1: Macromolecules that are vulnerable to intracellular degradation may benefit from oral delivery via a paracellular permeation enhancer such as 1-phenylpiperazine. Mice were anesthetized, and their intestines were surgically exposed and injected with a solution containing either FD70 or Cy5-labeled acylase (an 86 kDa protein) and one of the following treatments: 65 mg/kg PPZ, 200 mg/kg SDC, or PBS (untreated control). After 2 hours, blood was collected and the fluorescence was measured at 490/520 nm for FD70 and 646/662 nm for acylase-Cy5. For the mice treated with PPZ, the permeability relative to control was higher for acylase-Cy5 than for FD70. In contrast, SDC better delivered FD70 compared to acylase-Cy5. n = 3, error bars represent SEM.