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Review

The effects of excipients on transporter mediated absorption

Jonathan Goole[a,b,*], David J. Lindley[a], Wyatt Roth[a], Stephen M. Carl[a], Karim Amighi[b], Jean-Michel Kauffmann[b], Gregory T. Knipp[a]

[a] Department of Industrial and Physical Pharmacy, College of Pharmacy, Nursing and Health Sciences, Purdue University, West Lafayette, IN, USA
[b] Department of Pharmaceutical Sciences, Université Libre de Bruxelles, Brussels, Belgium

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ABSTRACT

Traditionally most pharmaceutical excipients used for peroral dosage forms have been considered to be inert, although they have been known to play an important role in governing the release of the active pharmaceutical ingredient (API) required for the desired therapeutic effect. Of considerable interest is the emerging data demonstrating that many of these "inert" excipients may produce subtle changes that could directly or indirectly alter the activity of membrane-spanning proteins such as transporters. In this way, excipients could be altering the overall ADMET properties of an incorporated drug thereby affecting its intended therapeutic efficacy and/or enhancing adverse side effects. Therefore, given this recent evidence, it seems necessary to review what has been reported in the literature on interactions of excipients with human physiological entities, particularly transporters. As of today, safety/toxicity evaluations are typically based on the appearance of gross morphological changes rather than the effects on a cellular level, the ability of excipients in modifying the pharmacological activity of an active drug could lead to toxicity evaluation in routine for each additive used in oral formulations.

Further knowledge on this subject will enable formulators to make more rational decisions in dosage form design and will help answer the question of whether certain excipients should be considered active pharmaceutical components of formulations.

Contents

Abbreviations: ABC, ATP-binding cassette transporters; ADMET, absorption, distribution, metabolism, excretion, and toxicity; ACE, angiotensin-converting enzyme; AIDS, acquired immune deficiency syndrome; AMP, adenosine mono-phosphate; AP, apical; API, active pharmaceutical ingredient; ATP, adenosine tri-phosphate; BBB, blood–brain barrier; BBMEC, bovine brain microvessel endothelial cells; BCECF, 2′-7′-bis′(carboxyethyl)-5-(6′)-carboxyfluorescein; BCRP, breast cancer resistance protein; BL, basolateral; Caco-2, epithelial human Caucasian colon adenocarcinoma; CERT, ceramide transfer protein; CHO, Chinese hamster ovary; CMC, critical micellar concentration; CsA, cyclosporin A; CYP, Cytochrome P450; DM-β-CD, dimethyl-β-cyclodextrin; DNA, deoxyribonucleic acid; DPPC, dipalmitoyl phosphatidylcholine; DPPE, dipalmitoyl phosphatidylethanolamine; DRM, detergent-resistant membrane; EDTA, ethylenediaminetetraacetic acid; FASSIF, fasted state simulated intestinal fluid; FM, flippase model; GIT, gastrointestinal tract; HBSS, Hank's balanced salt solution; HDL, high density lipoprotein; HVC, hydrophobic vacuum cleaner; LPS, lipopolysaccharide; LTC_4, leukotriene C; MCT, monocarboxylate transporters; MDCK, Madin-Darby Canine Kidney; MDR, multidrug resistance; MRP, multidrug resitance-associated protein; NaTC, sodium taurocholate; NSAID, non-steroidal anti-inflammatory drug; P-gp, P-glycoprotein; Panc-1, pancreatic adenocarcinoma cell line; PEG, polyethylene glycol; PEPT, peptide transporter; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; SM, sphingomyelin; ™, Traded Mark; URL, uniform resource locator.

* Corresponding author at: Laboratoire de Pharmacie Galénique et Biopharmacie, Université Libre de Bruxelles, Campus de la Plaine, CP 207, Boulevard du Triomphe, 1050 Brussels, Belgium. Tel.: +32 2 650 5252; fax: +32 2 650 5269.
E-mail address: jonathan.goole@ulb.ac.be (J. Goole).

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1. Introduction

Pharmacokinetics and pharmacodynamics (PK/PD) are processes that encompass drug absorption, distribution, metabolism, excretion, and response (ADMET). Absorption is the rate and extent at which drugs reach the systemic circulation from the site of administration. Distribution is the process of reversible transfer of a drug to and from the site of measurement, usually the blood or plasma (Rowland and Tozer, 1989). Metabolism involves all the biochemical processes that result in a chemical change to the drug compound including both the metabolism in the gut wall, the liver, and the blood circulation. Excretion is the process in which the drug is eliminated from the systemic circulation into bile, urine, feces, sweat, and air (Rowland and Tozer, 1989). Collectively, these parameters (ADMET) significantly affect the overall bioavailability of selected compounds in target populations.

As it can be seen in Fig. 1, dissolution rate, solubility and permeability are the three primary factors that influence drug absorption following oral administration (Amidon et al., 1995). If the rate of dissolution is the rate-limiting step in drug absorption, any factor affecting the dissolution rate will have an impact on bioavailability (Tong, 2008). This rate is mainly influenced by the solubility of the drug, which depends on its physicochemical properties (Amidon et al., 1995).

On the other hand, the dosage form components also play an important role in the dissolution rate of a particular drug. In addition to the API, excipients or "inert" materials are added to formulations to aid in further processing of the materials into its final dosage form and to achieve an optimum absorption and therapeutic effect (Badawy et al., 2006). Most of "inert" materials commonly used in the design of oral dosage forms are assumed to not influence API absorption. However it has been reported recently that they can have an effect on specific transporters located in the GIT.

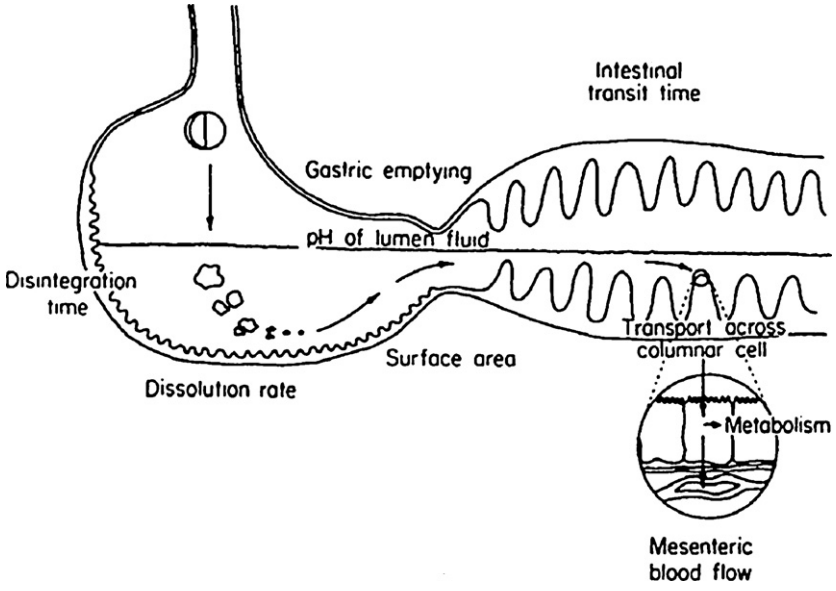

Fig. 1. Factors affecting the rate of absorption of drug from the gastrointestinal tract.

This review will attempt to summarize these interactions and their effect on the ADMET properties of pharmaceutical compounds. Since oral administration is likewise recognized as the major route of drug administration, we will focus our discussion only on excipients used in the formulation of oral dosage forms.

Although a plethora of information is available in the literature about the efficacy and safety profile of most excipients used for the design of oral dosage forms (not discussed in this review), little is known about the possible interactions of these compounds with transporter systems in the GIT and their effect on the ADMET properties of pharmaceutical compounds (Anderberg and Artursson, 1992). There have been several studies reporting that common excipients such as surfactants, lipidic compounds or solubilizing agents have been shown to mediate drug absorption by inhibiting efflux transporters that normally act to pump their substrates out of the cell. Although a large number of these studies have demonstrated increased absorption of these drugs and an overall improvement of their bioavailability through efflux inhibition, there has also been data suggesting that excipients could alter the influx process, thereby reducing the permeation of the active compounds. An incomplete understanding of these potential interactions prior to initiating the development of new oral dosage forms may lead to product failure in the clinical stages and significant financial loss to the innovator.

As such, due to the large number of excipients and the difficulty in understanding the underlying mechanisms by which excipients interact specifically with certain transporters, there has only been focus on a few of these interactions, mainly anticancer drugs and their efflux out of cells. This paper aims to review critical data in the literature available on excipient interactions with transporters to highlight the potential risks associated with the lack of understanding of the complexities of these interactions and their relevance to modulating drug ADMET.

2. Intestinal absorption modulating transporters

Absorption of nutrients/xenobiotics is governed and regulated by many processes. Of significant interest to scientists is delineating compound flux between the two major routes of drug permeation across membranes, paracellular and transcellular drug transport (Fig. 2) (Amidon et al., 1995). While ADMET involves transport/permeability across cellular barriers in numerous tissues, only intestinal absorption will be considered in this paper.

The epithelial cells within the GIT form intercellular junctional complexes that perform a variety of functions. These complexes include tight junctions, zonula adherens and desmosomes and form aqueous pores that allow the transport of dissolved solute only via passive diffusion processes. It has been reported that under physiological conditions, the passage across the tight junctions is restricted to molecules with molecular radii of less than 11 Å (Anderson and Van Itallie, 1995).

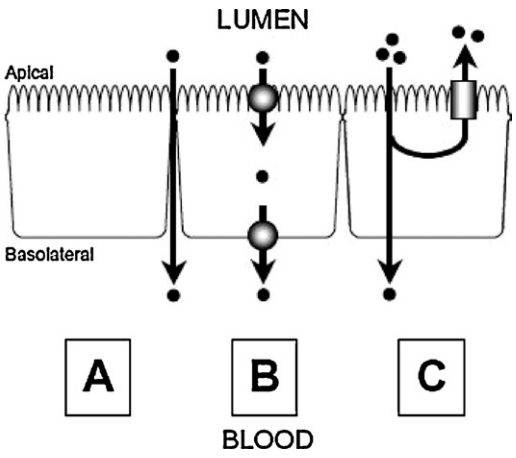

Fig. 2. Intestinal absorption pathways included paracellular transport (A), transcellular mediated transport (B), and passive diffusion with potential efflux process (C).

Generally, transcellular transport refers to the transport of nutrients/xenobiotics across the cell membrane. Although this can occur by a variety of different mechanisms, this type of transport involves the translocation of substrates within the extracellular space for further processing and transport inside the cell. Transcellular transport is a broad term that can be further divided into passive transcellular diffusion, endocytosis, and carrier-mediated processes (Rowland and Tozer, 1989; Hansen et al., 2005; Anderle et al., 2004).

3. Transporters overview

Depending on the direction and the category of the transported solutes, drug carrier-mediated systems can be classified as uniport, symport, or antiport transport mechanisms. Uniport is the carrier-mediated transport of a single solute molecule, while symport involves the transport of two solute molecules in the same direction, and antiport involves the transport of two solute molecules in opposite directions. Furthermore, tight junctional complexes between the cells in the GIT form an intercellular "zip-lock" seal that allows the cells to be polarized. In a general sense, this characteristic allows for a differential distribution of these transporters on the apical (AP) or luminal facing membrane as compared to the basolateral (BL) or blood facing membrane. In addition, the polarization of GIT epithelial cells allows for an intricate system where many nutrients/drugs are absorbed and subsequently metabolized or effluxed into the blood by a variety of mechanisms. Therefore, it is important to consider not only the transporters located on the apical membrane affecting initial absorption, but also those present within the cell and on the basolateral membrane affecting absorption and disposition (Rouge et al., 1996; Anderle et al., 2004). Furthermore, these transporters, on either membrane, can affect the uptake and/or efflux of drugs. Influx transporters act by facilitating the translocation of compounds into the cell while the efflux transporters pump materials out of the cell (Ho and Kim, 2005). On the apical membrane, efflux transporters prevent the entry of undesirable, potentially toxic materials into systemic circulation (Barbour and Lipper, 2008).

The complex nature of membrane physiology and the lack of predictive absorption methodologies is better understood when one considers the numerous functionalities of different drug transporters in mediating transcellular influx and efflux of xenobiotics. There are numerous classes of transporter proteins that have been identified to date, each with different substrate specificity, capacity and affinity, as well as specific tissue, cellular and temporal expression patterns (Bhardwaj et al., 2008). Several papers and book chapters have reviewed the influence of intestinal transporters on drug absorption and disposition and described each transporter in terms of expression, function, and regulation (Kunta and Sinko, 2004; Bhardwaj et al., 2008). In the present review, however, we will focus our discussion only on transporters that may be implicated in excipient–transporter interactions.

3.1. ATP-binding cassette (ABC) transporters

ABC transporters play an important role in the development of multidrug resistance (MDR) as these transporters are typically over-expressed in cancer cells. Moreover, MDR appears to be a major reason for failure of chemotherapy, since multiple

chemotherapeutic drugs of different classes are used to treat most cancers (Ambudkar et al., 2003). ABC transporters work to efflux their substrates by using the energy released by ATP hydrolysis for transport (Senior et al., 1995).

According to the Human Genome Organization, the ABC superfamily may be divided into seven subfamilies (A to G) among which, the ABCB and ABCC subfamilies contain the most widely investigated transporters influencing human intestinal absorption, P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRP), respectively (Higgins, 1991). Moreover, the isoform ABCG, also called the breast cancer resistance protein, has also appeared to be a key determinant of efficacy and toxicity of several substrates. The gene nomenclature on the ABC transporters can be found using the following URL: http://www.med.rug.nl./mdl/humanabc.html.

3.1.1. P-glycoprotein (P-gp; ABCB1)

P-gp was the first of the human ABC transporters to be described and characterized (Chen et al., 1986). P-gp is a virtually ubiquitous 170–180 kDa transmembrane efflux protein encoded by the MDR-1 gene in humans (Ambudkar et al., 2003). P-gp is located on the apical surface of mature enterocytes, canalicular membrane of hepatocytes, kidney cells, placenta barrier and endothelial cells of brain membrane, especially within rafts and caveolae characterized as detergent-resistant membranes (DRMs) (Balimane and Chong, 2008). P-gp has been shown to exhibit up to five substrate binding sites that allow the resistance of a wide variety of substrates (Loth et al., 2008).

Due to its multiple drug binding domains, P-gp possesses broad substrate/inhibitor specificity as it is involved in the transport of several hydrophobic, amphipathic, cationic and neutral molecules (Akiyama et al., 1988). P-gp homologues that transport drugs, such as human MDR1 and mouse mdr3 and mdr1, are designated as Class I or Class II. P-gp homologues that do not transport cytotoxic agents, e.g. human MDR3 and mouse mdr2, belong to Class III P-gp (Lo, 2000).

The only feature common to P-gp drug substrates appears to be that they are all hydrophobic, with a molecular weight of 200–300 Da (Ambudkar et al., 2003). Due to its ability to transport such a wide variety of drugs and its expression in most tissues, P-gp is widely recognized as the major efflux pump conferring multidrug resistance in human tissues.

It is important to notice that the level of P-gp expression increases from the upper to the lower part of the small intestine (Mouly and Paine, 2003). In order to understand how excipients might act to minimize the effects of P-gp, one must first understand the mechanism by which P-gp functions. P-gp has two proposed mechanisms of efflux: the hydrophobic vacuum cleaner (HVC) and the flippase model (FM). The HVC model assumes the presence of hydrophobic pores by which P-gp could efflux the substrate directly outside of the intestinal cell, avoiding any penetration of the compound into the cytoplasm (Gottesman and Pastan, 1993). Alternatively, the FM suggests that P-gp clears the substrate by interacting with it after its penetration inside the inner leaflet and flips it to the outer leaflet (Higgins and Gottesman, 1992).

Due to the large number of compounds that may interact with P-gp as either a substrate or inhibitor, it has been shown that drug–drug interactions may result in an increase in the absorption of one or more of these active agents, potentially leading to toxic side effects (Ho and Kim, 2005). In the same way, this kind of interaction may lead to decreased therapeutic efficacy. Since an oral dosage form is not exclusively composed of the therapeutic compound, excipients may interact with P-gp and so, increase or decrease the efficacy of the incorporated drug.

3.1.2. Multidrug resistance-associated protein family (MRP; ABCC)

To date, nine isoforms of MRP have been identified – MRP1 to MRP9 (Wang et al., 2004). However, only MRP1 and MRP2 are broadly expressed in the small intestine where they both act as efflux transporters (Peng et al., 1999). These isoforms of MRP show multiple binding sites for different drugs and have been described as gluthatione-dependent efflux pumps capable of transporting organic anion drug conjugates as well as intact anticancer drugs (Cole et al., 1992). Zimmermann et al. (2005) have also shown that the expression of MRP2 decreases from the duodenum to the colon.

3.1.3. Breast cancer resistance protein (BCRP; ABCG2)

Breast cancer resistance protein (BCRP) is an efflux transporter that belongs to the G subfamily of the human ATP-binding cassette transporter superfamily. Analysis of the BCRP peptide sequence reveals a single ATP/GTP-binding region and ABC signature motif within a relatively hydrophilic amino-terminal domain and a relatively hydrophobic carboxyl-terminal domain containing six putative transmembrane domains and four potential N-glycosylation sites (Doyle et al., 1998). BCRP is expressed at the apical membrane of the small intestine and colonic epithelium and on the canalicular membranes of hepatocytes and placenta syncytiotrophoblasts (Scheffer et al., 2000). Gutmann et al. (2005) have shown that the expression of BCRP is maximal in the duodenum and decreases progressively toward the rectum. BCRP may also be an important component of the blood–brain barrier, excluding xenobiotics from the brain (Eisenblatter and Galla, 2002).

3.2. Solute carrier transporters

The solute carrier transporters (SLC) are membrane transport proteins that include over 300 members organized into 47 families (Hediger et al., 2004). The SLC superfamily includes many functionally distinct transporters that include the proton dependent oligopeptide transporters (POT, SLC15A), organic anion transporters (OAT, SLC21A), organic cation transporters (OCT, SLC22A), nucleoside transporters (CNT, SLC28A; ENT, SLC29A) and the monocarboxylate transporters (MCT, SLC16A).

3.2.1. Proton/oligopeptide transporters (POT, SLC15A)

Due to the scientific improvements made in the pharmacogenomic area, a lot of peptide-like drugs are now therapeutically used in the treatment of numerous metabolic disorders such as diabetes, hypothyroidism, or in hormonal substitution therapy. Therefore, the POT-mediated transport of such compounds should be well-understood. The POT Superfamily consists of four distinct isoforms: peptide transporters 1 and 2 (PepT1 and PepT2) and peptide/histidine transporters 1 and 2 (PHT1 and PHT2). However, only PepT1, PHT1 and PHT2 have been shown to be expressed in the small intestinal enterocytes where PepT2 appears to be absent (Meredith and Boyd, 2000).

3.2.2. Organic anion transporters (OAT, SLC21A)/organic cation transporters (OCT, SLC22A)

Generally, a particular drug's solubility is greater in the ionized form, but has difficulty crossing biological membranes due to its high hydrophilicity. Conversely, the drug's non-ionized form is transported more readily across membranes due to its greater lipophilicity. However, it has been demonstrated that specific anion/cation transporters are involved in the absorption of ionic compounds across the intestinal membrane (Steffansen et al., 2004).

To date, OATs include five polyspecific isoforms that are capable of interacting with a broad range of therapeutic organic anions such

as NSAIDs, β-lactam antibiotics, antiviral drugs, diuretics, antitumor drugs and ACE inhibitors (You, 2004).

In addition to the transport of active drugs, it was demonstrated that OATs are involved in the intestinal transepithelial transport of excipients such as citrate in human (Sakhaee et al., 1992). Indeed, one or more organic anion transporters such as OAT2 and/or OAT4 mediate the Na^+-independent transport of citrate in Caco-2 cells.

On the other hand, to our knowledge, it was not reported that the OCTs are involved in the transport of compounds that can be used as excipients. Therefore, the description of this transporter family is behind the scope of this review.

3.2.3. Nucleoside transporters (CNT, SLC28A, ENT, SLC29A)

The nucleoside transporters consist of two distinct families that are characterized as either high affinity (SLC28) or low affinity (SLC29) according to the cellular transport of nucleotides (Kong et al., 2004). To our knowledge, it was not reported that the OCTs are involved in the transport of compounds that can be used as excipients. Therefore, the complete description of this transporter family is behind the scope of this review.

3.2.4. Monocarboxylate transporters (MCT, SLC16A)

To date, it has been established that the MCT family includes 14 members, each having unique tissue distribution. However, only isoforms 3-7 were shown to be expressed in the gastrointestinal tract by Northern blotting (Halestrap and Price, 1999).

4. Effect of excipients on transporters activity

Transporter proteins are integral proteins that function via either facilitated diffusion, or active, energy-dependent mechanisms to mediate transcellular flux of xenobiotics and nutrients (Amidon et al., 1995). Not surprisingly, the physicochemical properties of a compound greatly influence its interaction with transporters. For instance, excipient characterized by low water solubility cannot be dissolved in biological fluid and present a lower probability to interfere with the intestinal transport of the active drug. As explained previously, drug–drug interactions that influence transporters may affect the efficacy and/or the toxicity of a therapeutic agent. Moreover, some excipients may also influence the activity of transporters and thus, the ADMET properties of their substrates. Therefore, excipient selection should be an important factor to be considered in rational formulation design. We will focus our discussion on those that have demonstrated interactions with membrane transporters.

5. Classes of excipients

Due to their ease in manufacturing and high patient compliance, tablets are the most commonly used solid dosage form as compared to capsules or beads. APIs in formulations typically lack the properties necessary to be compressed into a tablet alone especially in term of flowing, filling and compression properties. In order to obtain adequate compressibility, the desired release properties, and the optimal stability, excipients are always included in the formulation.

Liquid dosage forms are convenient for patient populations who have difficulty swallowing tablets or capsules such as children, the elderly, and patients that are severely ill. Since disease compromised patients are more sensitive to therapeutic effect variations than healthy people, the study of possible interactions between transporters and excipients used in oral liquid dosage forms must be performed to avoid severe unexpected side effects.

5.1. Fillers

Fillers, also referred to as diluents, are added to solid formulations to provide bulk when the required dosage form components themselves are inadequate to give the tablet an appropriate weight and size. Fillers are also added to improve blend properties of materials such as their flowability and homogeneity as well as the solubility properties of the incorporated drug. The fillers commonly used are lactose, mannitol, calcium phosphate, starches, microcrystalline cellulose, sucrose, or sodium chloride (Mahato, 2007).

5.1.1. Transporter interactions

As discussed previously, compounds traverse biological membranes via different pathways (paracellular vs. transcellular) due to their diverse physicochemical properties. As such, utilizing these properties and a compound's known route of permeation, certain substrates can be used as 'markers' of specific transport pathways. For instance, mannitol is often used for determining monolayer integrity from intact tight junctional complexes such as in Caco-2 cells as it is transported predominately via the paracellular route. For instance, it has been shown that the apparent permeability coefficient of mannitol in Caco-2 cells (0.65×10^{-6} cm/s) can be increased by perturbing the tight junctions (Yee, 1997). Therefore, the inclusion of mannitol in solid dosage forms may result in competition with the API, if it is absorbed primarily via the paracellular pathway. This type of mechanistic interaction could theoretically be observed with any active substance absorbed through this pathway, e.g. nitroglycerin, isosorbide dinitrate, loperamide (Baird et al., 1997; Iga and Ogawa, 1997). Due to its poor water sorption properties, a mannitol is commonly used as diluent for lyophilized, buccal, and orodispersible preparations. Therefore, its potential competition with the API can lead to variable absorption of a variety of active pharmaceutical compounds used in this kind of formulations, e.g. nitroglycerin, isosorbide dinitrate, loperamide (Baird et al., 1997; Iga and Ogawa, 1997). However, as the absorption of mannitol occurs passively, competition with API remains theoretically putative.

5.2. Polymers

Polymers are broadly used in pharmaceutical formulations as binder, disintegrating agent, film-forming agents for coated tablets, for their adhesive properties, or as components of sustained-release and site-specific drug delivery systems. Due to their structural uniformity, the study of a broad range of polymers presenting different molecular weight, solubility or tensioactive properties should be conducted as they present a great risk of interaction with the biological membranes.

5.2.1. Transporter interactions

Depending on their molecular structure, polymers can act as surfactants or chelatants. Polyacrylates which are acrylic polymers exhibit both of these properties and demonstrate pronounced binding properties for bivalent cations such as zinc and calcium. Therefore, when included in formulations, they were able to prevent association of these ions with certain transporters, thereby inactivating their activities. These properties were utilized to enhance the Caco-2 permeation of the anticancer drug doxorubicin (Bromberg and Alakhov, 2003).

5.3. Surfactants

Surfactants are amphiphilic molecules that accumulate at interfaces between hydrophobic and hydrophilic compounds to reduce surface or interfacial tension. Due to the presence of both hydrophilic and hydrophobic regions, surfactants are commonly

used as emulsifying agents, solubilizing agents, detergents, and wetting agents. Two types of surfactants, ionic and nonionic, are commonly described in the literature. The increased hydrophobicity and the absence of charge make nonionic surfactants such as TPGS, Cremophor™, Pluronic®, polysorbate and Span® less toxic to biological membranes than ionic surfactants. In addition, nonionic surfactants possess a higher capacity to dissolve poorly water-soluble drugs. Because of the large number of surfactants used in the design of oral dosage forms, this discussion will focus only on those surfactants that are mentioned above.

5.3.1. Transporter interactions

Due to their amphiphilic structure, surfactants are able to interact with lipids in the cell membrane and change both its physical and functional properties. Indeed, surfactants have been found to reduce membrane viscosity and increase its elasticity (Martin et al., 1978), which enhances the absorption of compounds by both the paracellular and the transcellular routes (Tomita et al., 1988). In addition, changes in membrane fluidity can alter the microenvironment of the apically oriented transmembrane domains, and subsequently alter substrate recognition, substrate binding, and/or ATPase activity of efflux of transporters such as P-gp or the function of influx transporters such as PepT-1 (Ferte, 2000). It should be noted, however, that even though surfactants have a profound impact on membrane fluidity, none of the surfactants discussed below were reported to change the intrinsic passive permeability properties of the phospholipidic plasma membrane of the evaluated cell lines, regardless of the concentration range tested.

Vitamin E TPGS (D-α-tocopheryl polyethylene glycol succinate) is a water-soluble derivative of vitamin E containing a lipophilic, nonpolar head group (tocopherol succinate) and a hydrophilic polar PEG tail (Collnot et al., 2007). Using MDR1 transfected G185 cells, a derivative from the Swiss mouse embryo cell line NIH3T3, Dintaman and Silverman (1999) demonstrated that the presence of TPGS increased the sensitivity of the G185 cells to doxorubicin, vinblastine, paclitaxel, and colchicine and decreased the BL–AP permeability in a concentration-dependent manner from 0.001% to 0.0025% (w/w) TPGS. Above this concentration range, TPGS affected cell viability. Based on this data, it was hypothesized that TPGS increases the uptake of P-gp substrates by inhibiting the P-gp efflux process (Dintaman and Silverman, 1999). Moreover, they showed increased cellular concentrations of doxorubicin, vinblastine, paclitaxel, and colchicine even though TPGS was below its critical micelle concentration of 0.02% (w/w) in water at 37 °C, which suggests that micelle formation is not responsible for P-gp inhibition (Dintaman and Silverman, 1999). The exact mechanism by which TPGS acts on P-gp remains unclear; however, it seems that the structure and concentration play a major role in the P-gp inhibition activity of TPGS.

In a related study, uptake experiments using TPGS and 5-fluorouracil, which is not a substrate for P-gp, showed that TPGS does not affect cellular accumulation of 5-fluorouracil in both the NIH3T3 and G185 cell lines. This confirms the previous hypothesis that TPGS 1000 can reduce the ATPase activity of P-gp in a dose-dependent manner, but only in the presence of P-gp substrates (Collnot et al., 2007). Comparable observations in terms of P-gp inhibition, membrane fluidity and ATPase activity have been reported in Caco-2 cells for a similar conjugate, PEG-cholecalciferol polyethylene glycol succinate (CPGS) (Zhao et al., 2008).

Further evidence of surfactant mediated P-gp inhibition was demonstrated in a digoxin clinical trial in which twelve healthy human volunteers were administered immediate-release digoxin capsules 3 times a day. The bioavailability of digoxin, a P-gp substrate, was increased by 20% when the API was administrated with Cremophor™ RH40 (polyoxyl 40 hydrogenated castor oil), compared to surfactant free formulation (Tayrouz et al., 2003). In addition, *in vitro* experiments have shown that Cremophor™ RH40 inhibits both P-gp and the subfamily CYP3A in a concentration-dependent manner (Fig. 3), which explains the increase in bioavailability of P-gp substrates *in vivo* (Wandel et al., 2003). Moreover, Cremophor™ EL (polyoxyl 35 castor oil) was also shown to increase membrane fluidity resulting in the inhibition of P-gp and MCT in Caco-2 cells. It also increased AP–BL transport and decreased BL–AP transport of rhodamine 123 and benzoic acid, two well-known substrates of P-gp and MCT, respectively (Rege et al., 2002). It should be noted, however, that even surfactants within the same family can have dramatically different effects on xenobiotic uptake. This is illustrated by the fact that Cremophor™ EL increased the uptake of mitoxantrone in BCRP transfected MDCK-II cells, but Cremophor™ RH40 did not (Yamagata et al., 2007a).

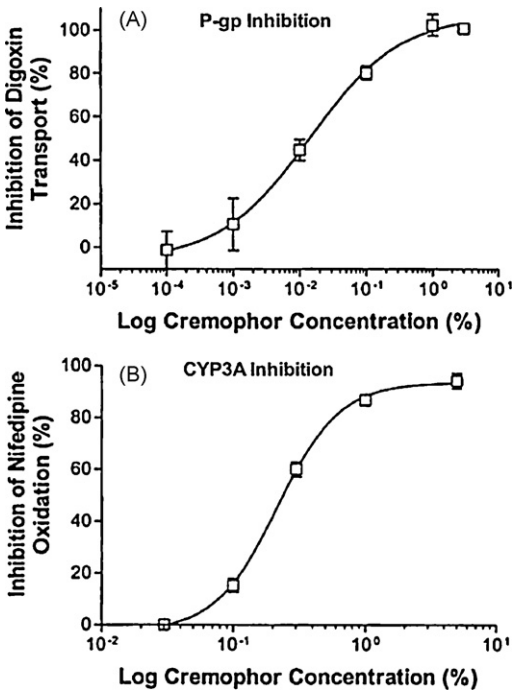

Fig. 3. Inhibition of P-gp mediated, tritium-labeled digoxin transport in Caco-2 cells (A) and CYP3A-mediated nifedipine oxidation in human liver microsomes (B) in the presence of increasing concentrations of Cremophor™ RH40 ($n = 3$) (from Wandel et al., 2003).

Polysorbate 80, another well-known nonionic surfactant, was able to inhibit P-gp mediated efflux of rhodamine 123 in a concentration-dependent manner (from 0.01 to 1 mM) that resulted in both increased AP–BL permeability and decreased BL–AP permeability in Caco-2 cells (Rege et al., 2002). Like Cremophor™ EL, polysorbate 80 altered the efflux properties of P-gp by fluidization of the plasma membrane. The alteration of the membrane was caused by monomeric surfactant molecules, since it was previously demonstrated that the apparent permeability coefficient in the BL–AP direction did not continue to decrease when the concentration of polysorbate 80 was greater than the CMC in HBSS (48–53 μM range) (Nerurkar et al., 1997).

In contrast, further experiments conducted by Katneni et al. (2007) concluded that polysorbate 80 did not inhibit P-gp mediated efflux. In the experiments conducted by Rege et al., polysorbate 80 was added to both the apical and basolateral chamber. Therefore, the carried-mediated process involved on each side of the cell monolayer could not be visualized. When polysorbate 80 was placed only in the basolateral chamber, the BL–AP transport of digoxin was inhibited whilst polysorbate 80 in the apical chamber did not change digoxin permeability (Katneni et al., 2007). As P-gp is located in the apical side, these observations suggest

Table 1
Effect of Pluronic® P85 on kinetic parameters of ATP-P-gp interaction.

Treatment	V_{max} (nmol mg^{-1} min^{-1})	K_m (mM)	V_{max}/K_m
Assay buffer	2.4 ± 0.36	0.4 ± 0.08	6
0.001% P85	2.4 ± 0.41	0.7 ± 0.05*	3.4
0.01% P85	2.4 ± 0.22	4 ± 0.9*	0.6
0.1% P85	1.1 ± 0.25*	0.9 ± 0.17*	1.2
1% P85	2.4 ± 0.33	0.4 ± 0.07	6

* $p < 0.05$ (from Batrakova et al., 2004).

a role for another efflux transporter, located in the basolateral side that is responsible for digoxin secretion in Caco-2 cells. However, more experiments should be conducted as digoxin might not be the only P-gp substrate having apical efflux mediated by another transporter. Indeed, another explanation could be that polysorbate 80 may be possibly taken up from the cell from the basolateral side and may gain access to the transport proteins on the apical membrane via intracellular space.

In agreement with the results observed for Cremophor™, polysorbate 20 was the only surfactant in the polysorbate family to increase the uptake of mitoxantrone in BCRP transfected MDCK-II cells, which is further evidence that structure specificity plays an important role in surfactant mediated inhibition (Yamagata et al., 2007a). These observations were confirmed *in vivo* as polysorbate 20 was able to increase the oral bioavailability of topotecan in wild-type mice, compared to BCRP (−/−) mice. It should be noted, however, that P-gp mediated efflux was inhibited by both polysorbate 80 and polysorbate 20 derivates in P-gp transfected MDCK cells, as observed with Cremophor™ RH40 and Cremophor™ EL (Yamagata et al., 2007b). Therefore, it seems that the activity of a surfactant depends on both its molecular structure and on the structural conformation of the specific transporter.

Pluronic® block copolymers, poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide), are other potent nonionic surfactant inhibitors of both P-gp and MRP (Miller et al., 1999). Pluronic® P85 was able to increase the AP–BL transport of some P-gp substrates such as fluorescein, doxorubicin, etoposide, and taxol both in Caco-2 cells and bovine brain microvessel endothelial cells (BBMEC). In addition, uptake was improved in a concentration-dependent manner with the optimal concentration of Pluronic® P85 below its CMC (<0.03%) (Batrakova et al., 1999). Concentrations higher than the CMC could involve a decrease in the AP–BL transport as the active substance could be incorporated into micelles. Similar results have been observed in the human pancreatic adenocarcinoma cell line (Panc-1), which is known to express only MRP. The AP–BL transport of fluorescein was increased when the API was incubated with Pluronic® P85 at a concentration lower than its CMC (Miller et al., 1999). Since the transport of rhodamine R123 was not affected, it seems that the response observed with fluorescein is attributable to the inhibition of MRP (Miller et al., 1999). Due to the fact that Pluronic® P85 could inhibit both P-gp and MRP, it was hypothesized that the mechanisms involved were due to the similar ATP-binding structure of the transporters. The rate of ATP hydrolysis in P-gp and MRP expressing membranes in the presence of Pluronic® P85 was determined using Mg^{2+} ATP as a substrate (Tables 1 and 2).

Table 2
Effect of Pluronic® 85 on kinetic parameters of MRP1- and MRP2-ATP interactions.

Treatment	V_{max} (nmol mg^{-1} min^{-1})	K_m (mM)	V_{max}/K_m
MRP1 control	4.2 ± 0.86	0.43 ± 0.08	9.8
MRP1 in 0.1% P85	3.3 ± 0.6*	0.43 ± 0.07	7.7
MRP2 control	2.7 ± 0.51	0.3 ± 0.05	9.2
MRP2 in 0.1% P85	1.9 ± 0.37*	0.49 ± 0.07	3.88

* $p < 0.05$ (from Batrakova et al., 2004).

The inhibition of ATP hydrolysis in P-gp by Pluronic® 85 was shown to be concentration-dependent as the minimal V_{max}/K_m ratio was obtained with only 0.01% of Pluronic® 85. On the other hand, the lowest V_{max}/K_m ratio was observed with a higher concentration of Pluronic® 85 (0.1%) for MRP transporters. Based on this data, it is apparent that certain excipients do not always act in the same concentration range, even on members of the same family. This phenomenon is most likely due to structural differences that change the vulnerability of each of the transporters to the surfactant. However, the mechanism of inhibition was found to be similar for both P-gp and MRP as Pluronic® seemed to induce ATP depletion in cells presumably by uncoupling of oxidative phosphorylation (Batrakova et al., 2004).

Moreover, even if the pathway of the interaction and the consequence remain similar, an ionic surfactant can involve another mechanism of ATP depletion. One example of this is the cationic surfactant Pluronic® L61, which interfered with ionic flux in the plasma membrane and caused compensatory ATP consumption by Na^+, K^+-ATPase, thus decreasing the intracellular ATP concentration and inhibiting P-gp (Krylova and Pohl, 2004).

5.4. Lipophilic compounds

Phospholipids – e.g. *lecithin* – and sterols – e.g. *cholesterol* – are often used with surfactants as emulsifying agents in the composition of liposomes. Moreover, cholesterol and phospholipid derivates such as sphingomyelin and ceramide, were found to be efficient in the formation of liposomes because they decrease the glass transition temperature which increases the fluidity and improves the stability of the liposomes during the manufacturing process (Socaciu et al., 2000). Liposomes are often administrated to increase the oral bioavailability of poorly water-soluble APIs such as polypeptides (Porter et al., 2007), immunoglobulins (Minato et al., 2003) or anticancer agents (Oku and Namba, 2005). Indeed, liposomes enhance the solubility of such lipophilic drugs and promote their intestinal absorption since cholesterol and phospholipids can be dissolved in the cell membrane.

5.4.1. Transporter interactions

Based on previous studies that have shown that an increase in membrane cholesterol can affect the fluidity of the membrane (Baggetto and Testa-Parussini, 1990) and decrease the activity of efflux transporters such as P-gp and MRP (Ferte, 2000), Cerf et al. (2007) have shown that an increased amount of cholesterol in the membrane downregulates MRP1 drug transport and ATPase activity. Because the amount of cholesterol could be carefully controlled, they used proteoliposomes in which drug transport and ATPase activity of MRP1 were preserved. They first observed that the ATPase activity of MRP1 was decreased by approximately 40% when the level of cholesterol in the proteoliposomes increased from 0 to 20–40%. The downregulation of the efflux transport was observed using proteoliposomes containing MRP1 and increasing amounts of cholesterol incubated with [^{3}H]LTC4 (150 nM, 100 nCi). The uptake of [^{3}H]LTC_4 into the liposomes was determined in the presence of 4 mM Mg-ATP or 4 mM Mg-AMP. Fig. 4 shows that only ATP is involved in the uptake of [^{3}H]LTC4. The uptake detected in the presence of Mg-AMP depended on the passive diffusion of cholesterol into the lipid bilayer and not on MRP activity. Moreover, in the presence of ATP, a molar ratio of 20% cholesterol significantly downregulated LTC_4 uptake, showing that the level of cholesterol could affect the functionality of the nucleoside-binding domains involved in ATP binding and hydrolysis (Cerf et al., 2007).

Shun and Liu (2007) have also demonstrated that cholesterol could improve the toxicity of some cytotoxic agents by affecting the

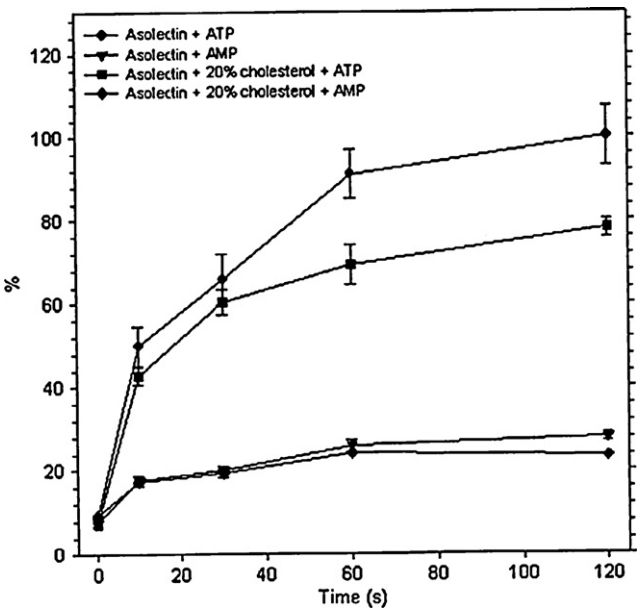

Fig. 4. Time-dependent uptake of [^{3}H]LTC4 into MRP1 proteoliposomes (from Cerf et al., 2007).

ATPase activity of P-gp in the plasma membrane. Due to the limited solubility of cholesterol in aqueous media, LDL, the natural carrier of cholesterol in the body, was used to obtain a sufficient concentration of cholesterol in cell culture medium. They first evaluated the concentration of cholesterol in the plasma membrane of human lymphoblastic leukaemia cells – CEM/T4 – and the drug-resistant cell line – CEM/VLB (Shun and Liu, 2007). It was demonstrated that the level of cholesterol was higher in the dug-sensitive CEM/T4 cells compared to the resistant CEM/VLB cell line. Interestingly, the level of cholesterol in the CEM/VLB cells reached the same value as that in the drug-sensitive CEM/T4 cells after incubation with LDL. These results help to corroborate the hypothesis according to which cholesterol changes membrane fluidity and could potentially reduce the activity of efflux transporters.

In order to visualize the result of this observation on the effective absorption of anticancer drugs, the uptake profiles of vinblastine and doxorubicin were determined in both CEM/T4 and CEM/VLB cell lines (Shun and Liu, 2007). It was shown that the intracellular levels of vinblastine and doxorubicin were significantly higher in CEM/T4 cells than in the resistant CEM/VLB cells. Moreover, after pre-treatment of the cells with LDL, the level of both anticancer drugs increased in both cell lines, showing an apparent decrease in efflux activity. Finally, Shun and Liu have demonstrated that ATPase activity of P-gp decreased in both cell lines following the incubation of 100 μg/ml LDL as a function of time (Fig. 5).

However, using Western blot analysis, the CEM/T4 cell line showed little expression of P-gp while the expression of P-gp remained higher in CEM/VLB cells regardless of LDL pre-treatment. These results suggest that cholesterol may affect the functionality of P-gp but not its expression in the resistant cell line CEM/VLB (Shun and Liu, 2007). This observation could be explained by the association of P-gp with cholesterol and sphingolipid-rich membrane microdomains called detergent-resistant membranes (DRMs) (Lavie et al., 1998). However, based on studies by London and Brown that showed cholesterol is responsible for the increased rigidity and reduced fluidity of the DRMs compared to surrounding membrane (London and Brown, 2000), Troost et al. (2004) have shown that the subcellular localization of P-gp in rafts was affected by an increase in cholesterol level in the plasma membrane which led to an increase in efflux activity.

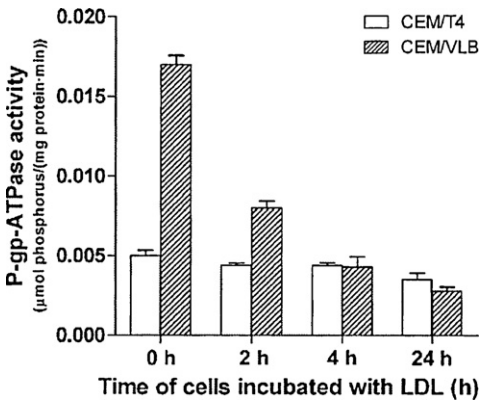

Fig. 5. The effect of LDL on P-gp-ATPase activity in the plasma membrane of CEM/T4 and CEM/VLB cells (from Shun and Liu, 2007).

To assess the presence of caveolin-1, a caveolar marker protein, in membrane microdomains, P-gp-over expressing L-MDR1 cells were fractionated. As shown in Fig. 6A the calveolin-1 control was found in both the sucrose dense fraction (fraction 5), which corresponds to DRMs, and detergent-soluble fractions (fractions 10 and 11). The immunoreactivity of caveolin-1 was undetectable upon cholesterol depletion in the DRM fraction, but reappeared after repletion. The immunoreactivity was detectable upon cholesterol depletion in the detergent-soluble fractions (10 and 11), however, it disappeared after repletion. Under normal conditions, P-gp is localized both in the DRM fraction and in the detergent-soluble fractions in significant proportion as compared with caveolin-1. Upon cholesterol depletion, P-gp was no longer expressed in the sucrose dense gradients suggesting that P-gp shifted into fractions 9 and 10. After cholesterol repletion, P-gp was redistributed in fraction 5 but, in contrast with the result observed for caveolin-1, remained expressed in fractions 9 and 10, even if the immunoreactivity decreased compared to the control (Fig. 6B).

The effect of cholesterol on P-gp function was determined by the efflux of verapamil in L-MDR1 cells and the control cell line LLC-PK1. Whereas in LLC-PK1 cells the intracellular level of verapamil did not change with concomitant cholesterol depletion, it increased by 59% in L-MDR1 cells. The observed reduction of transport function concurs with the dislocation of P-gp from DRMs to

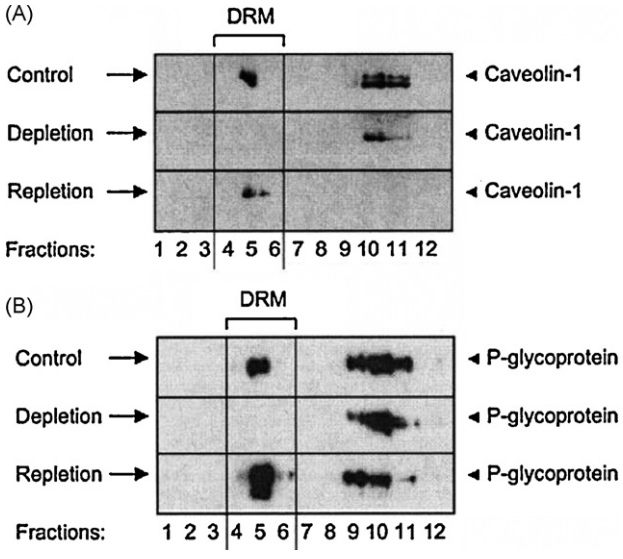

Fig. 6. Western blot analysis of discontinuous sucrose gradients for caveolin-1 (A) and P-glycoprotein (B) in L-MDR1 cells (from Troost et al., 2004).

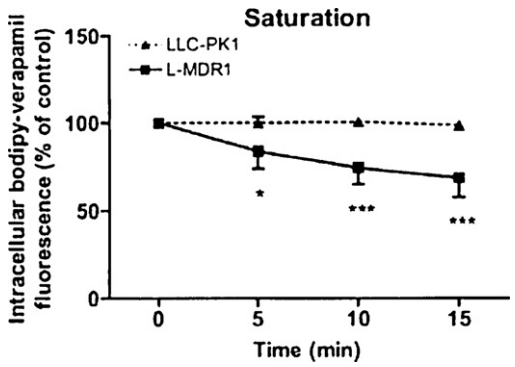

Fig. 7. Functional analysis of P-glycoprotein in LL-PK1 and L-MDR1 cells during cholesterol saturation with verampamil (1 μM) as a P-gp substrate (from Troost et al., 2004).

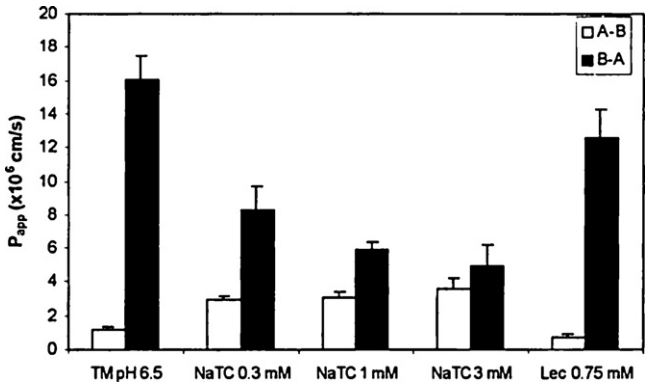

Fig. 8. Effect of lecithin (0.75 mM) and NaTC (0.3, 1 or 3 mM added at the apical side) on the absorptive (A–B) and secretory (B–A) transport of CsA (1 M). Bars represent average Papp value_SD ($n = 3$) (from Ingels et al., 2002).

detergent-soluble membranes. To confirm these results, it was also demonstrated that cholesterol saturation did not affect the level of verapamil in LLC-PK1 cells but significantly decreased in L-MDR1 cells (Fig. 7).

Since it is known that cholesterol can influence the rigidity of the plasma membrane, the idea that both MRP1 and P-gp require a specific lipid microviscosity to optimize their transport and ATPase activity cannot be excluded. In this way, exogenous cholesterol used in liposomes to treat some kinds of cancer could affect the intracellular drug concentration by modifying the localization and/or the morphology of efflux transporters through drug binding or ATPase activity. To date, it is nearly impossible to predict the exact influence of membrane cholesterol level on P-gp function as several studies have concluded. This could potentially be due to the presence of two functional populations of P-gp located in different regions in the plasma membrane (Barakat et al., 2005). The population located in the DRM region displays an optimal ATPase activity while the population in the solubilized-membrane fraction displays a lower ATPase activity. Moreover, Barakat and co-workers have shown that cholesterol depletion downregulated efflux transport in the DRM fraction but not in the solubilized-membrane fraction. As reviewed by Orlowski et al. (2006), the presence of P-gp in rafts and non-raft membrane domains also depends on the cell line and the fact that P-gp can shift from one domain to another *in vivo*. This inability to know exactly what happens in cholesterol-mediated P-gp efflux is evidence that further clarification must be provided to clarify the exact influence that cholesterol has when used as an excipient in liposomes to increase the bioavailability of the active ingredient.

Moreover, as reported by Takahashi et al. (2005), ABC efflux transporters are involved in the removal of cholesterol from the plasma membrane of peripheral cells. Indeed, the ubiquitous ABCG1 isoform mediates cholesterol efflux to pre-β-HDL and HDL (Vaughan and Oram, 2006). As it was previously shown that the sphingomyelin (SM) to cholesterol ratio influences membrane dynamics due to electron density, which depends on ordered DRMs in the plasma membrane (Pandit et al., 2004), Semple and co-workers have shown that the ABCG1-mediated efflux transport of cholesterol is correlated with cellular SM levels. To examine the effect of cellular SM content on the function of ABCG1, the mutant cell line CHO-K1 (LY-A), which has a missense mutation in the ceramide transfer protein (CERT), and its stable transformant cell line with human CERT cDNA (LY-A/CERT) were incubated with HDLl. Since the LY-A cell line has a CERT deficiency, the amount of SM in the plasma membrane of LY-A cells is lower than in their LY-A/CERT counterparts. Compared to mock-transfected LY-A and LY-A/CERT cells, the ABCG1-mediated efflux of cholesterol was significantly increased when the content of SM increased in the medium. Since sphingomyelin and cholesterol are both used in liposomes containing antitumor drugs (Semple et al., 2004), it seems useful to determine the level of both of these compounds in the plasma membrane after their release from the dosage form in order to correlate the possible influence of the sphingomyelin to cholesterol ratio contained in liposomes with therapeutic efficacy.

As sodium taurocholate (NaTC) is already known to enhance paracellular absorption by opening tight junctions (Johansson et al., 2002), it was used in orally administered liposomes to increase intestinal permeation of insulin (Degim et al., 2004). Further experiments showed that NaTC could also increase the transcellular absorption of certain APIs by affecting P-gp mediated efflux. This effect was discovered when Ingels et al. (2004) investigated the effect of simulated intestinal fluid on drug permeability across Caco-2 monolayers. The conventional transport medium for Caco-2 experiments is Hank's balanced salt solution, which is often buffered at pH 7.4. Since the pH of the intestinal lumen has been reported to be around 6.5 (Russel et al., 2003), the use of solutions buffered at higher pH values can give erroneous permeation results due to differences in ionization of the API. For this reason, fasted state simulated intestinal fluid (FASSIF) containing NaTC was investigated as a vehicle for the solubilization of the compound applied at the donor site in Caco-2 cells. ^{3}H-cyclosporin A (CsA) was used as a model drug to visualize the functional activity of P-gp efflux in Caco-2 cells. It was first demonstrated that the BL–AP transport of CsA decreased when transport medium was progressively substituted by FASSIF medium. This result suggested an inhibitory effect on efflux transporters by compounds present in FASSIF such as NaTC. Indeed, as shown in Fig. 8, the application of NaTC to the apical side of the monolayer resulted in a concentration-dependent increase of the absorptive and a concentration-dependent decrease of the secretory transport of CsA (Ingels et al., 2002).

Because they were shown to enhance the therapeutic efficacy of certain APIs, phospholipids have been increasingly used in liposomes to carry cancer chemotherapeutics such as epirubicin. Epirubicin is effluxed by Class I P-gp whereas Class III P-gp acts as a phospholipid translocase *in vivo*. Because there are overlapping physicochemical characteristics between the two different classes of P-gp, modulation of P-gp by phospholipids through substrate competition or other mechanisms may antagonize efflux properties and increase the cytotoxicity of epirubicin. Caco-2 cells were used to carry out the AP–BL and BL–AP transport of epirubicin in liposomes containing either dipalmitoyl phosphatidylcholine (DPPC) or dipalmitoyl phosphatidylethanolamine (DPPE). The flux of free epirubicin in the secretory direction was 2.29 times that of the absorptive direction. The DPPC and DPPE liposomal encapsulations markedly increased the intracellular accumulation of epirubicin by

both an increase in the AP–BL P_{app} (+72%/+107%) and a reduction of the BL–AP P_{app} (−45%/−52%), respectively. It was also observed that the intracellular concentration of epirubicin was higher in cells pretreated with empty liposomes than in the control group (Lo, 2000). Based on this data, it was hypothesized that phospholipids may enter the cell surface, induce a change in the composition and fluidity of the membrane, and thus modulate the activity of transporters such as P-gp. However, the modulation of P-gp by exogenous phospholipids may also be due to substrate competition. Because of the importance of the combined use of epirubicin with phospholipids, further studies should be conducted to elucidate the mechanism by which phospholipids act.

5.5. Polyethylene glycol

Polyethylene glycol (PEG) is an excipient commonly used in pharmaceutical formulations to increase the aqueous solubility of drugs intended for oral administration. These derivates exist in a variety of molecular weight grades, ranking from 200 to 35,000. Concentrations of PEG up to 40% (v/v) are considered safe and relatively non-toxic (Hugger et al., 2002a).

5.5.1. Transporter interactions

Polyethylene glycol (PEG) has been shown to interact with certain transporters; particularly efflux transporters such as P-gp (Hugger et al., 2002b). For instance, the efflux transport (BL–AP, S-to-M) of the P-gp substrate rhodamine 123 was significantly decreased when incubated with PEG 400, 2000 and 20,000 in Caco-2 cells. Moreover, it was demonstrated that the inhibitory effect on P-gp was concentration-dependant from 0.1 to 20% (w/v) (Shen et al., 2006). On the other hand, rhodamine 123 influx transport (AP–BL, M-to-S) was not significantly increased regardless of the concentration range (Fig. 9). Indeed, using an *in situ* closed loop method, an enhanced absorption of rhodamine 123 occurred across the rat jejunum when the concentration of PEG 20,000 increased from 1 to 20% (w/v) (Shen et al., 2006).

Another study illustrated the effect PEG 300 (20%, w/v) had on of the bidirectional transport of taxol and doxorubicin across Caco-2 cell monolayers compared to a PEG-free control (Hugger et al., 2002a). Although doxorubicin transport in the AP–BL direction did not increase with the addition of PEG 300 (20%, w/v), the BL–AP transport of the compound significantly decreased. In addition, taxol transport was substantially increased in the AP–BL direction and markedly decreased in the BL–AP direction. These results suggest that PEG 300 inhibits certain efflux transport systems (P-gp, MRPs) in Caco-2 cells that result in a net increase in taxol and doxorubicin permeability.

A study published shortly thereafter, in the same laboratory, demonstrated similar results in MDR1 transfected MDCK cells (Hugger et al., 2002b). The integrity of the monolayer of both Caco-2 and MDCK cells were confirmed by measuring mannitol flux after incubation with PEG of different molecular weights. The apparent reduction of the secretory transport seems to be correlated with the inhibition of efflux transporters.

As cholesterol was previously shown to inhibit the ATPase function of efflux transporters due to a decrease in the membrane fluidity (Baggetto and Testa-Parussini, 1990), it was postulated that PEG could also reduce the function of P-gp by affecting the membrane fluidity. It was observed that PEG could increase the rigidity of the plasma membrane of Caco-2 and MDR1-MDCK cells in a concentration range greater than 25% (w/v) due to a hydrophilic interaction with the polar head groups of the cell membrane (Hugger et al., 2002a,b). However, to date, no explanation has been provided to explain the apparent inhibition of the secretory transport of xenobiotics such as taxol and doxorubicin.

Wang et al. (2004b) have also demonstrated that PEG derivates such as PEG-32 lauric glycerides, PEG-35 castor oil, PEG-20 stearate, PEG-50 stearate, PEG-12 stearate and PEG-660 12-hydroxystearate also inhibit P-gp function in a logarithmic fashion that is dependent on excipient concentration. These results suggested that molecules with different structures could inhibit P-gp through similar mechanisms.

5.6. Cyclodextrins

Cyclodextrins (CDs) are enzymatically modified starches that have the ability to form inclusion complexes with a variety of lipophilic drugs allowing increased solubility and subsequent bioavailability (Uekama et al., 1998). The inclusion capacity depends on the molecular size of the hydrophobic molecule, the size of the cyclodextrin, and the lipophilicity of the molecule. The 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD) derivative is known to be one of the most effective cyclodextrins at improving the bioavailability of orally administered drugs with low aqueous solubility (Uekama et al., 1998).

5.6.1. Transporter interactions

The use of cyclodextrins as solubility enhancers led to the discovery of the inhibitory effect of DM-β-CyD on P-gp and MRP2 in Caco-2 cells and subsequent permeation enhancer property

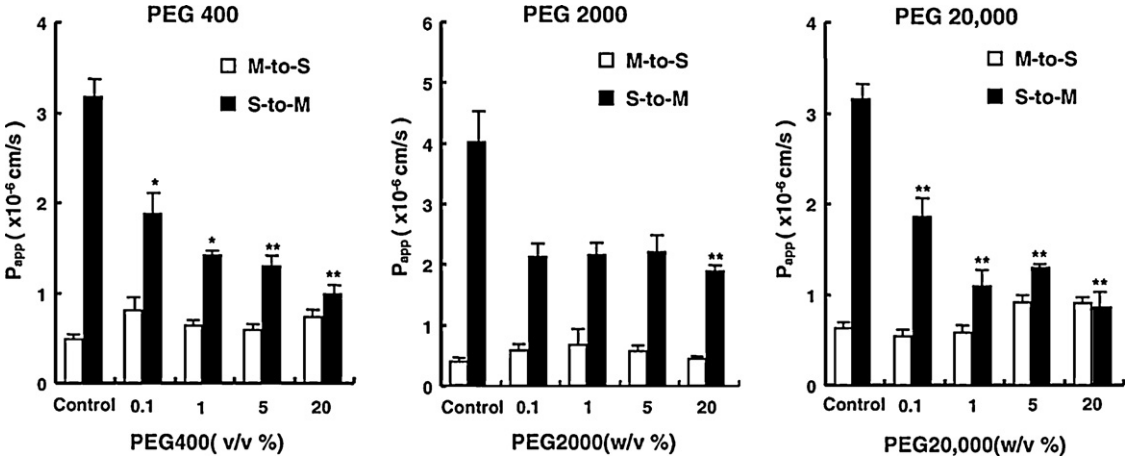

Fig. 9. Concentration-dependent effect of PEG 400, PEG 2000 and PEG 20,000 on apical-basolateral (AP–BL, M-to-S) and basolateral-apical (BL–AP, S-to-M)) transport of rhodamine 123 in the rat jejunum. The concentrations of PEG were 0.1–20% (v/v or w/v). Results are expressed as the mean ± S.E. of at least three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the control (from Shen et al., 2006).

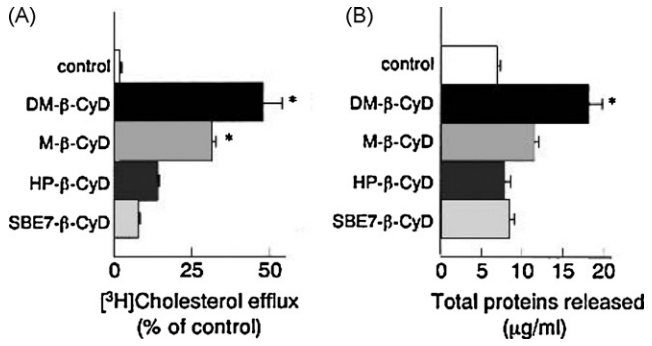

Fig. 10. Effect of β-CyD on the release of (A) [^{3}H]cholesterol and (B) total protein from Caco-2 cell monolayers (from Arima et al., 2004).

(Yunomae et al., 2003). It was noticed that a slight difference in the degree of substitution in methylated β-CyD may significantly affect cellular function. DM-β-CyD acts as a cholesterol-depleting agent and therefore, could potentially impair the activity of the efflux transporters in calveolae. An experiment conducted by Arima and co-workers has shown that the BL–AP and the AP–BL transport of rhodamine 123 in Caco-2 cell monolayers was inhibited and promoted, respectively. The same behavior has been observed using the MRP2 substrate BCECF. These alterations of P-gp and MRP2 function were due to the release of cholesterol from the monolayer. Fig. 10 shows that DM-β-CyD increased the release of [^{3}H]cholesterol by approximately 30-fold compared with the control. Moreover, the associated release of total protein was shown to be approximately 1.8-fold higher than the negative control (Arima et al., 2004). While this inhibitory effect was first shown in Caco-2 cells, Tilloy et al. (2006) have recently discovered that DM-β-CyD can also alter the function of P-gp in an *in vitro* model of the BBB and in MDCK cells. Although it was previously reported that β-cyclodextrins produced cell damage to erythrocytes (Kilsdonk et al., 1995), it was interestingly demonstrated that DM-β-CyD removed cholesterol from the plasma membrane in a concentration-dependent manner without affecting membrane integrity or tight junctions (Yunomae et al., 2003; Tilloy et al., 2006).

The action of DM-β-CyD on P-gp and MRP2 having been investigated, it was then shown that the inhibition of these efflux transporters allowed enhancing oral bioavailability of tacrolimus and CsA in rats (Arima et al., 2001).

In addition to transport studies performed on efflux transporters, evaluations on influx proteins were also conducted. To date, cyclodextrins have not showed significant effect on PepT-1-mediated absorption (Oda et al., 2004).

5.7. Suspending agents

A suspension is a heterogeneous system consisting of a dispersion of fine solid particles that are insoluble in a liquid medium (Park et al., 2007). The large surface area of the particles provides greater opportunity for dissolution and hence absorption of the dispersed drug. Excipients commonly used in suspensions are wetting agents – e.g. *surfactants*, flocculating agents – e.g. *electrolytes*, and suspending agents. The suspending agents increase the viscosity of the dispersion in order to avoid agglomeration and to delay the sedimentation of the dispersed particles. Suspending agents are often hydrophilic compounds, including cellulose derivates – e.g. *HPMC*, *HPC*, *CMC Na* – synthetic polymers – e.g. *Carbomer*, *Povidone* – and gums – e.g. *Xanthan gum*, *Carrageenan*.

5.7.1. Transporter interactions

Carrageenan is a member of the natural linear sulphated polysaccharide family that is widely used as a gel-forming polymer in solid or topical dosage forms or as a stabilizing agent in suspensions due to its pseudoplastic properties. However, several studies have shown that carrageenan can induce inflammatory reactions such as paw oedema or pleurisy in rats and mice (Farges et al., 2006; Halici et al., 2007). Since inflammatory reactions are known to alter expression and activity of several efflux transporters (Hartmann et al., 2001), Kalitsky-Szirtes and co-workers studied the efflux of digoxin and amiodarone in jejunal sections isolated from lipopolysaccharide (LPS) treated rats. Using the reverse transcription-polymerase chain reaction process, they reported that the levels of MDR1 (P-gp) and MRP2 mRNA were significantly higher in the jejunum of the LPS-treated rats than in the control rats. Moreover, corresponding increase in the BL–AP efflux of both drugs were observed which resulted in significant decrease in the uptake of these compounds (Kalitsky-Szirtes et al., 2004). The inflammatory reaction did not alter the transport of mannitol, so it was concluded that the inflammation process induced transport modifications only via the transcellular route by affecting the activity of specific efflux transporters such as P-gp and MRP (Kalitsky-Szirtes et al., 2004).

5.8. Preservatives

Preservatives are added in pharmaceutical dosage forms to prevent the alteration of the incorporated drug(s) or excipients. Some preservatives such as alcohol derivatives, surfactants, or parabens prevent microbial growth whereas other preservatives such as vitamin E, vitamin C, and EDTA reduce chemical degradation due to oxidation.

5.8.1. Transporter interactions

E. coli transfected with the MDR1 gene from mice were able to express a stable transmembrane protein with an apparent molecular weight similar to that of unglycosylated P-gp (120–140 kDa) (El-Masry and Abou-Donia, 2003). This expression conferred multidrug resistance in the so-called *E. coli* leaky mutant strain similar to that observed in eukaryotic systems, allowing P-gp mediated efflux to be studied in *E. coli*. Expression of P-gp in the *E. coli* leaky mutant conferred significant resistance to mitomycin C, doxorubicin, rhodamine, and ciprofloxacin HCl. It was shown that ascorbic acid (vitamin C) increased the uptake of mitomycin without directly affecting P-gp activity. It seems that the antioxidant properties of ascorbic acid reduce the production of reactive oxygen species which are already known to induce MDR after generation by TNF-α (El-Masry and Abou-Donia, 2003). This hypothesis is in accordance with previous results that show that ascorbic acid inhibits cellular resistance to vincristine in human lung-cancer PC-9 by acting through an indirect pathway (reduction of reactive oxygen species) on the uptake of the drug and not directly on the efflux transporter (Chiang et al., 1994).

Since Ca^{2+} is involved in the regulation of the tight junctional permeability, it would seem plausible that EDTA could bind and deplete extracellular Ca^{2+} and thus increase the paracellular permeability of the intestinal cell layers (Bravo-Osuna et al., 2007). Peppas and Kavimandan mentioned that EDTA may be used as permeation enhancer by rending the paracellular pathway more permeable. Including EDTA in complexation hydrogels allowed the intestinal absorption of macromolecules such as insulin (Peppas and Kavimandan, 2006).

5.9. Taste maskers

Sweeteners such as glucose, sorbitol, acesulfame potassium, aspartame or saccharin are added to oral pharmaceutical dosage forms to make the ingredients more palatable, especially in chewable tablets such as antacids or in liquids such as cough syrup.

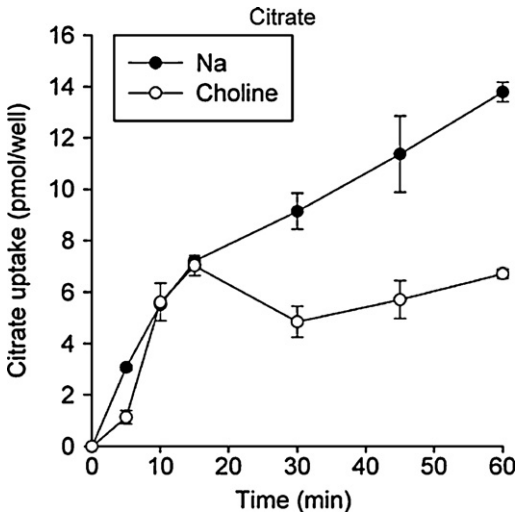

Fig. 11. Time course of uptake of citrate by Caco-2 cells ($n = 4$) (from Weerachayaphorn and Pajor, 2008).

Some compounds such as citric acid can also be incorporated into a dosage form in order to add an acidic, or sour, taste to the delivery system (Hewson et al., 2008).

5.9.1. Transporter interactions

Weerachayaphorn and Pajor demonstrated that the apical membrane of Caco-2 cells contains at least two sodium-independent organic anion transporters, OAT2 and OAT4, which were reported to not be expressed in normal enteric cell lines (Weerachayaphorn and Pajor, 2008). Experiments were conducted to evaluate the time dependence of citrate uptake in Caco-2 cells in the presence and absence of sodium over a 60 min time course. When sodium was not present, it was substituted by choline (Fig. 11).

It was shown during the first 15 min that transport of citrate was sodium-independent. After 1 h, however, citrate uptake capacity was 257 ± 18 fmol/(min well) ($n = 24$) in the presence of sodium and 149 ± 12 fmol/(min well) ($n = 29$) in its absence, which suggests a sodium-dependent process (Weerachayaphorn and Pajor, 2008). This experiment has shown that at least two transport mechanisms are involved in the transfer of citrate through Caco-2 cells. However, Weerachayaphorn and Pajor reported significant variability in expression of the sodium-independent citrate transporters. Even though an explanation was not provided, this observation can be corroborated by other data showing poor reproducibility observed in Caco-2 cells (Yee, 1997).

In order to confirm the variability observed in normal human intestinal tissue and CaCo-2 cells, they next used RT-PCR to examine for the presence of the three known Na^+-dependent di- and tricarboxylate transporters NaDC1, NaDC3 and NaCT in addition to the OATs members. Positive responses were obtained with NaDC1 and NaDC3 primers in normal human intestinal cell lines. In addition to NaDC1 and NaDC3, NaCT was found in Caco-2 cells (Weerachayaphorn and Pajor, 2008). Using hRPE cell lines transfected with both hNaDC1 and hNaCT, it was demonstrated that the sodium-dependent transport of citrate was mainly mediated by NaDC1 rather than by NaCT. On the other hand, inhibition studies performed on Caco-2 cells have shown that citrate transport was inhibited by substrates of the OAT family such as probenecid and bumetanide (Kobayashi et al., 2005) and by specific substrates of OAT4 such as taurine and estrone sulphate (Cha et al., 2000). As previously suggested, OAT2 and OAT4 can mediate the sodium-independent transport of citrate in Caco-2 cells. Moreover, using RT-PCR, Weerachayaphorn and Pajor (2008) have also demonstrated that OAT2 and OAT4 were not expressed in normal human intestinal tissue. These experiments have shown that the transcellular transport of citrate is mediated by sodium-dependent, NaDC1 and NaCT, and sodium-independent transporters, OAT2 and OAT4, in Caco2 cells whereas only sodium-dependent transporters are expressed in normal human intestinal cell lines. This reflects the altered metabolism and phenotype in cancerous cell lines.

Glucose is used in a broad range of oral liquid formulations including syrups. Due to ease of administration, syrups are commonly prescribed in the treatment of numerous pathologies such as bacterial/viral infections (Kearsley et al., 1997), inflammation diseases (Kimura et al., 1992), cold symptoms (Mizoguchi et al., 2007), and allergies (Rossi et al., 2005). Moreover, because syrups are easier to swallow than solid dosage forms, they are often used in paediatric and geriatric formulations (Seth et al., 1980). To date, very few studies have been conducted to investigate the influence of glucose on specific transporters. However, it has been shown that hyperglycemia may be an important factor in breast cancer cell proliferation as the prevalence of breast cancer is high in diabetic patients (Yamamoto et al., 1999).

Two isozymes of protein kinase C (PKC), PKC-α and PKC-β, are involved in the development of chemotherapeutic resistance in the MCF-7 human breast cancer cell line (Budworth et al., 1997). In order to determine if hyperglycemia is in fact a risk factor for breast cancer, the effect of glucose on the proliferation of the MCF-7 cell line and its multidrug-resistant variant MCF-7/ADR was evaluated. Yamamoto and co-workers have reported that the plasma concentration of glucose had no effect on the proliferation of the MCF-7/ADR cell line, but a hyperglycemic level (25 mM) signifi-

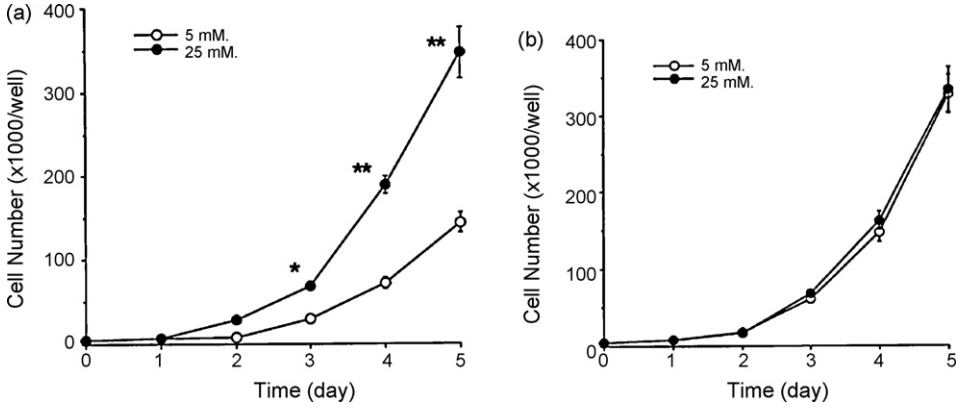

Fig. 12. The effects of glucose concentration on cell proliferation were examined in MCF-7 cells (A) and MCF-7/ADR cells (B). $^*p < 0.05$, $^{**}p < 0.01$ vs. control (5.5 mM glucose concentration), $n = 3$ (Yamamoto et al., 1999).

cantly increased cell proliferation of the MCF-7 cell line compared to the physiological level of glucose (5.5 mM) (Fig. 12).

Using [^{3}H]thymidine, it was shown that the difference in proliferation between MCF-7 and MCF-7/ADR cell lines was due to the effect of glucose concentration on DNA synthesis which alters the multidrug-resistant phenotype of MCF-7. In this way, high glucose concentration caused an increased in dThd incorporation in MCF-7 but not in MCF-7/ADR. Moreover, Western blot analysis has shown that high glucose concentration increased the level of PKC-βII only in MCF-7/ADR. Yamamoto and co-workers concluded that there was a link between high glucose-induced PKC-βII isozyme down-regulation with concomitant acceleration of cell cycle progress in MCF-7 cells.

In another study, Legen and Kristl have demonstrated that glucose facilitated the MRP2 mediated efflux of fluorescein by providing the ATP necessary for MRP2 function. This observation was corroborated when the addition of D-glucose on the apical membrane of rat jejunum increased the efflux of ketoprofen, ibuprofen and amoxicillin. However, it was also observed that in the presence of D-mannitol instead of D-glucose, the AP–BL transport of ketoprofen, ibuprofen and amoxicillin was significantly higher than the efflux process (Legen and Kristl, 2004; Legen et al., 2006). As observed in previous studies, these three drugs are characterized by passive transcellular absorption in addition to their active transcellular transport (Fagerholm et al., 1996; Legen and Kristl, 2003). To date, the influence of mannitol, which is known to be characterized by paracellular transport, on the AP–BL transport of ketoprofen and ibuprofen is not explained. It seems that more experiments need to be conducted to elucidate the exact mechanisms involved in the absorption and efflux of these widely used NSAIDs.

6. Conclusion

To date, safety/toxicity evaluations are typically based on the appearance of gross morphological changes rather than effects at a cellular level. However, it has been reported that inert substances such as excipients may produce subtle changes that could alter the activity of membrane-spanning proteins such as transporters. Indeed, it appears that excipients may alter efflux as well as influx processes of some important transporters involved in the intestinal absorption of APIs such as anticancer agents, antibiotics and antiviral compounds. Because these interactions interfere with absorption pathways, they may lead to therapeutic failure and/or toxic side effects, especially for drugs characterized by a narrow therapeutic window. Moreover, many of these drugs are used in severe diseases which have already compromised the health of the patient. In this way, unexpected influx or efflux transport processes may involve an additional risk due to the pathology of the disease. Elderly patients are subject to polytherapy. The potential interaction between the different treatments increases the probability of toxicity and makes the elderly patients more sensitive.

The data published in the literature have illustrated the lack of understanding associated with the interactions between common excipients and intestinal transporters. For instance, no data was found concerning excipients commonly used in self-emulsifying drug delivery systems, whereas these dosage forms are widely used on pharmaceutical companies. Due to the possible danger that could arise from this lack of information, it seems that preliminary cellular studies should be conducted prior to formulation development to avoid failure in the clinical stages. Unfortunately, these interactions seem to be specific to not only each excipient–transporter interaction, but to each cell line as well. In this way, the use of the Caco-2 cell line, which possesses inherent variability in terms of transporter expression, seems to be inadequate to perform interaction screening between excipients and transporters. Therefore, an appropriate cell model should be obtained after transfection of a gene encoding for the transporter of interest in cells showing poor to no expression of the specific transporter.

In addition to the specificity of the process, the large number of excipients and transporters that may be involved in absorption leads to considerable difficulty in performing systematic uptake studies prior to initiating the development of oral dosage forms. Nevertheless, further studies need to be conducted since the influence of some excipients on efflux transporters has not been reported yet. Indeed, Oda et al. (2004) mentioned that several pharmaceutical additives such as D-mannitol, microcrystalline cellulose, and carboxymethylcellulose calcium were able to increase methylprednisolone absorption by modulating P-gp without publishing the data.

To date, the study of physiological mechanisms involved in excipient–transporter interactions as well as their clinical consequences remains a fundamental area of research. One potential reason for this is that if excipients were officially considered active components of a formulation, then many pharmaceutical guidance documents would have to be completely revised. The regulatory assessment of bioequivalence for generic drugs would require special attention since excipients can dramatically alter the absorption process. As one might expect, the revision of these guidance documents would have a significant impact on health care costs. Therefore, the question should not be “Should excipients be considered as active compounds?” but “Are consumers willing to pay if excipients are considered active ingredients?”

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