Expert Opinion

Chemical modification and formulation approaches to elevated drug transport across cell membranes

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Drug delivery across cellular barriers, such as intestinal, nasal, buccal, alveolar, vaginal, ocular and blood–brain, is a challenging task. Multiple physiological mechanisms, such as cellular organisation, efflux, and chemical and enzymatic degradation, as well as physicochemical properties of the drug molecule itself, determine the penetration of xenobiotics across epithelial cell layers. Limited intestinal absorption of many novel and highly potent lead compounds has stimulated an intense search for strategies that can effectively enhance permeation across these biological barriers. This review discusses some of the approaches that have been, and are currently being, investigated for transepithelial drug delivery. Transdermal drug delivery requires a separate discussion on its own and is thus outside the scope of this review article.

Keywords: biological barriers, drug delivery, paracellular, permeability, prodrugs, surfactants, transcellular, transepithelial, transporter

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1. Introduction

The last few years have witnessed an explosive growth in our understanding of genomics, proteomics, signal transduction pathways and disease processes. This information has led to the identification of cellular targets and aided in the design of xenobiotics. A spurt in natural product research has also contributed to the identification of libraries of potential compounds. However, delivery of these molecules systemically employing non-invasive approaches has become a major challenge for the pharmaceutical scientists. Oral delivery is by far the most favoured route for drug administration. However, stability, solubility and transepithelial permeability often limit oral bioavailability. Although the pH of the gastrointestinal fluids and enzymatic degradation in the intestinal fluids, enterocytes and hepatocytes raises stability concerns, the efflux of lipophilic compounds by various efflux proteins, such as P-glycoprotein (Pgp), breast cancer resistant protein and multi-drug resistant protein, expressed on the apical membrane of epithelial cells, present a formidable barrier to transcellular permeation of a large number of therapeutic agents [1,2]. In addition, tight junctions between epithelial cells act as a barrier to the diffusion of hydrophilic compounds across the paracellular pathway [3,4]. Physicochemical properties of the compound itself may also play a major role in intestinal drug absorption.

Whilst research aimed at improving absorption across the intestinal epithelium is underway, drug delivery across various other epithelial membranes such as nasal, conjunctival, rectal, buccal, vaginal and alveolar are also being actively pursued as potential alternatives to the oral route. Transepithelial drug delivery across these non-invasive routes can not only circumvent the harsh chemical and enzymatic conditions of the gastrointestinal tract but also avoid hepatic first-pass metabolism.

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However, efflux proteins and tight junctions limit drug diffusion across these epithelial cell layers [5-10].

Two other physiological barriers (i.e., blood–ocular and blood–brain) also present similar challenges. The blood–ocular barrier, consisting of the blood–retinal and blood–aqueous barriers, prevent access of xenobiotics from the systemic circulation into the ocular chambers [11]. The blood–brain barrier (BBB), on the other hand, prevents diffusion of many therapeutic agents from the systemic circulation into the brain tissue [12]. Thus, an investigation of the transepithelial permeation enhancement approaches for drug delivery to the ocular and brain tissues has also become an active research area. Approaches to enhance permeation across these physiological barriers have overall been very similar and thus will be discussed together.

The following sections will discuss the mechanisms of drug permeation across epithelial layers, the barriers encountered and the approaches that have been and are being evaluated to enhance transepithelial drug transport. Transdermal drug delivery is outside the scope of this review article as it needs a separate discussion.

1.1 Mechanism of drug permeation across epithelial cells

Movement of molecules across cellular membranes can take place by passive or active transport mechanisms. Passive transport involves either transcellular diffusion (i.e., diffusion across cellular membranes) or paracellular diffusion, where small hydrophilic drug molecules can pass through the intercellular tight junctions. Various properties such as lipophilicity, charge, shape, molecular radius and size determine the passive diffusion rate [13]. Active transport, on the other hand, involves transporters. In this process, the permeant binds to the carrier proteins and is translocated across cell membranes. Active transport differs from passive mechanism in that it involves a carrier protein, is energy dependent, it is saturable and it can transport molecules against a concentration gradient. Conversely, passive transport does not require energy, can only transport molecules down a concentration gradient and does not demonstrate saturation as carrier proteins are not involved. Facilitated diffusion is another type of carrier-mediated process that differs from active transport in that energy is not required and transport against a concentration gradient is not favoured.

Receptor-mediated endocytosis is yet another mechanism by which selective molecules are ferried across cellular membranes. In this process, substrates bind to a receptor and the receptor–ligand complex undergoes endocytosis. Vesicular transport is another mechanism by which drug molecules can penetrate biological membranes.

1.2 Barriers to epithelial transport

Tight junctions of the intestinal epithelium, BBB, retinal pigmented epithelium, corneal and conjunctival epithelium and blood–aqueous and blood–retinal barriers diminish paracellular diffusion of drug molecules. Tight junctions consist of multiple proteins that are signalling molecules, such as occludin, claudin, junction-associated membrane protein, membrane-associated guanylate kinase and ZO-1, ZO-2 and ZO-3 [3,14,15].

Diffusion of small hydrophilic molecules across transepithelial barriers expressing tight junctions is extremely limited [3,4,15]. Interestingly, hydrophilic essential cellular nutrients such as amino acids, small peptides, nucleosides, nucleobases and certain vitamins are efficiently translocated across these epithelial barriers by specific nutrient transport systems.

A vast number of therapeutic moieties are lipophilic in nature, enabling rapid partitioning into the lipoidal cell layers and transcellular diffusion across biological membranes. However, efflux proteins expressed on epithelial cell membranes can minimise transcellular diffusion of a wide range of lipophilic molecules [1,2]. Such efflux proteins include Pgp, multi-drug resistant proteins and breast cancer resistant protein. These energy-dependent efflux transporters can bind with substrates on the inner leaflet of the cell membrane, as well as with substrates in the cell cytoplasm, and transport them back into the extracellular milieu, effectively protecting the cell from these agents, as well as diminishing transcellular diffusion.

Barriers to passive diffusion are depicted in Figure 1. Other than diffusional resistance presented by tight junctions and efflux proteins, factors such as enzymatic degradation also play a role in lowering drug bioavailability. This is especially significant in the intestinal epithelium where a large number of CYP enzymes are expressed.

The presence of these physiological barriers, whilst protecting the body from external agents can cause limited systemic, ocular and brain drug penetration. With the availability of a number of potential therapeutic candidates that demonstrate low transepithelial permeability, the problem has become a subject of intense research among pharmaceutical scientists. The following sections review some of the transepithelial penetration enhancement strategies that are currently being pursued.

2. Permeation enhancers

The use of permeation enhancers or absorption-promoting agents has been widely investigated with respect to their ability to promote drug absorption across epithelial cell membranes. These agents act through various mechanisms such as the disruption of tight junctions and/or membrane fluidisation and can be classified under various categories as discussed in the following sections.

2.1 Surfactants

Historically, surface active agents or surfactants have been evaluated with respect to their transepithelial permeation-enhancing effects. These agents can disrupt biological membranes and lead to enhanced transcellular as well as paracellular transport. Sodium deoxycholate [16,17], glycodeoxycholate

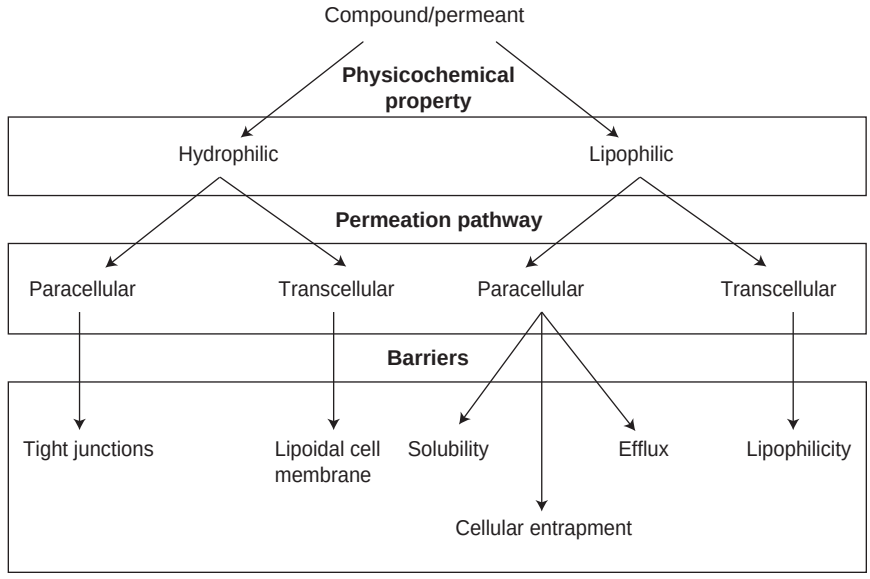

Figure 1. Barriers to transepithelial permeation of hydrophilic and lipophilic compounds.

[18-20] and sodium glycocholate [21-23], dioctylsulfosuccinate [24,25], Brij series [26-28], Tweens [27,29], Spans [30], dodecylmaltosides [28,31], sodium tauroglycholate [32] are just some of the surfactants that have been evaluated. Although these agents can enhance permeation of xenobiotics across oral, nasal, corneal, conjunctival, rectal and intestinal epithelia, on long-term use these agents may cause permanent damage to epithelial tissue [33]. Although these were initially considered to be very promising, cytotoxicity concerns have limited the use of surfactants as permeation enhancers [34,35]. Currently, surfactants are mainly employed as solubilisers rather than as permeation enhancers. However, it should be recognised, however, that even at very low concentrations some membrane permeation effects may be demonstrated by the surface active agents. In fact, quaternary ammonium compounds that are routinely employed as preservatives in ophthalmic formulations also fall under the classification of surfactants and have been observed to increase corneal penetration of topically applied compounds [16,36].

2.2 Chelators

The importance of extracellular Ca^{2+} in the maintenance of tight junctions in epithelial cells is well established [37]. Consequently, sequestration of extracellular calcium causes the disruption of tight junctions and prevents further expression of these junctions in confluent cell monolayers. EDTA has been classically employed in both *in vitro* and *in situ* experiments to demonstrate the effect of chelators on tight junction assembly, causing an enhancement of paracellular transport [38]. However, feasibility of modulating cellular tight junctions *in vivo*, using chelating agents, has not been clearly demonstrated. Non-specific chelation of potent and essential metal ions, variability in drug absorption and delivery to the desired site of action are some of the concerns that have limited the application of chelators as permeation enhancers.

2.3 Polymers

Recently, several polymers have been observed to possess permeation-enhancing properties. These agents act through various mechanisms, ultimately leading to the reversible opening of tight junctions or membrane fluidisation. Chitosan and pluronics probably hold the greatest potential as absorption-promoting polymers and are discussed in the following sections.

2.3.1 *Chitosan*

When protonated (pH < 6.5), chitosan (a non-toxic, biocompatible polymer) is observed to increase the paracellular permeability of peptide drugs across mucosal epithelia [39]. As chitosan is only soluble in acidic environments, its potential for enhancing absorption in the small intestine is limited. Chitosan derivatives, such as *N*-trimethyl chitosan chloride (TMC), a partially quaternised derivative of chitosan, is soluble in neutral and basic environments. TMC is prepared by the reductive methylation of chitosan. The degree of quaternisation can be altered by changing the number of reaction steps or reaction time. *In vitro* studies performed on Caco-2 cell monolayers demonstrated a pronounced reduction in the transepithelial electrical resistance (TEER) in the presence of TMC, an indication of the opening of tight junctions [40]. TMC can enhance permeation of hydrophilic compounds, such as [^{14}C]mannitol and [^{14}C]PEG 4000, across Caco-2 cell monolayers. Confocal laser scanning microscopy confirmed transport of large hydrophilic compounds via the paracellular

route: demonstrating disruption of tight junctions. Chitosan was observed to induce redistribution of the cytoskeletal F-actin, which leads to the opening of the tight junctions [40]. The degree of quaternisation of the polymer also plays an important role in its absorption-enhancing properties, especially in neutral environments where chitosan is ineffective as an absorption enhancer [40,41].

Younessi *et al.* evaluated a new quaternised chitosan, triethyl chitosan (TEC) [42]. *Ex vivo* experiments employing everted rat colon sac were conducted to determine the effect of TEC on the penetration of different molecular weight hydrophilic compounds (e.g., sodium fluorescein and brilliant blue) through colonic epithelia. These studies indicated a significant rise in the absorption of both of these compounds in the presence of TEC relative to chitosan. TEC, a cationic polymer, thus facilitates the permeation of sodium fluorescein and brilliant blue across the tight junctions [42].

In another study, Schipper *et al.* [43] demonstrated that chitosans are not taken up intracellularly, although they may bind tightly to the epithelium. In these studies, chitosans were observed to enhance apical, but not basolateral, cell membrane permeability, supporting the argument that chitosan affects the paracellular diffusional pathway. The authors also confirmed that chitosan induces a redistribution of cytoskeletal F-actin and the tight junction protein ZO-1. Interestingly, the addition of negatively charged heparin inhibited the absorption-enhancing effects of the chitosans, suggesting that these effects may be mediated via their polycationic nature. Moreover, the degree of acetylation had a significant effect on apical membrane permeability and tight junction structure of the Caco-2 cell monolayers [43].

Zerrouk *et al.* demonstrated that chitosan was more potent than cyclodextrins in enhancing glyburide permeability across Caco-2 cells. Cyclodextrins and chitosan demonstrated synergistic effects when employed as permeation promoters [44].

In vivo studies also demonstrated that TMC can elevate the absorption of the peptide drugs buserelin and octreotide after intraduodenal or jejunal administration [40]. Thanou *et al.* demonstrated that TMC improves the intestinal permeation of the peptide drug buserelin *in vitro* (Caco-2 cells) and *in vivo* (rats) [45]. Permeation was dependent on the degree of trimethylation of TMC. *In vivo* data demonstrated increased buserelin bioavailability (from 0.8 to 6 – 13%) following intraduodenal co-administration with 1.0% (w/v) TMCs.

A number of other studies also demonstrate the potential of chitosan derivatives as transepithelial absorption promoters [46-51].

2.3.2 Pluronics

Pluronics are block copolymers of ethylene oxide (EO), propylene oxide (PO) and EO. Each block can be of various chain lengths yielding different classes of pluronics and are represented by the formula $EO_xPO_yEO_z$, where x, y and z are the number of EO, PO and EO units, respectively. These compounds demonstrate surfactant-like properties, including membrane fluidisation. Demina *et al.* [52] studied the ability of 19 amphiphilic copolymers, including hyperbranched polyglycerols, pluronic and Brij surfactants, to accelerate flip-flop and permeation of the antitumour drug doxorubicin (DOX) in liposomes. The authors observed that not only bulk hydrophobicity but also the chemical microstructure of the copolymer determines its membrane-disturbing ability. Copolymers containing poly(propylene oxide) caused higher acceleration of flip-flop and DOX permeation than polysurfactants with aliphatic chains. The effects of copolymers containing hyperbranched polyglycerol were more pronounced, relative to copolymers with linear polyethylene oxide chains, indicating that a bulky hydrophilic block induced additional perturbances in the lipid bilayer structure. A good correlation between the copolymer flippase activity and a linear combination of copolymer bulk hydrophobicity and the van der Waals volume of its hydrophobic block was observed [52].

Erukova *et al.* studied the effect of pluronics on the permeability of several weak acids and bases through lipid bilayer membranes [53]. Pluronics facilitated the permeation of comparatively large molecules (such as 2-n-undecylmalonic acid and DOX) across lipid bilayers, whereas the permeation of small solutes (such as ammonium and acetic acid) remained unaffected. Translocation of large hydrophobic anions (tetraphenylborate) was also accelerated. The permeation-enhancing effect of these polymers was found to correlate well with the content of PO units, as well as with the proportion of poly(propylene oxide) block in the copolymer. The researchers proposed that pluronics accelerate the processes of solute diffusion within lipid bilayers (in a structure-dependent manner) rather than influencing the rate of solute adsorption/desorption on the membrane surface [53].

These copolymers can also modulate the activity of Pgp. Bromberg *et al.* evaluated microgels composed of crosslinked copolymers of poly(acrylic acid) and pluronics as possible permeation enhancers for DOX transport in Caco-2 cell monolayers [54]. Pluronics were chosen to represent the most hydrophobic (Pluronic L61 and L92 with average compositions of $EO_3PO_{30}EO_3$ and $EO_8PO_{52}EO_8$, respectively) and the relatively hydrophilic (Pluronic F127 with the average formula of $EO_{99}PO_{67}EO_{99}$) block copolymers. The weight ratio of pluronic to poly(acrylic acid) in the microgels was kept at 45:55. The researchers observed that the overall cellular absorption of DOX was enhanced as a result of inhibition of Pgp-mediated DOX efflux. Moreover, TEER measurements demonstrated that the microgels lowered TEER to ~ 80% of initial values. However, these effects were completely reversible, indicating cell viability after incubation with microgels. No significant enhancement of [^{14}C]mannitol transport by microgels was observed, relative to Carbopol 934NF (control polymer). A MTT assay confirmed that the microgels were not cytotoxic at the concentrations tested [54].

Another study by Johnson *et al.* demonstrated that the Pgp inhibitory effects of PEG and pluronic P85 block copolymer

(P85) were evident regardless of whether the excipients were added to the mucosal side, the serosal side or to both sides of the tissue [55].

With polarised bovine brain microvessel endothelial cells (BBMECs) monolayers as an *in vitro* model of the BBB and Caco-2 monolayers as a model for the intestinal epithelium, Batrakova *et al.* investigated the effect of P85 on the transport of the Pgp-dependent probe, rhodamine 123 (R123) [56]. The investigators observed that at concentrations below the critical micelle concentration, P85 inhibited Pgp efflux systems of the BBMEC and Caco-2 cell monolayers, resulting in an increase in the apical to basolateral permeability of R123. In contrast, at high P85 concentrations, the drug was incorporated into micelles, entered the cells and was then recycled back out to the apical side. As a result, a decrease in R123 transport across the cell monolayers occurred [56]. P85 was observed to modulate transport of various other Pgp substrates across BBMEC and Caco-2 cells [57]. In another study, co-administration of 1% P85 with radiolabelled digoxin in wild-type mice raised the brain penetration of digoxin (a Pgp substrate) threefold, and the digoxin level in the P85-treated wild-type mice was similar to that observed in the Pgp-deficient animals [58].

Using a wide range of block copolymers, differing in hydrophilic-lipophilic balance, Batrakova *et al.* [59] also demonstrated that lipophilic pluronics with intermediate length of PO block (from 30 to 60 units) and hydrophilic-lipophilic balance of < 20 were the most effective at inhibiting efflux in BBMECs. The methods used by these investigators included cellular accumulation studies with the Pgp substrate R123 in BBMECs to assess Pgp activity; luciferin/luciferase ATP assay to evaluate changes in cellular ATP; 1,6-diphenyl-1,3,5-hexatriene membrane microviscosity studies to determine alterations in membrane fluidity; and Pgp ATPase assays using human Pgp-expressing membranes. Pluronics with intermediate lipophilic properties showed the strongest fluidisation effect on the cell membranes along with the most efficient reduction of intracellular ATP synthesis in BBMEC monolayers [59].

The effect of pluronics on Pgp-mediated efflux was also demonstrated in other studies [60]. Broad specificity of these block copolymers with respect to drug permeation and efflux inhibition, and their effects on tight junctions, may offer a new strategy for developing formulations with a high degree of transepithelial drug permeation.

Various other agents and systems, such as polycarbophil [61], cyclodextrins [62], supersaturated systems prepared with antinucleant polymers [63], superporous hydrogels and superporous hydrogel composites [64], low molecular-weight methoxypolyethylene glycol-block-polycaprolactone diblock copolymer [65] and pH-sensitive hydrogel nanospheres composed of poly(methacrylic acid)-grafted-PEG, have also demonstrated efficacy in enhancing transepithelial transport [66].

2.4 Fatty acids

Short- and medium-chain fatty acids (MCFAs) have also been successfully employed as absorption promoters. Sasaki *et al.* examined the bioavailability of glycyrrhizin (GL) via extravascular (i.e., oral, rectal and nasal routes) when administered alone or in the presence of an absorption enhancer [67]. When GL was administered orally as a solution (30 mg/kg), rat plasma concentration of GL was extremely low. Fatty acids that were co-administered orally with GL produced a marked increase in GL absorption in the following order: sodium caprate > sodium laurate > sodium caprylate > sodium oleate.

In another study, Mori *et al.* evaluated the effect of labrasol and gelucire 44/14, which mainly consist of glycerides and fatty acids esters, and saturated fatty acids with various carbon chain lengths (C6 – C14) on the intestinal absorption of low molecular weight heparin (LMWH) as well as unfractionated heparin [68]. Anti-Xa activity (activity against clotting factor Xa) of the plasma samples collected was measured as a marker of the LMWH absorption. Administration of the LMWH formulation with labrasol, but not with gelucire 44/14, resulted in an increase in plasma anti-Xa activity above the critical level of 0.2 IU/ml that is necessary to produce anticoagulant activity. Saturated fatty acids also enhanced the intestinal absorption of LMWH, and the order of absorption-enhancing effect was C10 = C12 > C14 > C16 > C8 > or = C6. These results demonstrated that the absorption-promoting effect of the saturated fatty acids varies with the carbon chain length [68].

Kimura *et al.* investigated the effect of MCFAs on intracellular calcium (Ca^{2+}) levels and actin filaments in the Caco-2 monolayer. A site-dependent increase in intracellular Ca^{2+} levels caused by decanoic acid (C10) at 13 mM was observed by the authors. The other MCFAs did not significantly increase the intracellular Ca^{2+} levels. In addition, morphological changes of actin filaments were induced by only C10 among C8 – C14. The area in which actin filaments were depleted corresponded to elevated intracellular Ca^{2+} levels. The results suggest that the mechanism of absorption enhancement by C10 could be different from that by the other MCFAs, or that C10 causes additional physiological changes although the mechanism of the enhancement is the same as for the other MCFAs [69].

Mixtures of vancomycin hydrochloride (VCM) with labrasol were prepared to improve the oral absorption of VCM. Administration of VCM solution to rat ileum at a dose of 20 mg/kg did not result in detectable plasma VCM concentration. However, formulations containing 50% labrasol resulted in a C_{max} of 5.86 ± 0.97 µg/ml and an $AUC_{0\text{-}6h}$ value of 16.06 ± 1.78 µg.h/ml. A dose-dependent decrease in the C_{max} and $AUC_{0\text{-}6\,h}$ values was observed when the absorption enhancer dose was decreased by 50%. It is evident from this study that formulations containing labrasol can significantly improve intestinal absorption of the hydrophilic macromolecular drug VCM [70].

However, as with surfactants, fatty acids may also cause irreversible damage to cellular membranes. In an attempt to elucidate the mechanism of action of palmitoyl carnitine chloride (PCC), an effective enhancer of intestinal transport of

hydrophilic molecules, Duizer *et al.* studied the relationship between absorption enhancement, cell viability and tight junction protein localisation in Caco-2 cells and the rat small intestinal cell line IEC-18 [71]. In both cell lines, PCC caused a dose-dependent decrease in TEER and a concomitant increase in mannitol and PEG 4000 permeability. Transport enhancement was accompanied by an elevation in apical membrane permeability and a reduction in cell viability. At higher PCC concentrations (≥ 0.4 mM), the distribution pattern of the tight junctional protein ZO-1 varied and cells were unable to recover viability. Although PCC is an effective absorption enhancer for hydrophilic macromolecules, lytic effects on the cell membrane and reduced cell viability correlated with transport enhancement [71]. Other reports also indicate a toxicity potential that is associated with the use of fatty acids [72].

2.5 Ultrasound and iontophoresis

Application of iontophoresis and ultrasound has also been investigated as strategies for enhancing transepithelial drug permeation. Although these techniques have been mostly evaluated for transdermal drug delivery, some studies have demonstrated their use in enhancing drug permeation into the ocular and brain tissues.

Iontophoresis is a non-invasive technique and employs the principle of charge repulsion for driving charged drug molecules through cellular layers (Figure 2). Eljarrat-Binstock *et al.* evaluated corneal iontophoresis of gentamicin sulfate in healthy white rabbits by using drug-loaded disposable hydroxyethyl methacrylate hydrogel probes and a portable mini-ion device [73]. The iontophoretic treatment was performed with a current intensity of 1 mA for 60 s only. Peak gentamicin concentrations in the cornea (363.1 ± 127.3 µg/g) and in the aqueous humor (29.4 ± 17.4 µg/ml) were reached at 0 and 2 h after the iontophoretic treatment, respectively. The peak gentamicin concentrations after a single iontophoresis treatment were 12- to 15-times higher than those obtained after gentamicin injection or after topical eye drop instillation. Eljarrat-Binstock *et al.* also demonstrated that low current iontophoresis using drug-loaded hydrogel can generate high drug concentrations in posterior eye segments [74].

In a separate study, Frucht-Pery *et al.* evaluated the penetration efficiency of gentamicin into the rabbit cornea using iontophoresis with a hydrogel–gentamicin-containing probe [75]. Of 10 groups of 6 rabbits (one eye per rabbit), 8 underwent corneal iontophoresis with soft stable hydroxyethyl methacrylate hydrogel discs (80% water content) loaded with gentamicin sulfate, which were mounted on an iontophoresis probe. Current intensities were 0, 0.1, 0.3 and 0.6 mA, and durations of iontophoresis were 10 and 60 s. Two control groups received 1.4% topical drops of gentamicin every 5 min for 1 h (group 1) or subconjunctival injection of gentamicin 10 mg (group 2). Postiontophoresis, the concentration of gentamicin in the corneas ranged from high (88.60 ± 38.64 µg/ml) to very low (0.10 ± 0.89 µg/ml). Both the control groups and the groups that were treated with current intensity of ≥ 0.1 mA generated

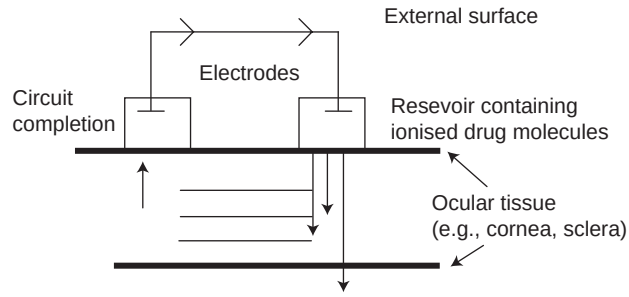

Figure 2. Schematic depiction of the iontophoresis technique.

therapeutic gentamicin levels in the cornea. The application of iontophoresis for 60 s or current intensity > 0.1 mA produced corneal gentamicin levels that were not substantially different from subconjunctival injection. A current intensity of ≥ 0.1 mA generated corneal gentamicin concentrations that were comparable to topical application, except when 0.6 mA was applied for 60 s ($p = 0.05$). Raising the current intensity or duration of iontophoresis significantly increased the gentamicin penetration into the cornea. Low or negligible drug levels were observed in the anterior chambers of rabbits. These studies demonstrated that iontophoresis using a hydrogel–gentamicin probe may deliver therapeutic concentrations of gentamicin in the cornea [75].

Halhal *et al.* demonstrated potential application of an iontophoresis device (Eyegate) in the treatment of various eye diseases [76]. A total of 17 patients received a penetrating keratoplasty at various intervals before active graft rejection was treated. Methylprednisolone sodium succinate 62.5 mg/ml was infused within the Eyegate ocular probe container and an electrical current of 1.5 mA was delivered for 4 min. After treatment, 15 of the 17 treated eyes (88%) demonstrated complete reversal of rejection. In two eyes, only a partial and temporary improvement was observed. The mean best corrected visual acuity of all 17 patients during the last follow-up visit was 0.37 ± 0.2 compared with 0.06 ± 0.05 before initiation of the iontophoresis treatment. The mean follow-up time was 13.7 months with a range of 5 – 29 months for the 17 patients. No significant side effects that were associated with the iontophoresis treatment were noted.

Mullenax *et al.* investigated iontophoresis as a method of accelerating drug absorption into the ocular anterior segment [77]. *In vivo* studies in rabbits assessed iontophoresis effects on the performance of dilators and constrictors. Direct current levels of 1 and 4 mA that were applied for 2-min durations yielded dilation time-history measurements. Drug samples processed through HPLC suggested minimal structural changes. Two dilating and constricting agents were used in a crossover design with 0.5 – 1.25 mA current levels and 20 – 60 s durations, with the dilation progress being recorded by digital photography. Initial studies showed faster, larger dilations and

quicker reversal by iontophoresis. Drug pH and HPLC retention times remained unchanged within this range, and the resistivity varied linearly as expected for increasing current. Tested ocular drugs exhibited no degradation when exposed to clinically useful iontophoretic currents.

A number of other studies have also demonstrated the applicability of iontophoresis for ocular drug delivery [78-83].

Cho *et al.* investigated whether ultrasound-induced mild hyperthermia (USHT, 0.4 Watts (W)/cm^2 at 41°C) could enhance drug absorption across brain capillary endothelial cells [84]. The researchers studied the effects of hyperthermia (41°C), USHT, Pgp modulator (PSC-833), and a combination of USHT and PSC-833 on the accumulation of the Pgp substrate R123 and non-Pgp substrates (sucrose, 2-deoxyglucose and antipyrine) in monolayers of primary BBMECs. USHT, through its thermal effect, produced a significant and comparable increase in R123 accumulation with PSC-833. The authors also demonstrated that USHT increases the permeability of hydrophobic (R123 and [^{14}C]antipyrine) and not hydrophilic molecules ([^{14}C]sucrose and 2-[^{3}H]deoxy-D-glucose). This enhanced permeability was reversible and size dependent, as USHT produced a much larger effect on cellular accumulation of [^{14}C]antipyrine than R123. Although USHT enhanced the membrane permeability, it did not affect the activities of Pgp or glucose transporters. USHT thus seems to be a reversible and non-invasive approach for raising BBB permeation of hydrophobic compounds, including Pgp-recognised substrates.

In another study, high-intensity focused ultrasound was employed to modify the normal feline and canine BBB permeability to a circulating vital dye; Evans blue [85]. Threshold doses (W s/cm^2) for focal permeability enhancement of the BBB in white matter and grey matter were: internal capsule (white matter) 340 – 680; thalamus (grey matter) ~ 1326; and caudate nucleus (grey matter) 2284 – 2952. In the presence of supra-lesioning ultrasound doses, the cross-sectional area occupied by the Evans blue was consistently larger than the attendant non-haemorrhagic lesion; thus suggesting that BBB changes may be inducible at sublesioning doses. The researchers concluded that high-intensity focused ultrasound may have a role in the treatment of brain tumours by either of the two mechanisms: i) direct, by the ultrasound, and ii) indirect, by an antineoplastic agent that is delivered via an ultrasonically modified BBB. Although ultrasound seemed to be a viable strategy to temporarily alter the barrier properties of the BBB, the mechanism of ultrasound action seemed to be due to changes in the permeability of neuronal membranes, leading to a chain of intracellular molecular reactions culminating in a subsequent general de- or hyperpolarisation of the membranes of neuronal populations, thereby altering the bioelectrical activity of the brain [86]. Such activity modulation can result in significant alteration in the normal brain function.

Zderic *et al.* studied ultrasound-mediated drug delivery across the corneal epithelium [87]. The effect of an application of 1-s bursts of 20 kHz ultrasound, at I(SAPA) of 14 W/cm^2 (I[SATA] of 2 W/cm^2), where I(SAPA) stands for spatial-average pulse-average intensity and I(SATA) stands for spatial-average temporal-average intensity, on corneal permeability of the antiglaucoma β-blockers atenolol, carteolol, timolol and betaxolol was evaluated. Corneal permeability in rabbits was raised by 2.6-fold for atenolol, 2.8 for carteolol, 1.9 for timolol and 4.4-fold for betaxolol (all p values < 0.05), after 60 min of ultrasound exposure *in vitro*. Differences between the treatment and control experiments were statistically significant after 10 – 30 min ultrasound exposure for all four drugs. However, ultrasound application seemed to produce epithelial disorganisation and structural changes in the corneal stroma.

In a subsequent study, Zderic *et al.* examined corneal epithelium cells that were exposed to ultrasound and observed them to be swollen and lighter in colour (indications of membrane rupture) relative to cells that were not treated with ultrasound [88]. It is likely that some of the surface epithelial cells sloughed off and cells in the inner layers of the epithelium seemed to be lighter in colour in some cases. In addition, vacuolar structures 3 – 10 µm in diameter were observed on the epithelial surface. No structural changes were observed in the stroma. Further studies are needed to optimise ultrasound parameters for a safe and effective treatment and careful investigation of the recovery of corneal structure and barrier function after ultrasound application, *in vivo*, is needed.

Although iontophoresis seems to be a promising method for improving ocular availability of drugs, particularly macromolecules, ultrasound seems to require further refinement so that cellular damage can be minimised.

The above sections highlight some of the integral formulation approaches that may be employed to achieve increased transepithelial penetration. Although, as discussed, many of these permeation-enhancing agents may not be useful for improving intrinsic transepithelial permeation characteristics of a drug moiety, they are routinely employed to increase drug solubility in the physiological fluids. An increase in solubility will result in higher concentration gradients and facilitate greater transcellular diffusion, leading to an overall increase in systemic bioavailability. Other formulation approaches attempt to decrease enzymatic degradation in the physiological environment to maintain higher free drug concentrations. In addition, dosage forms that by themselves may be able to cross the biological barriers have also been investigated. Microspheres [89] and nanoparticles [90,91], emulsions [92], microemulsions [93] and liposomes [94-101] are some of the dosage forms that have been successfully employed to enhance penetration across various epithelial barriers, including intestinal, nasal, blood–brain and the corneal epithelium. Another approach is the possibility of targeted drug delivery through the attachment of promoieties [102-104]. When Pgp-mediated efflux is identified to be responsible for poor absorption, inhibitors or competing substrates may be co-administered to modulate efflux [105]. Formulation components such as vitamin E D-α tocopheryl polyethylene glycol 1000 succinate

(TPGS), or specific inhibitors of Pgp and other efflux pumps, may also be included in the formulation. TPGS is also a known Pgp inhibitor. However, factors such as drug loading, drug entrapment, formulation feasibility, complicated manufacturing procedures, variability in absorption due to particle size and shape, stability in the gastrointestinal environment, formulation stability and so on present significant challenges to the formulator.

Thus, overall, formulation design provides significant opportunities for modifying transepithelial diffusion through improved solubility, decreased enzymatic degradation, diminished efflux and through the development of dosage forms that can penetrate these barriers better. However, so far, formulation approaches have not met with much success in enhancing intrinsic transepithelial permeation properties of drug moieties. This is primarily because of the toxicity issues that arise with the use of permeation enhancers. Ultrasound and ionotophoresis do show some promise but their applicability is site specific. Thus, for compounds whose absorption is limited by membrane permeation characteristics (e.g., hydrophilic xenobiotics), the use of formulation approaches may be limited.

2.6 Prodrugs

Chemical modification of the drug moiety is one of the most attractive options for enhancing the permeability of hydrophilic compounds across epithelial barriers that express tight junctions. Lipophilic prodrug derivatisation can impart sufficient permeability to the parent drug to enhance transcellular permeation. The derivatives may be cleaved within the cytoplasm of epithelial cells or may permeate both apical and basal membranes to reach the blood circulation. The promoiety is subsequently cleaved either in the blood or in the targeted tissue to liberate the free active drug [106]. One of the major disadvantages is that achieving the desired lipophilicity often requires compromising the aqueous solubility of the compound.

Recently, a transporter-targeted prodrug design has received significant attention. Epithelial cells express various nutrient transporters and receptors on both the apical and basal sides. Prodrugs or analogues designed to target these transporters/receptors can significantly enhance absorption of poorly permeating compounds. At the same time, both solubility and lipophilicity of the parent drug can be enhanced through proper selection of the promoiety. These prodrugs are recognised by the membrane transporters as substrates and are transported across the epithelia. An increase in lipophilicity also generates increased transcellular diffusion (passive). Figure 3 depicts the prodrug approach for transepithelial drug delivery. Although a number of nutrient transporters have been identified and characterised on various epithelia, peptide and amino acid transporters demonstrate the greatest promise with respect to drug delivery because of their wide substrate acceptability and high capacity.

2.6.1 *Peptide transporters*

Peptide transporters are perhaps the most versatile of all of the membrane carrier systems that have been discovered so far because of their capacity and broad substrate specificity. Two types of peptide transporters, PepT1 and PepT2, differing in tissue distribution, substrate specificity, affinity and transport capacity, have been cloned in mammals. These nutrient transporters accept substrates with a wide range of structures and are involved in the intestinal absorption of cephalosporins, 5-aminolevulinic acid [107], carnosine [108] and so on. Naruhashi *et al.* investigated the correlation between PepT1 mRNA and transport activity in the small intestine of fed and unfed rats [109]. A positive correlation was observed between PepT1 expression levels and cefadroxil permeability coefficients. The authors concluded that the intestinal transport of cefadroxil was directly proportional to PepT1 expression, suggesting that the expression level in the rat small intestine is the major determinant for the absorption of peptidomimetic compounds [109].

Studies have already demonstrated that enhanced transepithelial drug delivery may be obtained by targeting the peptide transporters through prodrug derivatisation. Valine ester prodrugs of acyclovir and ganciclovir, valacyclovir and valganciclovir, seem to be substrates of the intestinal peptide transporter, PepT1. Valine conjugation improved oral bioavailability of acyclovir by 3- to 5-fold [110] and that of ganciclovir by almost 10-fold [111]. Moreover, such derivatisation improved their aqueous solubility, allowing flexibility in formulation design [112].

Peptide transporters are also expressed on epithelial cells lining the retina [113] and lungs [114]. Recently, the functional expression of a peptide transporter on the rabbit corneal epithelium has been established [115]. Administration of peptidomimetic prodrugs of acyclovir (valacyclovir) led to enhanced corneal permeation of acyclovir and higher efficacy against herpes simplex virus type 1 corneal keratitis. The prodrug was rapidly cleaved in the corneal tissue to regenerate the parent drug acyclovir [115]. Similarly, valganciclovir and a dipeptide prodrug of ganciclovir (Val-Val-ganciclovir) interacted with the corneal oligopeptide transporter, which resulted in enhanced transport across the corneal epithelium and improved therapeutic activity against herpes simplex virus corneal keratitis [116].

2.6.2 *Amino acid transporters*

The absorption of amino acids from the gastrointestinal tract into the systemic circulation and subsequent uptake into tissues is mediated by amino acid transporters. Several subtypes of amino acid transporters have been defined based on their sodium dependence, charge and substrate specificity [117,118]. Amino acid transporters are expressed by almost all living cells and thus present a viable target for enhancing drug permeation. Its presence has been demonstrated on the corneal [119-121], retinal [122], intestinal [123-125] and airway [126] epithelia. The application of amino acid transporter-targeted drug delivery

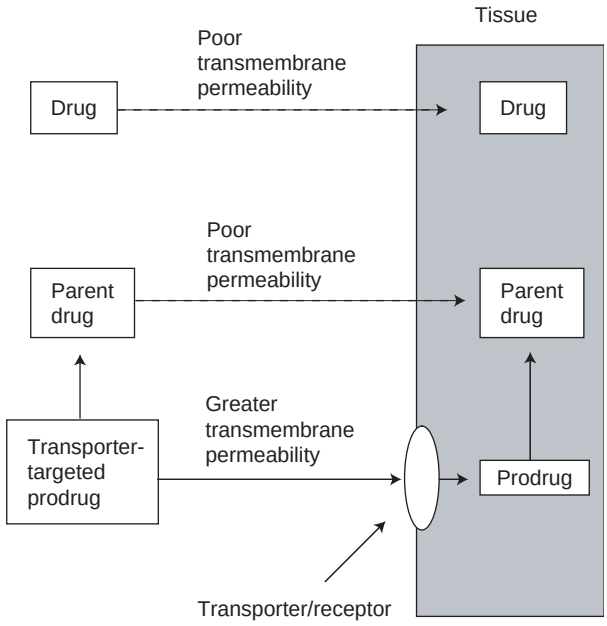

Figure 3. Schematic representation of the prodrug approach.

may provide enhanced absorption across these epithelial barriers as well as the BBB and blood–retinal barrier.

Several studies have demonstrated the use of large neutral amino acid transporters in drug delivery. Gabapentin (an analogue of GABA), is absorbed from the small intestine by the large neutral amino acid transporter. This transporter may be involved in CNS uptake of the drug [127]. Conjugation of L-glutamate with D-melphalan increased the absorption across the BBMEC monolayers via the involvement of glutamate transporters [128]. Other studies also demonstrate the use of amino acid transporter-targeted prodrug approach in enhancing drug transport across epithelial barriers [129-132].

2.6.3 *Receptor-targeted drug delivery approaches*

A number of receptors are expressed on the cell surface, some of which are responsible for the internalisation of nutrients such as folate, vitamin B_{12} and transferrin. These receptors are upregulated in rapidly dividing cells, such as cancer cells, and can thus be differentially targeted for drug delivery. In realising the importance of these receptors, several studies have been carried out with drug moieties that are conjugated to receptor ligands.

The folate receptor is an ideal candidate for tumour-targeted drug delivery because it is upregulated in many human cancers and because folate receptor density seems to increase as the stage/grade of the cancer worsens [133]. Conjugation of folic acid to various macromolecules results in receptor-mediated endocytosis into cells expressing the folate receptor. Both low molecular weight drugs and macromolecular complexes have been linked to folate receptors for tumour cell targeting. The conjugation of folic acid to macromolecules enhanced their delivery to folate receptor-expressing cancer cells *in vitro* in almost all cases, although *in vivo* studies have provided mixed results. Nevertheless, multiple results suggest that folate targeting can significantly improve the outcome of macromolecule drug therapy [134-136].

Recently, a number of studies have demonstrated the feasibility of transferrin receptor-targeted drug delivery [136-144]. Other receptors that were targeted for enhanced absorption and site-specific drug delivery include the asialoglycoprotein receptors, which are expressed specifically on the membranes of mammalian hepatocytes [145], and vitamin B_{12} receptors [146,147]. Besides these, a large number of receptors including insulin receptors [148] are also being actively investigated for gene therapy and targeting by researchers.

2.7 Evasion of P-glycoprotein-mediated cellular efflux by prodrug derivatisation

Pgp-mediated efflux has been recognised as a major factor limiting transepithelial permeation of a large number of lipophilic compounds. Recent reports indicate that prodrug derivatisation of lipophilic compounds may lead to circumvention of Pgp-mediated efflux. Two factors may be responsible for this phenomenon. In the first instance, when the prodrug is a substrate of a transporter, during its transition through the membrane leaflet it may be shielded from Pgp as the Pgp binding sites of the drug may no longer be exposed. As a result, Pgp-mediated efflux of the drug from the membrane leaflet will be significantly decreased, resulting in greater transepithelial penetration. The second reason behind circumvention of Pgp-mediated efflux could be that the prodrug no longer remains a substrate of Pgp or displays reduced affinity for the Pgp binding sites. Chemical modification can block or shield the Pgp binding sites of the drug moiety or result in configurational changes that reduces the prodrugs affinity for Pgp. This will thus lead to decreased interaction of the prodrug with Pgp and, hence, decreased efflux and increased permeation.

Differences in drug permeability from the lumen to blood and blood to lumen directions is a classical phenomenon that is associated with Pgp-mediated efflux. Drug permeation from the lumen to blood side is limited by Pgp expressed on the apical membrane (facing the lumen) of epithelial cells. As a result, permeation in this direction is decreased. On the other hand, drug penetration from the blood to lumen is not diminished as Pgp is not present on the basolateral (facing the blood side) membrane of most epithelial cells. Rather, Pgp aids in the translocation of substrates across the apical membrane. This results in drug permeability from the blood to lumen side or basolateral to apical side being much greater than from the apical to basolateral or lumen to blood direction.

Saquinavir (SQV) is a well-established substrate of Pgp. A recent study described the effect of transporter targeted SQV modification on its interaction with Pgp. In this study, Caco-2 cells, a model of the intestinal epithelium, were used

Table 1. Permeation-enhancing approaches: benefits and limitations.

Approach	Possible effects that lead to enhanced permeation	Limitations
Surfactants	Membrane fluidisation Inhibition of efflux proteins Improved solubility resulting in greater concentration gradients	May lead to membrane damage
Polymers	Opening of tight junctions Membrane fluidisation Improved solubility resulting in greater concentration gradients Inhibition of efflux proteins Protection from enzymatic degradation	May induce membrane damage
Fatty acids	Improved solubility resulting in greater concentration gradients Opening of tight junctions Membrane fluidisation	May lead to membrane damage
Ultrasound	Membrane perturbation	Cellular damage Non-specific effects
Iontophoresis	Charge repulsion acts as driving force	Patient compliance Side effect of chronic use Accessibility
Formulation design	Inhibition of efflux protein (co-administered agents) Use of particulate dosage forms for opening of tight junctions and protection from chemical and enzymatic degradation Emulsions/liposomal dosage forms for enhancing transcellular permeation and protection from chemical and enzymatic degradation Mucoadhesive dosage form for prolonged contact with epithelial tissue	Case-specific use Complex and cost intensive manufacturing protocols May be difficult to get the active and inhibitor to the same site at the same time
Prodrugs	Enhancement of transcellular absorption Transporter mediated translocation Improved solubility resulting in greater concentration gradients Evasion of efflux proteins	Case-specific use

to demonstrate the effect of prodrug derivatisation on permeability. Dipeptide prodrugs of SQV L-valine-L-valine-SQV (Val-Val-SQV) and L-glycine-L-valine-SQV (Gly-Val-SQV), targeting peptide transporters resulted in diminished interaction of the peptide prodrugs with Pgp, and resulted in an increased overall transport of SQV from the apical side to the basolateral side of Caco-2 monolayers. Transport studies demonstrated that Val-Val-SQV and Gly-Val-SQV exhibit enhanced apical to basolateral transport and diminished basolateral to apical transport compared with SQV [149]. The results clearly suggest that the affinity of SQV for Pgp had decreased.

In another study, Leontieva *et al.* [150] demonstrated significant differences in DOX, another well-known substrate of Pgp, and DOX-14-*O*-hemiadipate (H-DOX) accumulation in DOX-selected P388/R Pgp-positive drug-resistant murine leukaemia cell line (a subline of DOX-sensitive murine leukaemia cell line P388/S) in comparison to P388/S cells. Resistance to DOX is primarily due to Pgp-mediated efflux of DOX by the P388/R cells. When the uptake of DOX and H-DOX by the P388/R and P388/S cells were compared, DOX accumulation in P388/R cells was observed to decrease 38-fold compared with DOX uptake by the P388/S cells. This is a result of efflux of DOX by Pgp expressed on the P388/R cell membrane. On the other hand, only a fivefold decrease in H-DOX accumulation occurred, indicating a greater than sevenfold increase in H-DOX buildup in resistant cells. Increased accumulation of H-DOX in the DOX-resistant P388/R cells strongly suggest that H-DOX has a reduced affinity for Pgp compared with DOX. To verify the role of Pgp, the authors used ciclosporin A (CsA). This binds to the same Pgp site as DOX. Thus, in the presence of CsA, interaction of DOX with Pgp should be reduced, leading to an increased uptake of DOX. CsA did not alter the uptake or retention of DOX or H-DOX by sensitive P388/S cells (these cells do not express Pgp). However, incubation in the presence of CsA resulted in a 54-fold increase in DOX accumulation and only a 5-fold increase in H-DOX uptake in P388/R cells. The results thus verify the earlier observation that H-DOX has significantly decreased affinity for Pgp. This report also clearly demonstrates how chemical modification can lead to reduced Pgp-mediated efflux [150].

Several other studies also demonstrate the effect of chemical modification on Pgp-mediated drug efflux [151-155].

Thus, rational prodrug design can not only enhance passive diffusion, it can also lead to decreased recognition by Pgp and consequently reduce Pgp-mediated efflux and enhance absorption/bioavailability. The advantages and disadvantages

of the approaches that have been discussed are summarised in Table 1.

3. Conclusion

A number of strategies for enhancing transepithelial transport of poorly permeating compounds have been investigated over the last few years. However, careful attention needs to be paid to the nature of the drug moiety, the route of administration and co-administered substances, for successful selection of a particular approach. Surfactants and chelators initially showed a lot of promise as permeation-enhancing agents, but concerns over toxicity and specificity have dampened enthusiasm for these approaches. The use of fatty acids hold some promise, but enhancement of drug absorption seems to be highly case specific and caution needs to be exercised regarding epithelial cell damage. Among the polymers, chitosan is perhaps the most promising as it demonstrates very little, if any, toxicity at the concentrations that are necessary to produce enhanced permeability. However, all of these agents are useful for enhancing the solubility and stability of drugs in the physiological fluids and can lead to enhanced concentration gradients across epithelial membranes. This can result in enhanced drug diffusion across biological barriers. Thus, although these formulation approaches can improve systemic bioavailability, they cannot enhance intrinsic transepithelial penetration characteristics of the compound and may thus have limited use in situations where transmembrane diffusion is a rate-limiting factor. Prodrug derivatisation seems to be the only viable and versatile strategy for enhancing transmembrane permeation of drug molecules. As non-toxic ligands are employed in prodrug design, toxicity concerns are minimised with this approach. In addition, this strategy uses normal cellular transport mechanism to enhance transepithelial drug transport. Moreover, with proper prodrug design, both hydrophilicity and lipophilicity of drug molecules can be modulated to enhance transepithelial permeation of both lipophilic and hydrophilic compounds, respectively. In addition transporter-targeted prodrugs can also be employed to circumvent Pgp-mediated efflux and to enhance permeability, as well as solubility. With the discovery of a large number of membrane transporters/receptors, and the elucidation of their substrate specificity and structural configuration, rational prodrug design and targeting membrane transporters and receptors has become an active area of investigation.

4. Expert opinion

Transporter-targeted prodrug design seems to be one of the most viable options for enhancing drug permeation across epithelial barriers. However, to exploit the full potential of this approach, a clear understanding of substrate binding and translocation mechanism is warranted. In-depth research aimed at delineating transporter structure, binding domains and mechanism of interaction with substrates and structure–activity relationship is required. Moreover, the effect of commonly encountered polymorphism (of the transporters) on transepithelial transport needs thorough evaluation. Until the dynamics of transporter-mediated translocation is understood and factored into prodrug design, a combination of various transepithelial permeation-enhancement approaches will have to be adopted.

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