Part 3

Membrane transport of drug candidates

The aim of Part 3 is to provide an overview of drug transport across biological barriers. Tissues such as the intestine or the lung perform functions that are vital to the maintenance of the human body. One of the functions of such epithelial barriers is to separate the body from the exterior. In the intestine the epithelial cells separate ingested food from the systemic circulation, and the lung tissues separate inhaled air from the blood circulation. A normal function of biological membranes is thus to serve as a barrier. In drug delivery the biological membranes are normally viewed as barriers to drug transport, regardless of the fact that they may not always be barriers to all drug substances or candidates.

In Chapter 3.1 'Structure and function of absorption barriers', the focus is on the nature of the various epithelial and endothelial barriers. The following Chapters 3.2–3.6 provide a thorough description of the mechanisms moving molecules across membranes, and the concept of drug permeability. These chapters provide tools for understanding and describing transport via passive or active transport mechanisms. Since

membrane transporters are important for bioavailability of drug substances, classifications of transporters via classical functional concepts as well as genetic concepts are provided. The last chapter of this section is about the preclinical evaluation of drug transport. This chapter gives an industrial perspective on how drug candidates are evaluated in terms of transport properties as well as metabolism. The impact of transporters on bioavailability and potential risk of transporters as mediators of drug–drug interactions have also gained the attention of the regulatory authorities. A draft *Guidance for Industry* from the US Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER) states:

> . . . not every drug–drug interaction is metabolism-based, but may arise from changes in pharmacokinetics caused by absorption, distribution and excretion interactions. Drug–drug interactions related to transporters are being documented with increasing frequency and are important to consider in drug development (US Department of Health and Human Services, FDA, CDER and CBER, 2006).

Reference

US Department of Health and Human Services, FDA, CDER and CBER (2006). Guidance for Industry, Drug Interaction Studies – Study Design, Data Analysis, and Implications for Dosing and Labeling, Draft Guidance. http://www.fda.gov/CDER/guidance/6695dft.pdf (accessed 12 May 2009).

3.1

Structure and function of absorption barriers

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New drugs are normally formulated so they can be administered via non-invasive pathways. This is due to a number of diverse factors such as patient compliance, safety, production costs and product stability. The conventional oral tablet remains one of the most popular choices when selecting new formulations of a drug substance. However, a number of issues must be addressed when the drug substance is intended to permeate a barrier and act at a site distant to its dissolution site. The aim of this chapter is to provide an overview of morphology and permeability properties of the most important drug barriers in the body. This will hopefully provide the reader with a basic understanding of some of the factors that must be taken into account when designing new drug formulations for transepithelial delivery.

3.1.1 Epithelial morphology

Layers of specialised cells, epithelia, cover nearly all surfaces of the body, both external and internal. Epithelia constitute the barriers between the body and the external environment and have two main functions: (1) they protect the internal environment of the body from the external environment, and (2) they allow exchange of relevant nutrients, water, gases and waste products with the environment. Human epithelia are specialised so that the predominant exchange of gases takes place in the lungs, the predominant absorption of nutrients takes place in the small intestine, the skin is mainly protective, and so forth. However, when one tries to analyse the function of various epithelia, it must always be taken into account that the tissues serve these two functions: protection of the internal environment of the body and exchange of relevant compounds with the exterior.

Epithelia can be classified morphologically by (1) their number of cell layers, (2) the shape of the cells, and (3) the presence of apical surface specialisations such as cilia or keratin (Wheater *et al.*, 2006). This is shown in Table 3.1.1.

Table 3.1.1 Epithelial classifications; epithelia can be classified by numbers of cell layers, shape or specialisation of cells. Often an epithelium will be described with a combination of these terms

Number of cell layers	*Shape of cells*	*Specialisations*
1: Simple epithelium	Flat, irregular: squamous epithelium	Apical cilia: ciliated epithelium
>1: Stratified epithelium	Cuboidal: cuboidal epithelium Tall columnar: columnar epithelium	Keratin in apical layers: keratinising epithelium

As a general rule, simple epithelia normally perform functions such as absorption or secretion, whereas stratified epithelia to a larger extent serve protective roles. The morphologies of various types of epithelia are shown in Figure 3.1.1. Simple squamous epithelia are often found lining airways, in body cavities and blood vessels, permitting passive transport of liquids and gases between body compartments. Cuboidal epithelia are usually found in ducts and tubules of the kidney and glands. Columnar epithelia are found in highly absorptive epithelia such as the small intestine. The presence of keratin or cilia has traditionally been used to subdivide epithelia further. Keratin is found in the outermost layer of stratified, squamous epithelium, the skin for instance, and provides resistance to mechanical stress and protects the underlying tissue from loss of liquid. Cilia serve the function of generating a flow of liquid/mucus and are found in the airways as well as in other types of tissue. A specialised stratified epithelial type, the transitional epithelium, is found in organs that can stretch, such as the bladder or urethra. In transitional epithelia, the cell layers can slide over each other allowing distension and contraction.

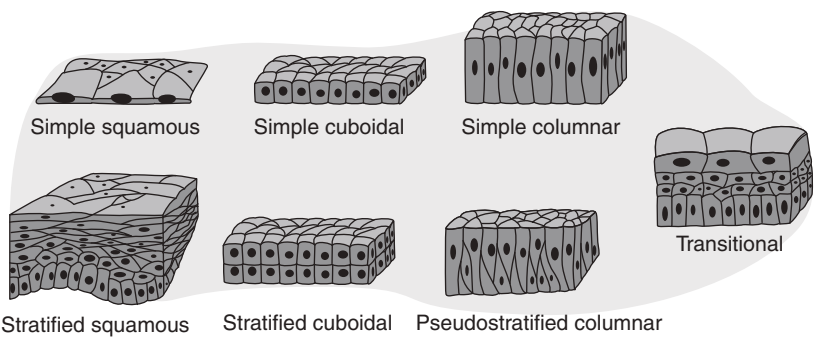

Figure 3.1.1 Morphologies of basic epithelial types.

Functionally, epithelia can also be classified as 'tight' or 'leaky' on the basis of their resistance to paracellular water and solute flow. The colon and collecting ducts of the kidney are typically tight epithelia where uptake of salts and water is transcellular. The small intestine and the proximal tubule of the kidney are examples of leaky epithelia where a substantial paracellular transport can occur. Both tight and leaky epithelia possess tight junctions, but differ in their permeability. This is also reflected in the electrical resistance of the epithelia, with leaky epithelia having transepithelial resistance values below $200\,\Omega\,cm^2$ and tight epithelia having resistances above this value (Marrero *et al.*, 1998).

3.1.2 The epithelial cell and tight junctions

Epithelial cells generate boundaries between the body and its external environment. In order to maintain a constant intracellular environment, the cells must be able to allow controlled passage of water, nutrients and waste products. Two important features of epithelial cells make this possible: (1) tight junctions, and (2) polarity of protein expression. Absorptive epithelial cells are illustrated in Figure 3.1.2.

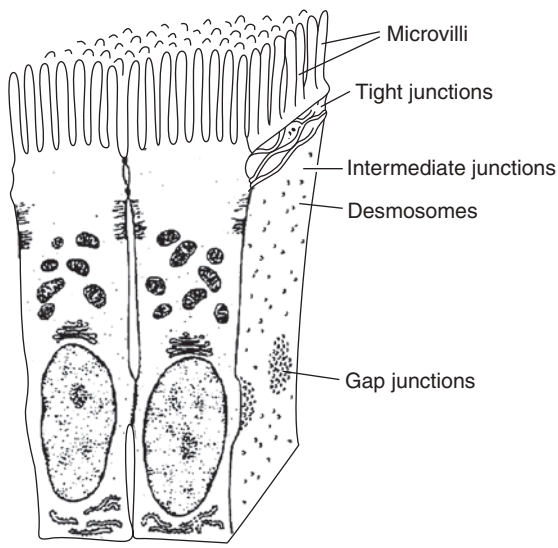

Figure 3.1.2 Absorptive epithelial cells. Tight junctions define the border between the apical and lateral membranes, and enable polarised expression of proteins. Adapted from Weinstein RS, McNutt NS (1972). Cell junctions. *N Engl J Med* 286: 521–524.

Tight junctions connect epithelial cells at their apical end. Epithelial cells can also be interlinked via a set of protein contacts such as gap junctions, desmosomes, integrins etc. Three types of membrane domains can be distinguished: (1) apical plasma membrane facing the exterior, (2) lateral plasma membrane facing neighbouring cells, and (3) basal plasma membrane. The latter two are normally treated together and termed the basolateral membrane. The epithelial cells rest on 'the basement membrane', a filamentous protein/polysaccharide network secreted by the epithelial cells and by the underlying connective tissue. Beneath the basement membrane lie connective tissue and blood vessels. The barrier function of epithelia is determined by the passive permeability of the tight junctions and of the epithelial cells, as well as the selective barrier properties of the cells, which are determined by a range of transport proteins. The tight junctions constitute a barrier for paracellular diffusion of molecules, but also a barrier for lateral diffusion of membrane proteins within the membrane. Epithelial cells can therefore maintain a polarised membrane protein expression pattern, enabling vectorial transport of water and nutrients. Important specialisations in absorptive epithelia are the microvilli, small projections of the plasma membrane, which can significantly increase the nominal surface area for absorption.

3.1.3 The gastrointestinal tissue barriers

The gastrointestinal (GI) tract is a long tube traversing the body. Its primary function is breakdown of food to absorbable entities, uptake of nutrients and water, and excretion of indigestible food components. The GI tract is divided into segments with different functions and is illustrated in Figure 3.1.3.

The oral cavity is designed for chewing and wetting food components. The epithelia in the mouth are mainly protective; however, a number of glands secrete liquid along with some digestive enzymes. The main function of the oesophagus is to move food to the stomach by peristaltic muscle action. The role of the stomach is to digest and solubilise food particles and kill microbes. This is accomplished by release of pepsin and hydrochloric acid. The stomach also regulates release of dissolved food into the small intestine through the pyloric sphincter. Digestion continues in the small intestine, aided by enzymes released from the exocrine glands of the pancreas as well as by enterocytic enzymes, (intestinal peptidases are summarised in Table 2.4.3, Chapter 2.4). The

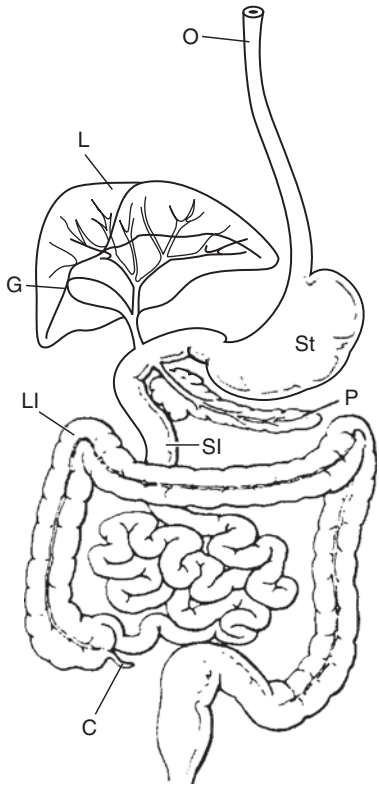

Figure 3.1.3 The GI tract (mouth and pharynx not included). C, colon; G, gall bladder; L, liver; LI, large intestine; O, oesophagus; P, pancreas; SI, small intestine; St, stomach. Adapted from Eckert R, Randall D, Augustine G (1988). *Animal Physiology. Mechanisms and adaptation*, 3rd edn. New York: WH Freeman and Company.

small intestine is responsible for uptake of most of the food constituents such as proteins, carbohydrates and fat. The colon is the site for reabsorption of water from the intestinal contents, and the primary role of the rectum is to cause defecation, via muscle action. A detailed description of the anatomy of the GI tract is beyond the scope of the present text; however, in the following we shall focus on GI barrier structures that are relevant in drug absorption and delivery.

3.1.3.1 The oral cavity, pharynx and oesophagus

The primary function of the epithelial barriers of the oral cavity is to provide an environment for wetting and chewing food particles, while maintaining a barrier for micro-organisms and protection against

mechanical stress due to chewing and mastication. The oral cavity is thus covered with stratified, squamous epithelia, which can withstand moderate mechanical abrasion and is an effective barrier for large hydrophilic molecules and micro-organisms. Glands open up into the oral cavity and moisten tissue as well as food contents. This protects the epithelium of the oral cavity from desiccation and lubricates the food bolus before passage to the stomach. In terms of drug delivery, the oral cavity raises some challenges. The transit time of a food bolus is normally very short, so formulations for the oral cavity are usually designed to act on the buccal or sublingual epithelia, where they can avoid being swallowed. However, the continuous flow of saliva will tend to carry drug substance further down the GI tract. The mouth also is well equipped with sensory nerves, and drug formulations should thus act as quickly as possible in order to avoid compliance problems. The advantages of drug delivery from the oral cavity to the blood are the avoidance of hepatic first-pass metabolism of the drug, and avoidance of the acidic environment in the stomach, which may cause breakdown of certain drug substances.

The pharynx and the oesophagus mainly serve as a passage way from the mouth to the stomach, and due to the fast passage time and low permeability of the epithelia, they will not be treated further.

3.1.3.2 The stomach

The function of the stomach is to initiate digestion of food components, to grind food components into chyme and to release the chyme for further digestion and absorption. The epithelium of the stomach is a simple columnar epithelium, its main role being the secretion of a protective mucus layer. Specialised gland cells also produce hydrochloride and precursors of digestive enzymes. Very little absorption occurs in the stomach; there are, however, some notable exceptions. Alcohol can be absorbed to some extent through the stomach wall, as well as some weak acids, including the common drug substance acetyl salicylic acid (aspirin), which are more lipid soluble as their neutral species rather than anionic species.

The main role of the stomach is to control the rate of chyme influx into the small intestine. The gastric emptying rate is increased by gastric distension and decreased by duodenal distension. Duodenal fat or acid content also decreases gastric motility, and a number of other sensory inputs might also influence the emptying rate, e.g. pain, depression and fear. Variances in gastric emptying rates may have a large impact on the bioavailability of drug substances. Functional characteristics of the

Table 3.1.2 Functional characteristics of gastrointestinal segments influencing drug dissolution and absorption; reproduced from Daugherty AL, Mrsny RJ (1999). Trans cellular uptake mechanisms of the intestinal epithelial barrier. Part one. *Pharm Sci Technol Today* 4: 144–151

GI segment	*Surface area (m^2)*	*Segment length (m)*	*Residence time (h)*	*pH of segment*
Stomach	3.5	0.25	1.5	1–2
Duodenum	1.9	0.35	0.5–0.75	4–5.5
Jejunum	184.0	2.8	1.5–2.0	5.5
Ileum	276.0	4.2	5–7	7.0–7.5
Colon and rectum	1.3	1.5	1–60	7.0–7.5

stomach segment influencing drug and nutrient dissolution and absorption are shown in Table 3.1.2.

3.1.3.3 The small intestine

The chyme is passed from the stomach into the small intestine, via the pyloric sphincter. Functional characteristics of the various intestinal segments influencing drug and nutrient dissolution and absorption are shown in Table 3.1.2. The small intestine is a coiled tube, approximately 7–8 m long, consisting of three segments: the duodenum, the jejunum and the ileum. The duodenum is a short structure (35 cm) where the bile-pancreatic duct empties its contents of bile, digestive enzymes and bicarbonate. It is followed by the jejunum (~2.8 m) and the ileum (~4.2 m). There is no clear-cut distinction between the small intestinal segments. The small intestine is specialised in absorbing nutrients. It is highly folded with large folds (plicae) extending into the intestinal lumen. The surface is covered by small finger-like projections (villi) (see Figure 3.1.4), and the epithelial cells covering the villi are covered with microvilli.

The degree of folding decreases along the length of the small intestine. The total surface area of the small intestine has been estimated at ~300–500 m^2. A meal will normally be digested and absorbed before it reaches the terminal end of the jejunum. The intestine therefore has a considerable overcapacity.

The villi are covered with a simple columnar epithelium consisting mainly of absorptive enterocytes. The intestinal epithelium is generally considered to be a leaky epithelium with resistances of the order of 100 $\Omega\,cm^2$ (Greger, 1996). The small intestinal epithelium mediates

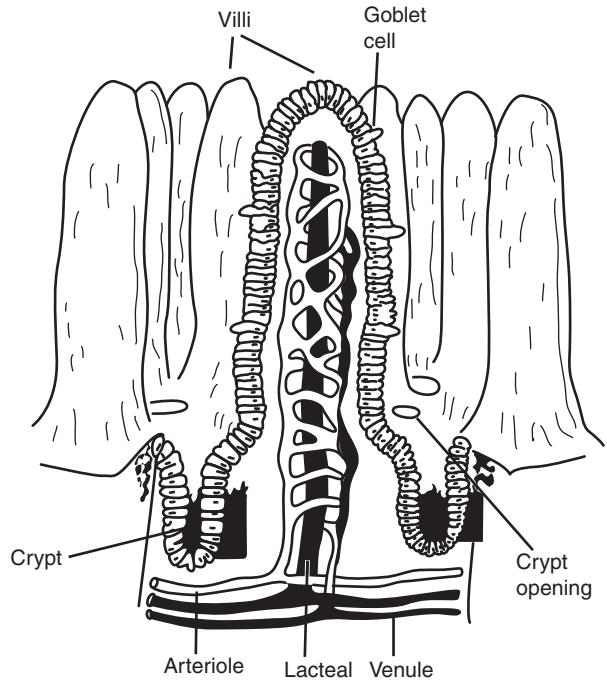

Figure 3.1.4 Villi of the small intestine. Enterocytes are generated in the crypts, where division occurs. They migrate towards the tip of the villi, becoming progressively more mature. Adapted from Eckert R, Randall D, Augustine G (1988). *Animal Physiology. Mechanisms and adaptation*, 3rd edn. New York: WH Freeman and Company.

absorption of dietary protein and carbohydrates via a range of membrane transport proteins. The uptake mechanism of lipids is still controversial. Although the classical point of view is that lipids diffuse passively into the intestinal cells, a number of reports have indicated that lipid transport proteins may mediate the uptake (Kampf and Kleinfeld, 2007). Smaller compounds may also pass paracellularly via the 'leaky' tight junctional complexes. Molecules as large as mannitol may cross paracellularly, and mannitol is often used as a marker for paracellular transepithelial flux in intestinal drug transport studies.

The huge surface area, the relatively long transit time, and the wide range of nutrient transporters present, make oral administration an obvious first-choice strategy for new formulations. However, some factors regarding the intestinal barrier must be taken into consideration with respect to oral administration. The drug candidate in question must either be stable in the acidic environment of the stomach, or protected by an

acid-resistant coating that disintegrates when the formulation reaches the small intestine. The drug candidate should also be able to withstand attack from the digestive enzymes present in the lumen of the small intestine. The physicochemical properties of the drug candidate should accommodate uptake by passive diffusion, or be a substrate for one of the intestinal transporters. Furthermore, the drug candidate should not readily undergo first-pass metabolism in the liver, since compounds taken up from the small intestine enter the portal vein, from where they are transported directly to the liver before reaching the systemic circulation.

3.1.3.4 The colon and the rectum

The colon is a tube, ~120 cm long, consisting of three relatively straight segments: the ascending, transverse and descending colon. The function of the colon is to store and concentrate undigested matter and propagate the luminal content towards the rectum. This is reflected in the morphology of the colon, where plicae or villi are absent, and the intestinal tissue is thicker. Absorption in the colon is mainly limited to absorption of sodium, chloride and water, but vitamins produced by colonic bacteria are also absorbed. Intestinal contents can reside in the colon for 18–24 h, and are moved towards the rectum by muscular action. Colon transit time varies considerably depending on age, nutritional status and lifestyle factors. The contents are passed to the rectum. The main function of the rectum is to mediate defecation. The rectum is a thick, highly vascularised structure with no role in absorption of nutrients. However, due to the high vascularisation and its accessible epithelial surface, drug substances can be taken up from the rectal lumen if they are able to permeate passively across the epithelium, i.e. have suitable lipophilicity.

3.1.4 The respiratory tract

The main role of the respiratory tract is to mediate exchange of gases (oxygen, carbon dioxide) between the blood and the atmosphere. Minor specialised functions are also present, such as olfactory organs in the nasal cavity and the vocal cords in the larynx. The respiratory tract can roughly be divided into the conductive part and the respiratory part. Air is taken up via the nose into the nasal cavity, where it is heated and larger particles are trapped. Air moves via the trachea, the bronchi and the bronchioles to the respiratory part, consisting of the respiratory bronchioles, the alveolar ducts, alveolar sacs and finally the alveoli.

3.1.4.1 The nose

The nose and the nasal cavity are designed to take up air, clean it of particles and humidify it. Air is inhaled via the nostrils and passes into the nasal cavity and via the nasopharynx to the trachea. The nasal epithelium is divided into two major cell types, mucus-producing goblet cells and cilia-covered cells that move the thin mucus layer towards the nasopharynx. The epithelium is a pseudostratified columnar ciliated epithelium with goblet cells immersed. The submucosa is extremely vascularised, providing a means of heating the inhaled air towards body temperature. Submucosa furthermore contains glands that secrete mucus and liquid, which aids in humidifying inhaled air. The nasal cavities have an area of $\sim$150 cm^2. The nasal epithelium has properties that make it an obvious target for transepithelial drug delivery, e.g. a mucus layer which might trap particles, a fairly thin epithelial barrier without keratinisation, extensive submucosal vascularisation and no hepatic first-pass metabolism. However, nasal drug delivery also offers a number of challenges. Formulations must be deposited in the nasal cavities and stay there long enough to permit absorption; formulations must not interfere with ciliary function or irreversibly alter the epithelial integrity. The olfactory epithelium is situated in the upper part of the nasal cavity and covers $\sim$10 cm^2. It has been suggested that drug substances taken up in the olfactory epithelium might pass directly from the nasal cavity to the brain, thus circumventing the blood–brain barrier (Behl *et al.*, 1998; Illum, 2004). However, the existence of this proposed phenomenon is still controversial (Merkus and van den Berg, 2007).

3.1.4.2 The lung

The lungs are composed of a number of different tissue structures, forming two large sacs situated in the thorax. The upper parts of the lungs are tubes of decreasing diameter (trachea and bronchi), which primarily serve to conduct air to the lower parts. Gas exchange occurs in a number of small sacs at the end of the bronchioles, called alveoli. Gas exchange occurs in the alveoli, where the large surface area ensures rapid exchange. The maximal area available for gas exchange in the adult lung ranges from 50 to 100 m^2. Lung epithelial morphology depends on function. The trachea and bronchi, which function as conductive tubes, are covered with a columnar ciliated epithelium. The alveoli are lined with two types of epithelial cells; type I and II pneumocytes. Type I pneumocytes are large squamous cells, constituting by far the largest part of the alveolus surface

area. Type II pneumocytes are smaller rounded cells, secreting surfactant. Exchange of gases occur across type I pneumocytes, while type II pneumocytes posses a number of transport proteins involved in fluid and salt transport. The alveoli are surrounded by a network of capillaries in close contact with the alveolar wall. The type I pneumocytes and the endothelial cells of the capillaries share a basement membrane, and the thickness of the 'air–blood barrier', pneumocyte – basement membrane – endothelial cell, is ~0.6 μm (Brain, 2007).

Systemic drug administration via the lungs is an attractive alternative to the more conventional oral administration pathway, due to the large absorbing surface area and direct access to the systemic circulation, thus avoiding hepatic first-pass metabolism. Especially for proteins, the pulmonary pathway seems to be a good alternative to oral delivery, since proteins are normally broken down rapidly in the GI tract (see Chapter 2.4). However, lung delivery involves the use of inhalation devices with the risk of large dose variations due to low compliance.

3.1.5 The skin

The skin is a continuous protective epithelium covering the external surface of the body. The skin of an adult is approximately 2 m^2 and is the largest organ in the body. Skin thickness varies greatly in an individual, from <1 mm on the eyelids to ~5 mm on the upper back. Major roles of the skin are to prevent water loss from the organism, and to provide a physical and immunological barrier against pathogens. The skin also provides protection against mechanical, chemical and thermal stimuli, and participates in thermoregulation. Properties of the skin vary both at the level of the individual and between individuals. Age, race and diseases are all causes for skin variations. The outer boundary of the skin is the epidermis, a squamous stratified keratinised epithelium. The epidermis rests on the dermis, a thick vascularised layer containing glands, nerves and hair follicles. Epidermis and dermis structures of the skin are shown in Figure 3.1.5.

Below the dermis is the hypodermis, the subcutaneous layer of adipose tissue and larger blood vessels. The epidermis is the actual barrier towards the outside environment, and it is across this cell layer that drug substance permeation must occur. The barrier of the epidermis resides in the outer layer, the stratum corneum, where dead keratinised cells constitute a barrier for water and hydrophilic compounds (Elias, 1991). The strateum corneum can be considered a protein-lipid matrix, which

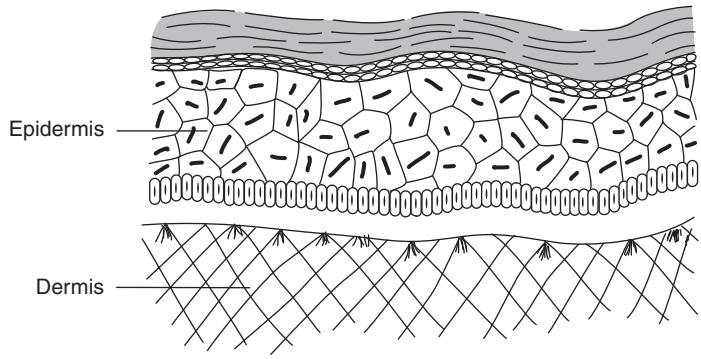

Figure 3.1.5 Epidermis and dermis of the skin. The proliferative layer of the epidermis generates cells, which gradually move upwards to end up as flattened, water-impermeable keratinised cells at the outermost boundary (stratum corneum).

restricts the passive diffusion of both lipophilic and hydrophilic compounds (Curdy *et al.*, 2000). This barrier has a very high transepithelial resistance, estimated to be of the order of 550 $k\Omega\,cm^2$ (Kalia and Guy, 1995), indicating that the strateum corneum has a very low paracellular passage of ions (and larger hydrophilic compounds). However, this resistance drops markedly upon hydration. This is often exploited when designing formulations for skin administration. Once a compound has crossed the strateum corneum it may diffuse in the intercellular spaces to the dermis. From the dermis, drug substances can escape to the systemic circulation via blood vessels. Drugs might also act locally on nerve endings, inflammation and skin diseases.

The major challenge of drug administration via the skin is to overcome the barrier residing in the strateum corneum. The fact that permeability is increased upon hydration is exploited in formulations such as creams and patches. Another approach commonly used is increasing the strateum corneum permeability of candidates by formulating them as prodrugs with higher lipophilicity than the parent compound (Fang and Leu, 2006) or to include permeability enhancers in the formulations. Prodrugs are further described in Chapter 2.4.

3.1.6 Barrier tissues in the brain

The capillaries of the brain make up a very effective barrier for transport of drug substances and solutes between the blood and the cerebrospinal fluid. In the brain, the endothelial cells of the capillaries form an extremely tight continuous monolayer, as opposed to normal continuous

capillaries with a substantial higher permeability, or fenestrated capillaries with pores allowing for exchange of fluid and solvents.

The brain endothelial cells are linked together via extensive tight junctions (see Figure 3.1.6). The presence of tight junctions makes the tissue practically impermeable for larger hydrophilic molecules (Brightman and Reese, 1969), with the exception of some nutrient molecules, which are transported through the cells via nutrient transporters (Boado *et al.*, 1999; McAllister *et al.*, 2001; Paulson, 2002). Lipophilic molecules, which in other tissues normally would pass by simply diffusing through the lipid bilayers of the cells, are extruded by the activity of efflux pumps: transport proteins situated in the apical membrane of the brain endothelial cells. Thus the blood–brain barrier provides a 'passive' physical barrier, i.e. the tight monolayer of cells, and an 'active' barrier, i.e. the efflux pumps. Efflux pumps are present in other barrier tissues as well, but in the blood–brain barrier they play a predominant role in determining drug pharmacokinetics.

The transepithelial electrical resistance (TEER) across the blood–brain barrier is $>1500\,\Omega\,cm^2$ (Crone and Olesen, 1982; Butt *et al.*, 1990), with experimental estimates reaching levels as high as $6000–8000\,\Omega\,cm^2$ (Smith and Rapoport, 1986; Butt *et al.*, 1990). This is due to the very low ionic conductivity of the junctional structures in the blood–brain barrier in combination with the low ion permeability of the endothelial cells.

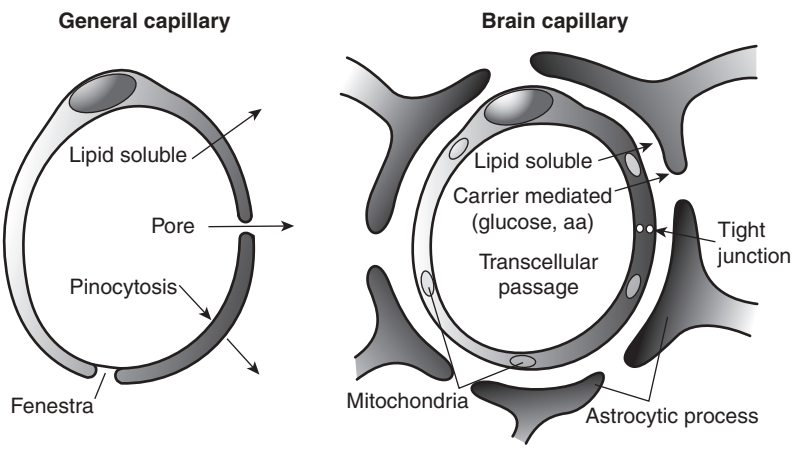

Figure 3.1.6 The blood–brain barrier endothelial cells as compared to fenestrated capillaries. aa, amino acids.

The blood–brain barrier is considered the most important challenge factor in the development of central nervous system (CNS) drugs (Pardrige, 2007). In order to cross the blood–brain barrier, a drug substance can either cross paracellularly, transcellularly via diffusion through lipid bilayers, or transcellularly via membrane transport proteins. Drug substances with physicochemical properties that enable them to diffuse through lipid bilayers might, however, be apically extruded by the battery of efflux pumps extruding lipophilic substrates (Leslie *et al.*, 2005). Drug substances permeating via uptake transport proteins might show limited uptake due to saturation of the uptake transporters.

The most accurate method of determining the blood–brain barrier permeability of a drug substance is the *in situ* perfusion method (Reichel, 2006). However, this method is costly and not suited for screening studies. Over the last two decades, a number of research groups have therefore tried to generate *in vitro* blood–brain barrier models, but with variable success. Attempts have included endothelial cells isolated from bovine brain (Rim *et al.*, 1986, Shah *et al.*, 1989), astrocyte-conditioned media (Arthur *et al.*, 1987), co-cultures of endothelial and glial cells (Stanness *et al.*, 1999), and generation of blood–brain barrier cells from stem cells (Weidenfeller *et al.*, 2007). However, most attempts have failed to reproduce the tightness of the tissue, with TEER values of the models ranging from 30 to 100 $\Omega\,cm^2$. The generation of an *in vitro* model of the blood–brain barrier is thus a major challenge for both industry and academia.

The extreme tightness of the blood–brain barrier and the activity of efflux proteins make administration of drug substances to the brain a great challenge. It has been estimated that up to 98% of the hit compounds coming out of traditional high-throughput screening programmes would fail to cross the blood–brain barrier due to low permeability (Pardridge, 2002). Screening programmes, based on Lipinski's rule of five and focusing on selecting substrates with blood–brain barrier permeability, have been implemented, thus selecting substrates with 'CNS-likeness' (see Figure 3.1.7).

Thus CNS drugs have a smaller optimal range of molecular weight, lipophilicity, and hydrogen-bond donors as well as hydrogen-bond acceptors compared to general therapeutics.

However, these screening criteria still remain rough guidelines and should be combined with knowledge of the substrate profiles of the brain efflux transporters.

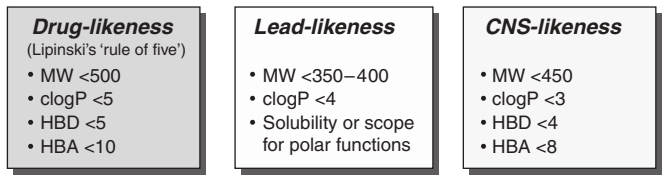

Figure 3.1.7 High-throughput screening criteria. The original 'rule of 5' and its modification for lead and CNS compounds, respectively. MW, molecular weight; clogP, calculated log*P*; HBD, number of hydrogen-bonding donors; HBA, number of hydrogen-bonding acceptors. For Lipinski's rule of five see Chapter 2.4.1.

Another factor, which makes prediction of blood–brain barrier permeability a difficult task, is the fact that blood–brain barrier properties might vary during disease conditions. Overcoming the obstacle of brain drug administration thus remains one of the major challenges in the field of drug delivery.

3.1.7 The eye

The eye is a complex structure, composed of many different cell types and tissue components. The design of formulations for topical delivery to the eye is challenging. The globe of the eye (the eyeball) is shown in Figure 3.1.8. The inner part of the eye (the anterior and the posterior chambers) is protected anteriorly from the environment by the corneal and the scleral epithelium. The cornea is a transparent 0.5–0.7 mm five-layer-thick membrane, consisting of an outward-facing epithelial layer, a thick fibrillated stroma, and finally an endothelium lining the anterior chamber. The cornea is covered by a film of lacrimal fluid at the exterior surface. Corneal epithelium is of the squamous-stratified type, with extensive networks of tight junctions resulting in low paracellular passage and high electrical resistance.

The remaining part of the eye exposed to the exterior, including the inside of the eyelids, is covered by conjunctival epithelium. The conjunctiva is a stratified columnar epithelium which is leakier than the cornea. However, transcorneal permeation is the main route of drug substance entrance from the lacrimal fluid to the anterior chamber (Urtti, 2006).

The posterior part of the eye is shielded from xenobiotics in the blood by the blood–retinal barrier, which shares some similarities with the blood–brain barrier described above (Antonetti and Wolpert, 2003). The blood–retinal barrier is composed of the retinal pigment epithelium and the endothelial cells of the retinal vasculature.

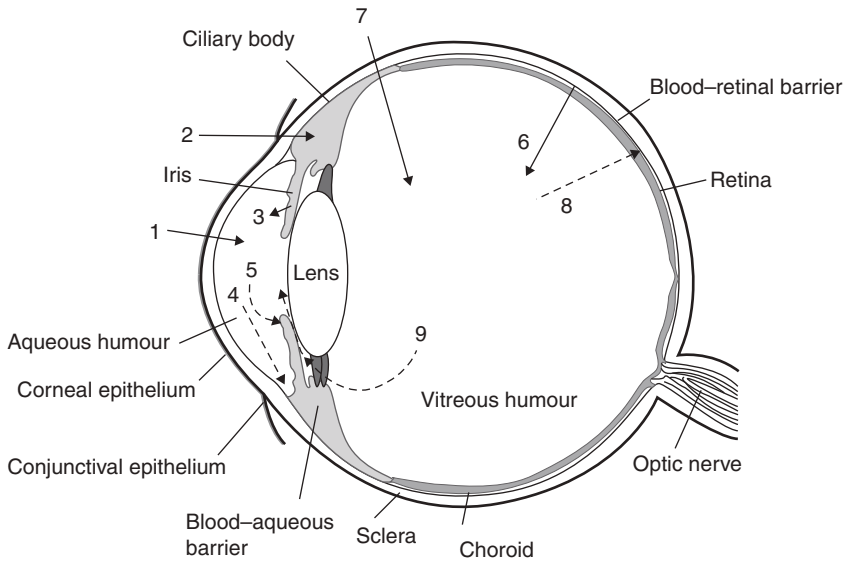

Figure 3.1.8 Schematic representation of the eye and an overview of drug entrance and elimination pathways (depicted by arrows). (1) Transcorneal permeation; (2) permeation via the conjunctiva and sclera; (3) transport over the blood–aqueous barrier; (4) elimination from the aqueous humour into the trabecular meshwork and Schlemm's canal, a drainage system; (5) elimination from the aequeous humour, across the blood–aequeous barrier into the blood; (6) transport from the blood into the posterior part of the eye, across the blood–retina barrier; (7) intravitreal drug administration; (8) drug elimination from the vitreous humour, across the blood–retina barrier; (9) drug elimination from the vitreous humour to the anterior chamber.

The anterior part of the eye can be treated with topical fluid formulations, for example eye drops. However, drainage from the ocular surface is fast, and the bioavailability of topically administered eye drops is low. Bioavailability to the posterior segments after topical administration of eye drops is generally close to zero due to fast clearance, low corneal permeability and long diffusion pathway. Systemic drug delivery to the posterior segment is hindered by the blood–ocular barriers, consisting of the blood–aqueous barrier and the blood–retina barrier. The posterior segment of the eye can generally only be reached via massive intravenous dosing or intravitreal administration. However, not all drug substances can be administered at high systemic concentrations due to systemic side effects, and intravitreal administration has its limitations due to its invasive nature. Therefore many posterior segment diseases, for example diabetic retinopathies and neural changes induced by glaucoma,

lack effective treatments (Urtti, 2006). Consequently ocular drug delivery to the posterior part of the eye remains a challenge.

3.1.8 Conclusions

The epithelial tissues of the body represent barriers for topical and systemic treatment by drug substances. Internal epithelia, i.e. endothelia, act in addition as barriers between the systemic circulation and the target such as the brain and the posterior segments of the eye. Detailed knowledge of the barrier in question will aid the formulation scientist in making proper choices of dosage forms and in selecting relevant preclinical studies such as permeability studies across relevant cell culture models or excised tissue models of the barrier in question. Such preclinical studies can be used to select and discard drug and formulation candidates at an early stage in the pharmaceutical drug development process.

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3.2

Passive diffusion of drug substances: the concepts of flux and permeability

Birger Brodin, Bente Steffansen and Carsten Uhd Nielsen

Experimental studies of the movement of molecules in solutions and molecular transport across artificial or biological barriers are used by the pharmaceutical scholar in a variety of contexts, ranging from simple diffusion and dissolution studies, to complex *in vivo* pharmacokinetic investigations. Movement of molecules in solutions and molecular transport across barriers may be described mathematically, and knowledge about these descriptions will aid in the design of experiments and interpretation of data. A number of textbooks explain the mathematical background necessary for transport studies (for example Schultz, 1980; Steen-Knudsen, 2002). In the present chapter, however, focus will be on presenting only the most commonly used equations and explaining the parameters involved, and the circumstances under which these equations can be applied. The aim of this section is thus to provide a basic framework of concepts describing transport of drug substances across biological barriers, hopefully enabling the reader to choose appropriate experimental models and data analysis for a given problem related to flux and permeability studies.

3.2.1 How do molecules move in solution? The concepts of flux, migration and diffusion

Mass transport of molecules in a solution or molecular transport across a barrier is normally measured by fluxes. The flux of a solute is simply defined as the mass or number of molecules moving through a given cross-sectional area during a given period of time (Equation 3.2.1):

$$J = \frac{m}{A\,t} \tag{3.2.1}$$

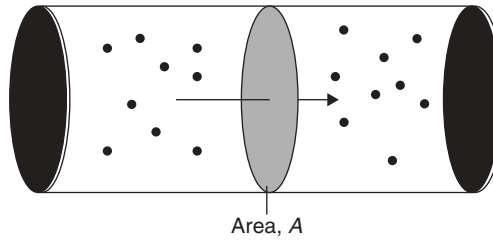

Figure 3.2.1 Flux, i.e. movement of molecules (•) through cross-sectional area (*A*) in a given time period (*t*).

where J is the flux of a mass of compound m, moving through a cross-sectional area A during time t as illustrated in Figure 3.2.1. The unit for a flux value could thus be mol $cm^{-2}\,min^{-1}$, or alternatively $\mu g\,cm^{-2}\,h^{-1}$.

Movement of molecules in solution or molecular transport across barriers can be caused by migration or diffusion. Migration is movement of molecules caused by an external force that is acting on each of the solute molecules. Such external forces can be gravity, electrical fields (in case of charged solutes) or hydrodynamic flow. Diffusion is the random thermal movement of molecules in a solution, and thus diffusion may only cause a net transport of molecules in the presence of a concentration gradient.

The velocity of diffusion is related to the diffusion coefficient of a solute, a constant related to the properties of a given molecule in a given solvent. The diffusion coefficient (D) is dependent on the size of the solute molecule and the viscosity of the solvent as described by the Stokes–Einstein equation (Equation 3.2.2):

$$D = \frac{RT}{6\pi\eta N_0 r_A} \tag{3.2.2}$$

where R is the gas constant, T absolute temperature, r_A the radius of spherical solute, N_0 Avogadro's number, and η the viscosity of the solution. Thus the diffusion coefficient decreases with increasing molecule size and increasing viscosity of the solvent.

Diffusional flux can be described by the relationship commonly known as Fick's law (or Fick's first law), normally accredited to the German physiologist Adolf Fick (Equation 3.2.3):

$$J(x,t) = -D\frac{\delta C(x,t)}{\delta(x)} \tag{3.2.3}$$

Fick's Law is a partial differential equation, describing a flux, J, down a concentration gradient, δC, in a plane over time, t, for a solute, x, with a diffusion coefficient D. This version of Fick's law is rarely used for interpreting simple transport studies. However, when assuming a time-independent linear concentration profile (Equation 3.2.4), Fick's law becomes more straightforward to use, as we shall see later.

In transport studies conducted in biopharmaceutical science or in preclinical development, most experimental designs aim at eliminating migrational flux components to solely study diffusional fluxes. Migrational fluxes will not be described further in this chapter, apart from being mentioned in relation to electrical fields in Sections 3.2.5 and 3.2.6.

3.2.2 Fluxes across barriers and the permeability coefficient

The most common biopharmaceutical use of flux studies is transport investigations of drug candidates or prodrug candidates, across a barrier tissue, such as small intestinal cell culture models or tissue models. A typical setup for the conduction of this type of experiment will include a donor compartment with a defined initial concentration of compound, a defined volume, a barrier structure with a defined cross-sectional area and thickness, and a receiver compartment with a defined initial concentration and a defined volume (see Figure 3.2.2).

Stirring should be complete in both donor and receptor compartments, in order to ensure that there is no concentration gradient within the two compartments, and thus the only gradient present is across the barrier structure separating the two compartments. Fluxes are then measured simply, by taking samples from the receiver compartment at given time points after addition of the test compound to the donor solution. In the simple situation, where the flux across the barrier only moves an insignificant amount of solute test compound from the donor chamber, the concentration gradient across the barrier is essentially constant, and the flux will thus be of zero order, i.e. be constant, since flux occurs as a function of the concentration gradient. In such cases, a simple version of Fick's law can be used to relate fluxes and concentration gradients (Equation 3.2.4):

$$J = P\,(C_{\text{donor}} - C_{\text{receiver}}) \tag{3.2.4}$$

where P is the permeability coefficient, C_{donor} and C_{receiver} are the concentrations of the drug substance in the donor and receiver compartments, respectively, and J is the flux from the donor to receiver compartment of the drug substance in question. Occasionally the notations P_{apparent} (P_{app})

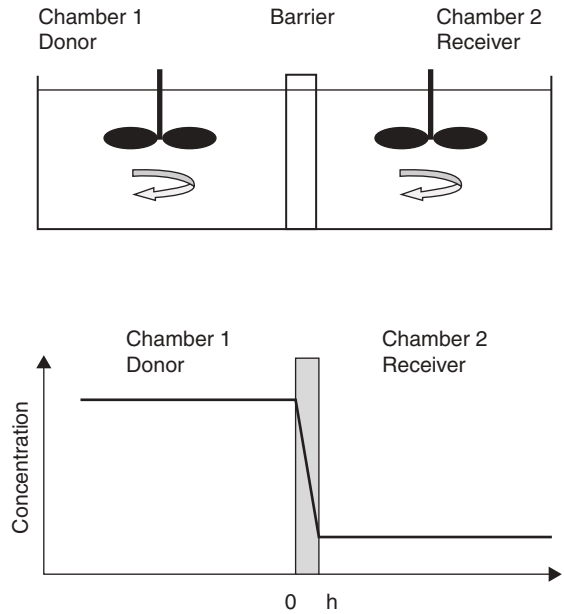

Figure 3.2.2 Top: a two-compartment system for transepithelial flux measurements. A barrier (a tissue or an artificial membrane) is bathed in stirred solutions. Bottom: concentration gradient in the two-compartment system. Normally perfect stirring is assumed, i.e. it is assumed that there are no concentration gradients within each of the two compartments and that the whole concentration drop occurs across the barrier.

or $P_{\text{effective}}$ (P_{eff}) are used when dealing with experimentally obtained permeability coefficients, in order to underscore their vulnerability to bias due to experimental conditions. Assuming that the concentration gradient, $C_{\text{donor}} - C_{\text{receiver}}$, across the barrier is linear and constant, i.e. time independent, Fick's law can be simplified to the equation shown in Equation 3.2.4 and further simplified if it is assumed that the drug substance concentration in the donor compartment is constant and that the concentration in the receiver chamber is virtually zero as compared to the donor concentration. The concentration gradient thus becomes equal to the concentration of the drug substance in the donor chamber at time zero, as shown in Equation 3.2.5:

$$J = P\, C_{\text{donor}} \Leftrightarrow P = \frac{J}{C_{\text{donor}}} \tag{3.2.5}$$

It follows from Equations 3.2.4 and 3.2.5 that the flux is proportional to the concentration gradient and that the permeability is simply the constant that relates flux and concentration gradient. It follows that the

permeability of a drug substance in a given barrier can be estimated from a simple flux experiment at a given concentration gradient, and that the determined permeability value can readily be compared with other permeability values obtained at other concentration gradients. The obtained permeability value can thus be compared with permeability values obtained from a similar experimental setup and, on basis of previous experience, the drug substance in question could be categorised in terms of permeability.

However, a number of experimental considerations must be made, before the estimated permeability value is valid.

1 The concentration gradient should be constant or nearly constant throughout the experiment. Normally, experiments are performed under sink conditions, i.e. the concentration in the receiver compartment is initially zero and is assumed to increase insignificantly during the time course of the experiment. The acceptable increase in $C_{receiver}$ depends on the required precision, but we suggest that the concentration gradient should not change more than 10% during the experiment. The change in concentration gradient is easily measured, either by measuring the concentration in the donor and receiver chamber before and after the experiment, or by using the measured flux value to estimate how much compound has moved during the experiment. If, in fact, the concentration gradient changes considerably, data can be processed assuming non-steady-state conditions, see Section 3.2.4.
2 The concentration gradient should be the only gradient present across the tissue. This implies that concentrations of all other compounds than the drug should be kept similar in the donor and the receiver compartment, that no hydrodynamic gradient should exist and that no electrical gradient should be present when permeabilities of charged drug compounds are measured.
3 It should be ensured that the transport of drug is purely passive. This is normally performed by investigating drug transport at a range of concentration gradients. If transport is purely passive, a linear relationship between flux and concentration gradient will be observed. For active transport see Chapter 3.4.
4 Unstirred water layers surrounding the barrier in question should be minimised. In experimental practice, this is normally accomplished by choosing the highest possible stirring rate, i.e. a rate that minimises concentration gradients in the donor and receiver compartment but does not damage the barrier (properties).

Permeability data may be used for a number of purposes. The permeability constant obtained in a cell model or an *in vitro* tissue model can be used to predict the bioavailability of a given drug substance, or permeabilities of a series of related drug candidates can be compared for selection of a drug candidate with a high permeability. Permeability comparisons must, however, be done with caution. Table 3.2.1 shows the permeabilities of a number of compounds in the Caco-2 cell model and intestinal tissue, respectively. It is evident that the relative ranking of compounds is meaningful, but the absolute values vary between models.

The permeability is a constant that consists of a number of model-specific variables, i.e. the diffusion coefficient of the drug in the barrier (D), the thickness of the barrier (h) and the partition coefficient of the drug into the barrier (α) (Equation 3.2.6, see also Chapter 2.1 for a discussion on partition coefficients).

$$P = \frac{\alpha D}{h} \tag{3.2.6}$$

When comparing permeability data obtained in different experimental setups such as for example intestinal epithelial cell culture models and *in situ* perfusion models, the lipid composition may vary between the two systems, causing different values of D and α. The thickness of the barrier, h, i.e. the height of the cell layer can also vary. Variations in

Table 3.2.1 Permeabilities of drug and reference compounds in Caco-2 cells and human intestine

Compound	*Permeability in Caco-2 cells, apical–basolateral* ($cm\,s^{-1}$)	*Intestinal permeability in rat (r) or human (h)* ($cm\,s^{-1}$)	*Bioavailability (%)*
Mannitol	0.069×10^{-5}	0.3×10^{-5} (r)	Low
Atenolol	0.13×10^{-5}	1.5×10^{-5} (h)	45
Ranitidine	0.01×10^{-5}	1.5×10^{-5} (r)	~50
Terbutaline	$\sim 0.1 \times 10^{-5}$	3.0×10^{-5} (h)	65
Ondansetron	1.8×10^{-5}	n/a	100
Metoprolol	3×10^{-5}	15×10^{-5} (h)	100
Antipyrine	5×10^{-5}	50×10^{-5} (h)	100

Notes: Values are taken from Gan *et al.* (1993); Collett *et al.* (1999); Rege *et al.* (2001); Lennernas (1998); Brusewitz *et al.* (2007); Laitinen *et al.* (2003).
n/a, not available.

the thickness of the unstirred water layers surrounding the tissues can furthermore cause differences in permeability values obtained in the two models. In order to underline that permeability estimates can be prone to errors, it is quite common to use the notation $P_{\text{effective}}$ (P_{eff}) or P_{apparent} (P_{app}) when referring to permeability estimates from flux experiments.

3.2.3 Unstirred water layers

In simple two-compartment systems, barrier permeabilities, estimated from flux measurements, are determined assuming that the concentrations in compartments 1 and 2 are constant throughout the individual compartments. The only concentration gradient present will thus be the gradient across the barrier. However, this simplification does not always hold true. In some cases the permeability measurements are dependent on the stirring conditions. This phenomenon is due to the presence of unstirred water layers close to the tissue, and it is caused by local concentration gradients in the solutions surrounding the tissue. Unstirred water layers can be viewed as two additional barriers in the transport pathway, a barrier in compartment 1 and a barrier in compartment 2 (see Figure 3.2.3).

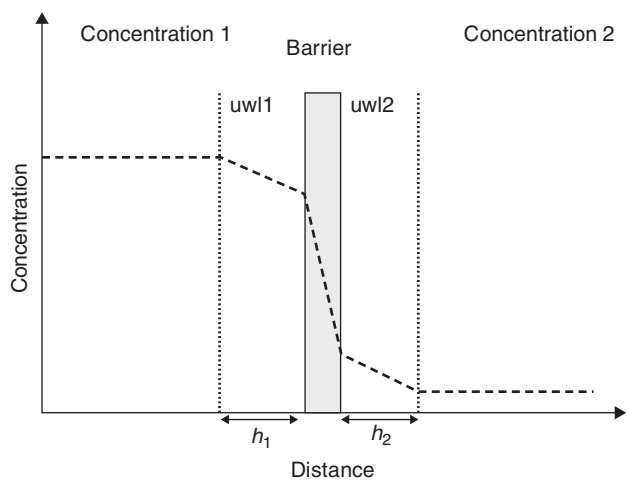

Figure 3.2.3 Unstirred water layers (uwl) can be present close to the tissue barrier due to imperfect stirring of the experimental solutions. The unstirred water layers can be regarded as two extra barriers, with thickness h_1 and h_2, in series with the tissue barrier.

Steady-state flux across an unstirred water layer can be described by the simplified Fick's expression, and an equation can easily be set up describing the measured permeability as a function of the real barrier permeability and the permeabilities in the two unstirred water layers (Equation 3.2.7):

$$\frac{1}{P_{\text{eff}}} = \frac{1}{P_{\text{uwl1}}} + \frac{1}{P_{\text{barrier}}} + \frac{1}{P_{\text{uwl2}}} \Leftrightarrow \frac{1}{P_{\text{eff}}} = \frac{h_1}{D_{\text{uwl1}}} + \frac{1}{P_{\text{barrier}}} + \frac{h_2}{D_{\text{uwl2}}} \quad (3.2.7)$$

where P_{eff} is the actual estimated permeability across the tissue and the unstirred water layers, $P_{\text{uwl1}}/P_{\text{uwl2}}$, h_1/h_2 and $D_{\text{uwl1}}/D_{\text{uwl2}}$ are the permeabilities, thickness and diffusion coefficients of uwl1 and 2, respectively, and P_{barrier} is the true permeability of the tissue barrier.

However, the permeabilities, P_{uwl1} and P_{uwl2} are not readily determined, and therefore the real permeability is not easily derived. A simpler approach for dealing with unstirred water layers is to determine the permeability (P_{eff}) at a range of different stirring rates, and since the estimated permeability will approach the true permeability asymptotically, the true permeability can be estimated from a mathematical fit of the obtained data. An alternative approach is to choose an experimental setup and just compare P_{eff} values under identical conditions, while knowing that they might be underestimated due to the presence of the unstirred water layers.

This possible influence of unstirred water layers must be kept in mind when absolute permeabilities are compared between different experimental setups with different stirring rates and different barrier structures.

3.2.4 Fluxes across a barrier under non-steady-state conditions

In experiments with highly permeable compounds, the transport of a drug candidate from the donor compartment to the receiver compartment cause a first-order decrease of the concentration of drug candidate in the donor chamber and an accompanying increase in its concentration in the receiver chamber (see Figure 3.2.4). In this situation, the concentration gradient cannot be considered constant, and the permeability cannot be calculated directly from Equations 3.2.4 or 3.2.5.

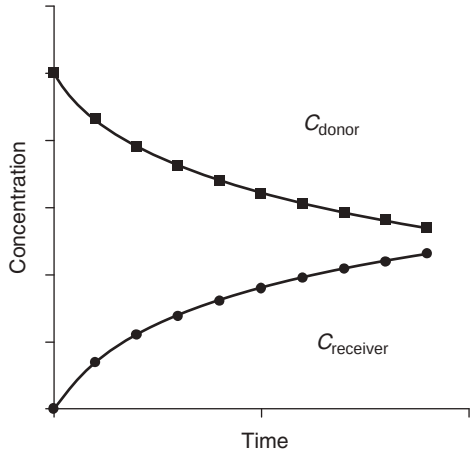

Figure 3.2.4 Non-stationary diffusion of a solute from a donor compartment to a receiver compartment, in a two-compartment system with equal volumes of solution on both sides of the barrier, and initially only solute present in the donor compartment. Both C_{donor} and $C_{receiver}$ will approach the same value asymptotically.

This is observed when flux of a lipophilic drug candidate is measured across a barrier tissue for a sufficiently long time period. The problem can be solved in two ways, either by keeping the volumes of the donor and receiver compartments very large, i.e. by constantly flushing the donor and receiver compartments with fresh experimental solutions (a flow-through system), or alternatively by treating the obtained data using a set of equations that take the changing concentration gradient into account. When a significant flux occurs across a barrier with a constant permeability, the concentration of drug candidate in the donor chamber will decrease with one-phase exponential decay, and the concentration in the receiver chamber will increase with one-phase exponential association. When the concentration of drug is plotted in a log diagram against time, the slope of the curve will be related to the permeability.

The calculation of the permeability from non-stationary fluxes demands knowledge of C_{donor} at time zero ($C_{donor,\ t=0}$), the volumes of the donor and receiver compartments (V_{donor} and $V_{receiver}$), the tissue area (A) and the concentration of at least one (but ideally 3–5) receiver samples at different time intervals (C_t). From these input values, other values can be derived, for use in the calculation, such as the total mass of compound in the system (m_{total}), which is equal to the mass initially added to the donor compartment ($m_{i,\ t=0}$) and can be calculated as $C_{donor,\ t=0} \times V_{donor}$. Then

the following equations can be applied:

$$C_\infty = \text{the final concentration in both compartments} = \frac{m_{i,t=0}}{V_{donor} + V_{receiver}} \tag{3.2.8}$$

$$\frac{C_\infty - C_{receiver,t}}{C_\infty} = e^{-kt} \tag{3.2.9}$$

$$k = AP\frac{C_{donor} + C_{receiver}}{C_{donor} + C_{receiver}} \tag{3.2.10}$$

The constant k can be isolated mathematically or found by plotting time and concentration data in a logarithmic plot, and P can be isolated easily from Equation 3.2.10.

When applying this treatment to experimental data, it must be verified that mass balance exists, as lipophilic drugs often adsorb to pipettes and glass and plastic surfaces.

3.2.5 Fluxes of a charged solute in the presence of an electrical potential gradient

The estimation of a permeability value as described in Equations 3.2.4 and 3.2.5 implies that the only factor responsible for mass transport across the barrier in question is the concentration gradient of the solute. If an electrical potential gradient is present across the tissue and the solute is charged, the driving force for mass transport is a combination of the electrical and chemical gradients, and the estimated permeability will be influenced by the electrical potential gradient (see Figure 3.2.5).

The presence of electrical fields generated by an electrically active tissue can be dealt with in two ways, either by setting up the barrier tissue in an Ussing chamber setup, allowing voltage clamp of the barrier and thereby reducing the electrical potential to zero by applying current from an external current source, or by measuring the electrical potential gradient during the experiment and estimating the permeability by using Equation 3.2.11 (also known as the Nernst–Planck equation):

$$J = zPv\frac{C_2 - C_1 e^{zv}}{1 - e^{zv}} \tag{3.2.11}$$

where J is the flux of charged compound from compartment 1 to compartment 2, z is the charge of the substrate, P is the permeability of the compound, C_1 and C_2 are the concentrations of the compound in

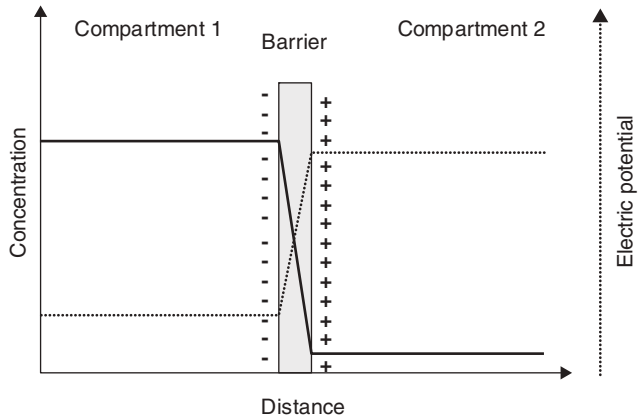

Figure 3.2.5 Flux of a charged solute across a barrier with an electric potential difference between compartment 1 and compartment 2. If an electrical potential exists across a barrier, it will impose a force on charged drug molecules. The direction of the force will depend on whether the compound is positively or negatively charged. When drug transport is studied across electrically active epithelia or across cell membranes, or when currents carried by an electrophoretic process should be predicted, electrical fields must thus be taken into consideration.

compartments 1 and 2, respectively, and v is the normalised electrical potential difference across the barrier (Equation 3.2.12):

$$v = \frac{V\mathrm{F}}{\mathrm{R}T} \tag{3.2.12}$$

In this equation, v is the potential difference across the barrier (with compartment 1 as reference), F is Faraday's number, R is the gas constant and T is the absolute temperature. Using Equations 3.2.11 and 3.2.12, one can either calculate the permeability of a charged compound in the presence of an electric field, by measuring values of flux, J, and potential, V, and then calculating P from Equation 3.2.11, or calculate how much compound will be moved by a given electrical field across a tissue with a given permeability, by inserting the potential V and the permeability in the equation.

3.2.6 Use of flux ratios to analyse transport mechanisms

The transport mechanism of a compound across a given tissue barrier can be analysed in terms of flux ratios, in order to investigate whether the transepithelial transport is active, i.e. energised by ATP-consuming

pumps, or passive (see Section 3.3.1). The flux ratio equation deals with unidirectional fluxes of radiolabelled compounds (Ussing, 1949; Dawson, 1977). A unidirectional flux can be defined as a flux from one compartment to another, across a barrier, measured by radiolabel, without considering counterflux of the same compound. If a charged compound does not interact with other compounds and its transepithelial transport is solely driven by the electrochemical potential difference across the barrier, then the ratio between the unidirectional fluxes can be described as follows:

$$\frac{J^{1-2}}{J^{2-1}} = \left[\left(\frac{C_1}{C_2}\right) e^{\left(\frac{-zFV}{RT}\right)}\right] \tag{3.2.13}$$

where J^{1-2} and J^{2-1} are the fluxes from compartment 1 to 2 and vice versa, V is the potential difference between compartment 1 and 2 ($V_2 - V_1$), C_1 and C_2 are the concentrations in the respective compartments, z is the charge of the compound, F is Faraday's number, R is the gas constant and T is the absolute temperature. What Equation 3.2.13 actually states is that the ratio between the unidirectional fluxes should equal the concentration ratio times a factor describing the electric gradient across the barrier. It follows from the equation that for a non-charged solute, this factor becomes 1, and the flux ratio will thus equal the concentration ratio. A flux-ratio analysis is thus a very simple method of investigating whether a transepithelial transport process is passive or energised.

3.2.7 Conclusions

Mass transport across a barrier, e.g intestinal epithelium or other pharmaceutically relevant barriers, can be caused by diffusion or migration. Transport by simple diffusion can be measured as flux, and characterised by a permeability for the transported solute. The permeability can be calculated using the measured flux and the concentration gradient. Permeabilities can be compared between a series of related compounds in order to select drug candidates in a screening process, or used to predict *in vivo* bioavailability of a given drug substance. Care must be taken in experimental design and when interpreting permeabilities, in order to distinguish permeabilities of compounds with carrier-mediated flux components from permeabilities of compounds that are solely driven by diffusion.

3.2.8 Examples

Example 3.2.1: calculation of the permeability of a drug compound in the Caco-2 cell intestinal model

The apical to basolateral (equivalent to lumen–blood) flux, J^{a-b}, of a new drug candidate **x**, has been measured across 21-day-old Caco-2 cell monolayers grown on permeable filter support. **x** has been added to the apical solution at a concentration of 50 mM. Samples have been taken from the basolateral solution at intervals of 15 min. The concentration of the compound has been measured using radiolabelled **x**. At time zero and at the end of the experiment, donor samples have been taken (see Table 3.2.2).

Experimental parameters:
Volumes of the experimental solutions: apical volume = 0.5 ml, basolateral volume = 1.0 ml. Sample volume, receiver solution (basolateral) = 100 µl; sample volume, donor solution (apical) = 20 µl. Cross-sectional area of tissue = 1 cm^2.

Calculations:
In order to obtain the flux across the tissue, the amount of substance which has moved per time unit must be calculated. This is done, first by calculating the amount (mass) of substance present in the receiver solution at time = t, by multiplying the sample concentration by the volume of

Table 3.2.2 A theoretical data set, showing concentration values sampled at different time points, and the derived flux values

Sample number	*Time, t (min)*	*[Drug **x**] in donor sample (mM)*	*[Drug **x**] in receiver sample, (mM)*	*Mass of Drug **x** in receiver solution (nmol)*	*$Mass_{total}$ (nmol)*	*Flux (nmol $(cm^2\ min)^{-1}$)*
1	0	50	0	0	0	0
2	15	–	0.02	20	20	1.33
3	30	–	0.07	70	72	3.47
4	45	–	0.15	150	159	5.80
5	60	–	0.25	250	274	7.67
6	75	–	0.35	350	399	8.33
7	90	–	0.44	440	524	8.33
8	105	–	0.51	510	638	7.60
9	120	48	0.58	580	759	8.07

experimental solution, i.e.:

$$C_n \times V_r = \text{mass} \tag{3.2.14}$$

However, since substance has been removed from the receiver solution, every time a sample has been taken, a correction must be introduced. The total mass that has crossed the barrier at a given time is thus:

$$\text{Mass}_{\text{total}} = V_s \left(\sum_{n=1}^{n} C_{n-1} \right) + C_n \times V_r \tag{3.2.15}$$

where $C_1, C_2 \ldots C_n$ are the sample concentrations in samples $1, 2 \ldots n$, V_r is the volume of the receiver solution and V_s is the volume of the sample.

The data can be plotted, either in a plot of accumulated **x** versus time (Figure 3.2.6, graph 1) or as a plot of flux of **x** versus time (Figure 3.2.6, graph 2). Both these types of plots have advantages and drawbacks. Graph 1 simply displays the mass of substance per area which has crossed the cell monolayer at a given time. The flux can then be found as the slope of the linear part of the relation. In this example, the flux is 0.0081 μmol $(\text{cm}^2\,\text{min})^{-1}$ or 8.1 nmol $(\text{cm}^2\,\text{min})^{-1}$. The intercept with the x axis is called the *lag time* and in the example shown this is 25.5 min. The lag time is obtained by setting $f(x) = 0$.

Graph 2 in Figure 3.2.6 displays the same data set, but with the flux instead of accumulation on the y axis. Thus the bar at the x value 15 min is the mean flux during time 0–15 min, the bar at 30 min is the mean flux at 15–30 min etc. The flux, or transport rate, reaches steady state after

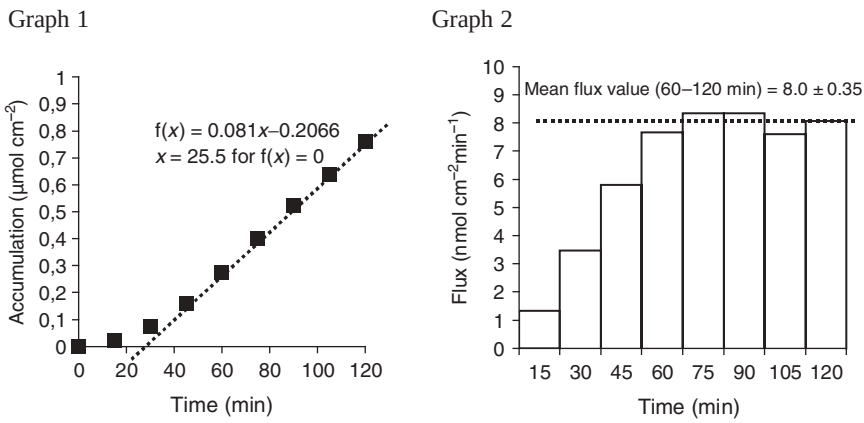

Figure 3.2.6

~60 min, as judged by visual inspection of the data. The steady-state flux can thus be determined as the mean of the flux values obtained in the steady-state period (60–120 min). The value 8.0 nmol cm^2 min^{-1} is obtained from the present data set, a value corresponding fairly well with the determination from graph 1. When plotted in a graph like graph 2, parameters such as the time of steady-state and possible depletion of donor compound will be more visible to the investigator, whereas graph 1 demands fewer calculations and yields the lag-time.

The permeability can now be calculated using Equation 3.2.5 (thus ignoring the slight concentration drop in the donor chamber during the experiment):

$$P = \frac{J\,\frac{\text{nmol}}{\text{cm}^2\ \text{min}}}{C_{\text{donor}}\,\frac{\text{mmol}}{\text{l}}} = \frac{8\ \text{nmol l}}{50\ \text{mmol cm}^2\ \text{min}} = 0.16\,\frac{10^{-9}\,\text{l}}{10^{-3}\ \text{cm}^2\ \text{min}}$$

$$= 0.16\,\frac{10^{-9}\,10^{3}\ \text{cm}^3}{10^{-3}\ \text{cm}^2\ \text{min}} = 0.16\ 10^{-3}\,\frac{\text{cm}}{\text{min}} = 9.6\ 10^{-3}\,\frac{\text{cm}}{\text{h}} = 2.6\ 10^{-6}\,\frac{\text{cm}}{\text{s}} \tag{3.2.16}$$

Example 3.2.2: calculation of the permeability of a drug in the Caco-2 cell intestinal model under non-steady-state conditions

The apical to basolateral (equivalent to lumen-blood) flux, J^{a-b}, of a new drug compound **y**, has been measured across 21-day-old Caco-2 cell monolayers grown on permeable filter support. As in the previous example, **y** has been added to the apical solution at a concentration of 50 mM. Samples have been taken from the basolateral solution at varying intervals, see below. The concentration of the compound has been measured using radiolabelled compound **y**. At time zero and at the end of the experiment, donor samples have been taken (see Table 3.2.3).

Experimental parameters:
Volumes of the experimental solutions: apical volume = 0.5 ml, basolateral volume (at $t = 0$) = 1.0 ml. Sample volume, receiver solution (basolateral) = 10 µl; sample volume, donor solution (apical) = 10 µl. Cross-sectional area of tissue = 1 cm^2.

Calculations:
In order to obtain the flux across the tissue, the amount of substance that has moved per time unit is calculated as in the previous example. The flux values indicate that a large percentage of the added dose has moved across

Table 3.2.3 A theoretical data set, showing concentration values sampled at different time points under non-stationary flux conditions

Sample number	*Time, t (min)*	*[Drug* **y***] in donor sample (mM)*	*[Drug* **y***] in receiver sample (mM)*
1	0	50	0
2	10	–	8
3	30	–	14
4	90	16.6	16.6

the barrier during the measurement period, therefore a non-steady-state flux analysis is performed.

The concentration of compound **y** at time infinity in both compartments is calculated, using Equation 3.2.8:

$$C_\infty = \frac{m_{i,t=0}}{V_{donor} + V_{receiver}} = \frac{50\,\text{mM}\,0.5\,\text{ml}}{0.5\,\text{ml} + 1\,\text{ml}} = 16.7\,\text{mM} \qquad (3.2.17)$$

The concentrations measured can be fitted to Equation 3.2.9:

$$\frac{C_\infty - C_{receiver,t}}{C_\infty} = e^{-kt} = \frac{16.7\,\text{mM} - C_{receiver,t}}{16.7\,\text{mM}} = e^{-kt} \qquad (3.2.18)$$

The k value can be estimated graphically or by isolation, and can be used to calculate the permeability according to Equation 3.2.10:

$$\begin{aligned} k = 62.7\ 10^{-3}\,\text{min}^{-1} &= AP\frac{V_{donor} + V_{receiver}}{V_{donor}V_{receiver}} \\ &= 1\,\text{cm}^2\,P\,\frac{0.5\,\text{ml} + 1\,\text{ml}}{0.5\,\text{ml}\,1\,\text{ml}} \Leftrightarrow P = 62.7\ 10^{-3}\,\text{min}^{-1}\frac{\text{cm}^3}{3\,\text{cm}^2} \end{aligned} \qquad (3.2.19)$$

obtaining a P value of $0.0209\,\text{cm}\,\text{min}^{-1}$ or $3.5 \times 10^{-4}\,\text{cm}\,\text{s}^{-1}$.

Example 3.2.3: flux ratio analysis

The unidirectional fluxes of a novel drug substance, testicine, have been measured in Caco-2 cell monolayers. The monolayers have been incubated with 1 mM of isotope-labelled testicine in both the apical and basolateral compartment, and steady-state fluxes have been determined. The apical and basolateral test solutions are identical. Testicine has a negative charge. The monolayers are mounted in an Ussing chamber, allowing for

measurement of the potential difference (V) from the apical to the basolateral side.

The following parameters are obtained during the experiment:

$$J_{\text{apical–basolateral}} = 15\,\text{nmol} \times \text{cm}^{-2} \times \text{min}^{-1}$$

$$J_{\text{basolateral–apical}} = 10\,\text{nmol} \times \text{cm}^{-2} \times \text{min}^{-1}$$

$$V = V_{\text{basolateral}} - V_{\text{apical}} = 10\,\text{mV} = 0.010\,\text{J C}^{-1}$$

Temperature = 20 °C

F, R and T have the values:

$$\text{F} = 96\,487\,\text{C mol}^{-1}$$

$$\text{R} = 8.3144\,\text{J (K mol)}^{-1}$$

$$T_{20\,^\circ\text{C}} = 293\,\text{K}$$

The experimentally determined flux ratio thus becomes:

$$\frac{J^{1-2}}{J^{2-1}} = \frac{15}{10} = 1.5 \tag{3.2.20}$$

and the calculated flux ratio becomes

$$\left(\frac{C^1}{C^2}\right) e^{\left(\frac{-zFV}{RT}\right)} = \left(\frac{1}{1}\right) e^{\left(\frac{-(-1)\ 96487\ \text{C mol}^{-1}\ 0.01\ \text{J C}^{-1}}{8.31144\ \text{J K}^{-1}\text{mol}\ 293\ \text{K}}\right)} = 1.49 \tag{3.2.21}$$

The flux ratio analysis thus indicates that no active transporters are involved in the transepithelial transport process, which appears to be driven solely by the transepithelial electrochemical gradient.

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3.3

Carrier-mediated transport kinetics

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Over the last few decades it has become clear that a number of drug substances are transported across epithelial or endothelial biological barriers in a saturable way. For many of these compounds it has been shown that the explanation for this saturability is that they are substrates for transporters. Transport of these compounds is thus mediated by transport proteins present in the biological membrane. Such proteins exert physiological functions in which they transport endogenous molecules or exogenous nutrients, toxins or vitamins from one compartment to another. They are specialised transporters in terms of bringing or excluding one class of compounds from the exterior to the interior of the human body, or from one interior compartment to another. Thus, besides being saturable they are also selective and specific in their recognition of substrates. Nevertheless, some drug substances are transported by one or more transporters and must in this way mimic the biological presence of their 'natural substrates'. Transporter-mediated transfer of a compound from one compartment to another may influence its pharmacokinetics. In some cases, if the concentration of drug substance is lower than the maximal transport capacity of the transport system then its transport is controlled by transporter. In other situations, drug transfer may be limited if the concentration needed is much higher than the capacity of the transport system. It is therefore necessary for the scientist working in molecular biopharmaceutical settings to be able to describe the kinetics of drug transfer via a transport protein, to investigate whether a drug candidate is a substrate for a transporter or not, and, furthermore, to evaluate the impact of these observations on the usefulness of a given new drug candidate. In the following sections carrier types and functions will be described and the basic equations for describing transport of molecules via a carrier protein will be given, with a special focus on carriers located in the intestine.

3.3.1 Carrier function and mechanisms

The chemical and physical properties of cell membranes make them practically impermeable for ions and hydrophilic compounds. The cell relies on specialised transport protein for exchanging ions and hydrophilic nutrients with its surroundings. The transport proteins are typically integral proteins, which span the lipid bilayer of the cell membrane multiple times. The cell maintains an asymmetric distribution of ions due to two fundamental types of membrane transport protein – carriers and channels. The concentrations of ions outside and inside the cell are listed in Table 3.3.1.

Carriers are functionally characterised by their substrates, their driving force and their substrate specificity. The transport of substrate via a carrier is passive in nature since the transport rate is dependent on the concentration gradient of the transported substrates. However, if the carrier is viewed in the dynamic cellular system, it may, for symports or antiports, be secondary or tertiary dependent on an active transporter in order to maintain the gradients for the co-transported substrate. In general, carriers must be able to perform at least four functions in order to move a substrate across the cell membrane, i.e. transport the substrate; these are illustrated and explained in Figure 3.3.1.

Depending on the nature of these processes, the carrier is classified. In the following the concepts of carriers – uniports, symports, antiports and active transporters – will be illustrated, using examples that are relevant for the biopharmaceutical student or scientist. Channels do not

Table 3.3.1 Approximate concentrations of important ions inside and outside a generalised mammalian cell

Ion	*Concentration (mM)*		*Equilibrium potential (mV)*
	Inside the cell	*Outside the cell*	
Na^+	5 to 15	150	91 to 62
K^+	150	5.5	−88
Cl^-	9	125	−70
Ca^{2+}	10^{-4}	1 to 2	129 to 132
Mg^{2+}	0.5	1 to 2	9 to 19
[a]H^+	4×10^{-5}	1.6×10^{-4} to 4×10^{-5}	−37 to 0

Notes: Equilibrium potential (mV) is calculated at 37 °C using the Nernst equation.
[a]The proton concentration inside the cell is calculated based on an intracellular pH of 7.4, and for outside the cell on a pH range of 6.8 to 7.4.

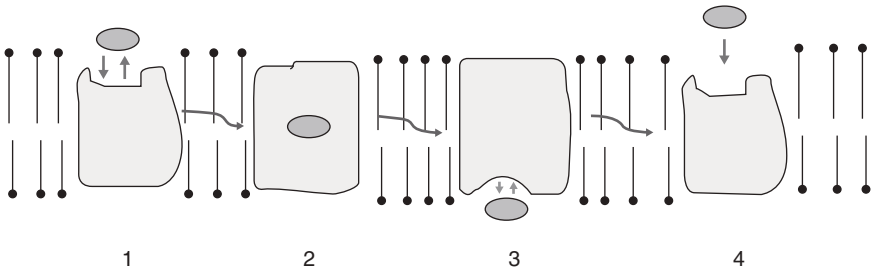

Figure 3.3.1 Illustration of the conformational changes associated with translocation of substrate:

1 recognition of the substrate is the process where the substrate binds to the carrier
2 translocation of the substrate is the process where the carrier undergoes a conformational rearrangement with the result of moving the substrate from outside the cell to the inside of the cell
3 release of the substrate where the substrate is dissociated from the carrier and present in the cytosol of the cell
4 recovery of the conformation of the carrier to the orientation in which it is ready to bind another substrate.

need to bind the substrate in order to transport the solute across the membrane. Channels form hydrophilic pores that the solute, typically inorganic ions, can pass through from one compartment to another.

3.3.1.1 Uniports

Uniports are carriers that only move one substrate in one direction in the transport process (Figure 3.3.2). This implies that there is no additional

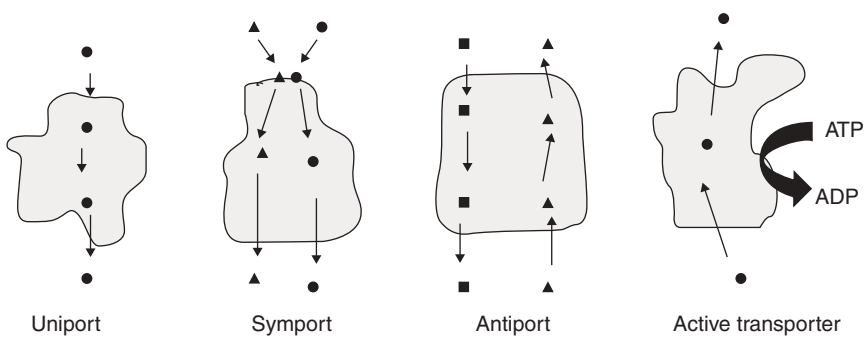

Figure 3.3.2 Illustration of the various forms of carriers.

ion or substrate involved in the translocation of a substrate across the cell membrane. The transport of a neutral substrate is thus dependent only on the concentration gradient of the single substrate, and the concentration of substrate outside and inside the cell will define the rate of substrate transfer via the carrier. In other words the transport process will occur downhill, i.e. down the concentration gradient, and no intracellular accumulation occurs. However, the carrier will still perform a vital function since it facilitates the transfer of a substrate that is otherwise not normally transportable from the outside/inside of the cell to the inside/outside. For charged compounds, the electrical potential across the cell membrane may influence the transport of a compound. A combination of a chemical and electrical gradient is termed an electrochemical gradient. Typical uniports are found in the solute carrier family 2, SLC2, of glucose transporters. SLC2 family members facilitate the diffusion of glucose across membranes of different tissues, and they are referred to as glucose transporters or GLUTXs, where X is a number indicating the isoform of the transporter. Other uniport carriers are found in the facilitative nucleoside transporter family, SLC29, which transports nucleosides, and the Na^+-independent, system L-like amino acid transporter family, SLC43, which transports neutral amino acids. Different isoforms of these transporters are found in different tissues and have different localisations at the cellular level of polarised cells, i.e. at the apical or basolateral membranes.

3.3.1.2 Symports

Carriers that have two or more substrates moving in one direction during the transport process are called symports (see Figure 3.3.2). This means that in addition to the substrate, which could be an amino acid, a dipeptide or a nucleoside molecule, there is also an ion or substrate involved in the translocation process across the cell membrane. The transport of a substrate is thus dependent on both the concentration gradient of the substrate itself and the concentration of co-substrate. The typical symport substrate would be a solute such as an amino acid, a di/tripeptide or a nucleoside, and typical co-substrates are ions such as Na^+ or H^+. The rate of transport via the carrier will depend on both the concentration gradient of the substrate and the concentration gradient of the co-substrate(s). The total driving force for the transport process is thus the sum of the electrochemical gradients working on the cellular system. This enables the cell to accumulate the substrate intracellularly by utilising the gradients of the co-substrates. This is also known as uphill

transport or a concentrative capacity of the transport system. This is seen for members of the proton oligopeptide cotransporter family, SLC15, where di/tripeptides are transported along with protons (Brodin *et al.*, 2002). In the small intestine the H^+/peptide transporter, PEPT1, transports di/tripeptides from the intestinal lumen into an enterocyte along with a proton. The influx of protons causes a decrease in intracellular pH, which activates the Na^+/H^+ exchanger, and proton efflux occurs by exchanging protons for sodium ions. This maintains the intracellular pH around approximately 7.4, and, due to the slightly acidic microclimate of the intestine (pH ~6.8), the pH gradient is maintained. Since the total driving force is responsible for the transport of di/tripeptides via PEPT1, the cell is able to accumulate an amount of peptide substrate inside the cell – even though the concentration gradient of the peptide is in the opposite direction. As mentioned earlier, symports may have two substrates moving in the same direction. In addition to a dependence on the amino acid gradient, $ATB^{0,+}$ (SLC6A14) from the solute-linked carrier family 6, which transports neutral and cationic amino acids is also dependent on both sodium and chloride as driving forces. Thus three substrates are moving in the same direction. This involvement of two co-substrates makes the concentrative capacity of the carrier higher than for an amino acid carrier coupled to sodium alone (Ganapathy *et al.*, 2005).

3.3.1.3 Antiports

Carriers that have two substrates moving in opposite directions during the transport process are called antiports (see Figure 3.3.2). For antiports, one substrate involved in the translocation process will exchange another substrate across the cell membrane. The transport of a substrate is thus dependent on both the concentration gradient of the substrate itself and the concentration of co-substrate. The typical example of an antiport would be the Na^+/H^+ exchanger (NHE), for which a Na^+ ion is exchanged for a H^+ across the cell membrane. The rate of transport via the carrier will depend on the concentration gradient of the substrates going in opposite directions. Antiports are also involved in the exchange of amino acids across the basolateral membranes of cells.

3.3.1.4 Active transporters

In the previous sections the different types of carriers were described. Carriers are passive in nature and the transport rate of substrate depends

on the electrochemical gradients involved, which determine the driving force for the transport process. However, other integral proteins exist where the translocation of substrates is coupled to the direct consumption of cellular ATP. Transport proteins utilising ATP for transporting their substrates are called active transporters, or primarily active transporters or pumps. The hydrolysis of ATP: $ATP^{4-} + H_2O \rightarrow ADP^{3-} + H^+$, yields energy ($\Delta G' = -30\,kJ\,mol^{-1}$), which is utilised in the transport process for transporting the substrate in the opposite direction to the chemical gradient. As seen in Table 3.3.1, the intracellular concentration of Na^+ is low compared to the extracellular concentration; nevertheless, one of the cell's most fundamental functions is the active transport of Na^+ out of the cell and influx of K^+ via Na^+/K^+-ATPase. Another class of active transporters that is important for the biopharmaceutical student or researcher is the active multidrug transporters. These transporters are described in Chapters 3.4 and 3.6. Briefly, they use the energy from the hydrolysis of ATP to transport a wide range of endogenous and therapeutically active molecules out of various cells and are called efflux transporters.

3.3.1.5 Carriers and active transporters in the dynamic cellular system

Carriers and transporters may be described functionally when viewed alone. However, in the dynamic cell the carriers or transporters work in concert with other carriers and ion channels. Even though the transport of a substrate via a carrier is passive in nature, the maintenance of gradients responsible for their transport in a dynamic cell system may be dependent on active transporter(s). Therefore the carriers in a cell system may be described according to their dependence on active transporters. This is illustrated in Figure 3.3.3.

In situation 1 the true active transporter is illustrated. The transport of substrate is coupled to the hydrolysis of ATP, and is dependent on the ATP level and the concentration of substrate. In situation 2 the transport of substrate via a symport is illustrated. In the case of glucose, the influx of glucose is coupled to the transport of Na^+ and occurs via the sodium-dependent glucose transporter SGLT1. The Na^+ ion moving into the cell is transported across the basolateral membrane via the active Na^+/K^+-ATPase. The transport of glucose via SGLT1 is thus secondarily dependent on an active transporter, and at the cellular level it is a secondarily active transporter. Glucose is either consumed inside the cell or

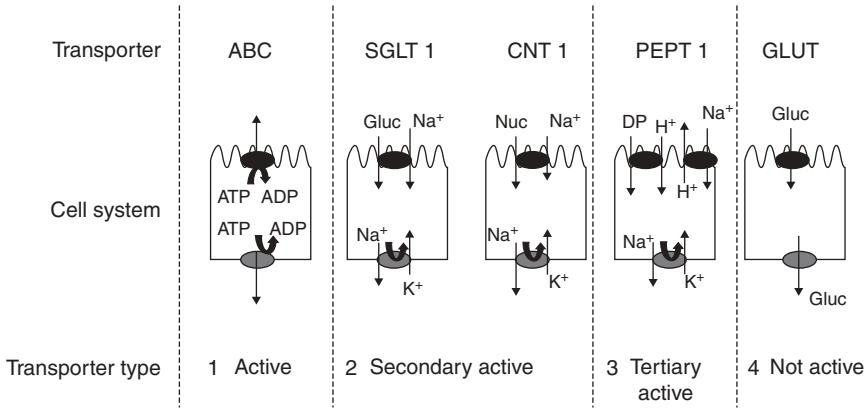

Figure 3.3.3 Illustration of the various forms of carriers working in a dynamic cell. Gluc, glucose; Nuc, nucleotide; DP, dipeptides; ABC, ATP-binding cassette family transporter; SGLT1, the sodium-dependent glucose transporter; CNT1, the concentrative nucleotide transporter; PEPT1, the di/tripeptide transporter. These transporters are discussed further in Chapter 3.4.

transported across the basolateral membrane via the uniport GLUT5. In situation 3 the intestinal peptide transporter, PEPT1, is a carrier responsible for the cellular accumulation of di/tripeptides. The influx of substrate is dependent on protons, and if no other mechanisms were working in the cell the intracellular environment would eventually exist in equilibrium with the extracellular fluid, and the concentration of substrate and protons will be the same on each side of the cell membrane. In the dynamic cell system, the protons are exchanged across the apical membrane for sodium, thereby creating a sink for protons and resulting in an uphill transport of substrate. The Na^+ ion moving into the cell ion is transported across the basolateral membrane via the active Na^+/K^+-ATPase. The influx of peptide and protons is thus indirectly dependent on the functional activity of the active Na^+/K^+-ATPase, and in the dynamic cell PEPT1 is termed a tertiary active transporter. The influx of peptide substrate is tertiarily dependent on an active transporter. At the transporter level alone, PEPT1 is a symport (see Section 3.3.1.2 Symports), and at the cellular level it is a tertiary active transporter. The peptide substrate does not stay in the cell; it is either metabolised to amino acids or transported out of the cell across the basolateral membrane. In situation 4 the influx and efflux of substrate in a dynamic viable cell is mediated by two identical or distinct carriers, and the total cellular transport is not directly or indirectly dependent on any active transporters.

3.3.2 Description of carrier-mediated transport kinetics

The identification of carrier type and how the carrier operates at a cellular level is normally necessary for an appropriate mathematical description of the transport of substrate. In this section transporter kinetics are discussed, whereas non-saturable kinetics are described in Chapter 3.2.

3.3.2.1 The carrier seen as an enzyme

The mathematics used to describe transport via carriers is derived from the kinetics known for enzymes since the early 1900s. For enzymes the reaction between one substrate, S, and enzyme, E, forming a product, P, is initially given by:

$$\mathrm{E} + \mathrm{S} \leftrightarrow \mathrm{ES} \rightarrow \mathrm{E} + \mathrm{P} \tag{3.3.1}$$

In contrast, the simplified transport of one substrate via a transport protein, T, is initially given by:

$$\mathrm{T} + \mathrm{S} \leftrightarrow \mathrm{TS} \rightarrow \mathrm{T} + \mathrm{S} \tag{3.3.2}$$

The enzyme mediates the bioconversion of a substrate to a product, whereas the transport protein moves the substrate from one compartment to another without changing the structure of the substrate. Originally described for enzymes, the initial velocity is given by the Michaelis–Menten equation:

$$V_{\mathrm{o}} = \frac{V_{\max}[S]}{K_{\mathrm{m}} + [S]} \tag{3.3.3}$$

V_{o} is the initial velocity (molarity/time), $V_{\max}$ is the maximal velocity at a constant enzyme concentration (molarity/time), S is the substrate concentration (molarity), and K_{m} is the equilibrium constant when V_{o} is equal to one-half of $V_{\max}$. Equation 3.3.3 is also used for transport proteins present in tissues or cell cultures and the velocity is sometimes normalized with respect to the transport area, thereby yielding the flux, J (moles/area/time), across the system:

$$J_{\mathrm{o}} = \frac{J_{\max}[S]}{K_{\mathrm{m}} + [S]} \tag{3.3.4}$$

In the following, the derivation of the Michaelis–Menten equation is given under steady-state conditions. According to Equation 3.3.2, the

following reactions are involved in transport of a substrate: under steady-state conditions in which the concentration of transport protein is maintained constant and the transporter–substrate complex is constant:

$$T + S \overset{k_1}{\longleftrightarrow} TS \overset{k_2}{\longleftrightarrow} T + S \tag{3.3.5}$$

If the amount of substrate is lower than the amount required to completely occupy all the transport proteins present, a population of free transporters (transporters not binding a substrate) must exist:

$$[\text{transporter}]_{\text{free}} = [\text{transporter}]_{\text{total}} - [\text{transporter}]_{\text{bound}} \tag{3.3.6}$$

If we look at a situation where the rate constant for the release of substrate, k_2, is higher than k_{-2} (rate constant in the opposite direction to k_2), the following can be stated:

$$(k_{-1} + k_2)[TS] = k_1([T] - [TS])[S] \tag{3.3.7}$$

Collecting the rate constants yields an equilibrium, which is here termed the Michaelis–Menten constant:

$$(k_{-1} + k_2)/k_1 = K_m = (([T]_{\text{total}} - [TS])[S])/[TS] \tag{3.3.8}$$

Solving this equation for [TS] gives the following steps:

$$([T]_{\text{total}} - [TS])[S] = [TS]K_m \tag{3.3.9}$$

$$[TS]K_m = ([T]_{\text{total}}[S]) - ([TS][S]) \tag{3.3.10}$$

$$[TS]([K_m + [TS]) = [T]_{\text{total}}[S] \tag{3.3.11}$$

And finally:

$$[TS] = ([T]_{\text{total}}[S])/[K_m + [S]) \tag{3.3.12}$$

Since the velocity of the transport process depends on the rate of the release of substrate from the transporter:

$$V = k_2[TS] \tag{3.3.13}$$

The expression of the rate equation is obtained by substituting the value of [TS] from Equation 3.3.13 into Equation 3.3.12:

$$V = (k_2[T]_{\text{total}}[S])/(K_m + [S]) \tag{3.3.14}$$

And, since $k_2[T]_{\text{total}}$ equals the maximal velocity at a given transporter concentration, $V_{max} = k_2[T]_{\text{total}}$, Equation 3.3.14 can be rearranged

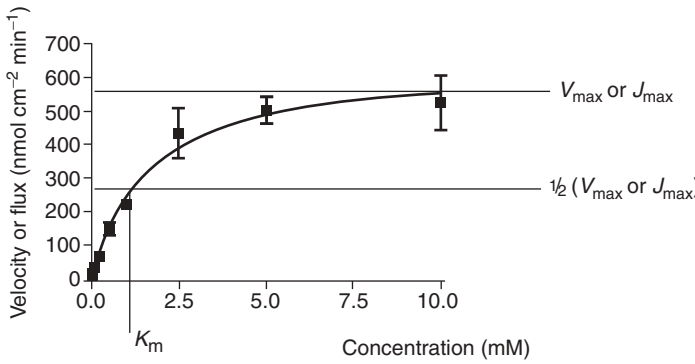

Figure 3.3.4 Illustration of a Michaelis–Menten curve. The velocity or flux for a saturable transport process is described as a function of the total concentration of substrate at one given transporter concentration.

to give the familiar Michaelis–Menten expression given earlier in Equation 3.3.3:

$$V_o = \frac{V_{max}[S]}{K_m + [S]} \tag{3.3.15}$$

The velocity's dependence on concentration is illustrated in Figure 3.3.4.

3.3.2.2 The carrier seen as an adsorption surface

The carrier transport derived based on enzyme kinetics is one way of viewing transporter kinetics; another is to view the carrier as a surface available for adsorption of substrates at discrete points (Neame and Richards (1972). The carrier is seen as composed of a number of mobile adsorption sites in which the substrate is adsorbed at one surface and later released from another surface. By using the Langmuir adsorption equation, the derivaton of an expression describing the transport rate via a carrier yields basically the Michaelis–Menten-type equation given in Equation 3.3.3.

3.3.2.3 The maximal velocity and the amount of enzyme present

In Figure 3.3.4, the typical Michaelis–Menten curve is illustrated. It can be seen that as the concentration is increased the velocity of the transport process is also increased. However, two important observations can also

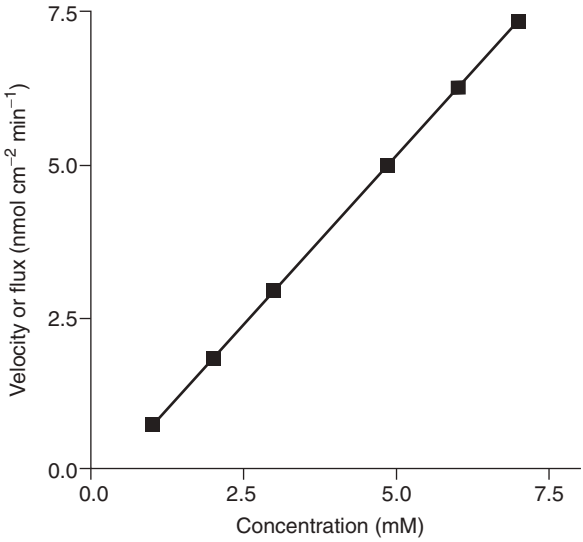

Figure 3.3.5 Illustration of the dependence of velocity on the transporter concentration: the effect of the transporter concentration, $[T]_{total}$ (mM), on the velocity, *V*, (M/time) of a transporter-mediated transport at a fixed constant concentration of substrate, [S].

be made: (1) as the concentration of substrate is increased to well above the concentration equal to K_m, the velocity, *V*, gradually approaches a constant velocity at which it is constant and equal to the maximal velocity, V_{max}, at the given amount of transporters present; (2) as the concentration is increased the change in velocity, i.e. the slope of the curve in Figure 3.3.4, decreases gradually to zero. It is important to remember than V_{max} is dependent on the total number of transporters present, as illustrated in Equation 3.3.14. This is particularly important when evaluating experimental results obtained from different experimental system (see Sections 3.3.3 and 3.3.4). If the amount of transporter varies between the experiments, the velocity will vary accordingly, and problems will arise when the analysis of the experiments is conducted. As seen in Figure 3.3.5, there is a linear relationship between the velocity of the transport process and the amount of transporter present in the experimental or cellular system.

3.3.2.4 The Michaelis–Menten constant

The Michaelis–Menten constant is an equilibrium constant, which is experimentally determined from the initial concentration of substrate at half-maximum velocity, or at half-saturation of T with S. As seen

in Equation 3.3.8, the constant is based on how well a substrate binds to its transport protein. However, the constant may be dependent on substrate binding to at least two conformations of the transport protein, which in simplified terms mean its conformations at intra- and extracellular positions. The K_m value may be viewed as an indicator for how good a substrate is for a given transporter. However, since both k_1 and k_2 (Equation 3.3.8) may be rate-determining parameters in the expression for K_m, a change in K_m may for a transporter be a result of binding of substrate in the two different binding situations. From Equation 3.3.8 it is also seen that the K_m value is independent of the absolute amount of transporter present in the investigated system. The amount of transporter must, of course, be constant in the experiments giving the velocities at given concentrations.

3.3.2.5 What happens if other ligands or substrates are present?

The kinetics of transporter-mediated transport of a single substrate via one transporter are described above. However, transporters normally have several substrates, which are structurally similar, and therefore the transport of a given substrate will be influenced by the presence of other substrates. Moreover, lead series of novel compounds from a drug discovery programme or newly developed compounds from rational synthetic design are often investigated or even targeted for their ability to interact with transporters. In the following section the kinetics and experimental tools for investigating interactions of leads and drug candidates with transporters are described.

3.3.2.5.1 Substrate, ligand, or transportate?

First, let us look at the types of interaction between a compound and a transporter that we are likely to encounter. In traditional enzyme terminology, the term 'substrate' is used for a molecule that which binds to the enzyme and undergo a series of reactions, which results in the formation of a product. The term 'ligand' is used in the literature on receptors, where a molecule binds to a receptor, which subsequently initiates a cascade of biological responses. On the other hand, an inhibitor is a molecule that binds to an enzyme (or receptor) and interferes with the enzyme's activity by preventing either the formation of the ES complex or the generation of a product. How does this translate into looking at transporters? In terms of transporters, a substrate is a molecule which is moved

from one compartment to another by the transporter (see Section 3.3.2.1). Hence, the term 'substrate' in the field of transporters has a slightly different meaning from the term 'substrate' for an enzyme. Nevertheless, the term 'substrate' is most often used for a molecule that is moved from one compartment to another by a transporter, i.e. translocated. The term 'transportate' (Brandsch *et al.*, 2004) has been suggested for a molecule translocated by a carrier protein, and this may more appropriately describe the process; however, the future will show if this term is being widely accepted in the field. Normally, a compound that binds to a transporter without being translocated is referred to as a 'ligand'. However, the term 'ligand' brings associations with a molecule that binds to a receptor and elicits a biological response, and therefore the term 'inhibitor' may be a more precise one to use.

3.3.2.5.2 Competitive inhibitors

In preclinical studies as well as molecular biopharmaceutical research, experiments are often performed in order to investigate whether a novel compound is binding to a given transport protein. This is frequently tested against a known substrate. The first analysis of binding assumes that the substrate and the novel compound (here termed I for inhibitor) interact with the same binding site of the transporter:

$$T+S \Leftrightarrow TS \Leftrightarrow T+S \tag{3.3.16}$$

$$T+I \Leftrightarrow TI \tag{3.3.17}$$

This kinetic scheme, in which I binds to the transport protein, is known as competitive inhibition. Conceptually, in a competition assay, we then look at a new compound as an inhibitor. However, by measuring the transport of S via T, we can easily extract information with regard to whether I decreases the transport of S in a concentration-dependent manner or not. The different types of reversible inhibition can then be distinguished experimentally, by investigating inhibitor effects on the kinetic parameters for the transport process. These inhibitor effects are summarised in Table 3.3.2. However, from competition assays one may not able to distinguish between an inhibitor and a transportate, but only whether a new compound binds or not to the transporter. Initially we do not know if the binding is reversible or irreversible, but that can be tested by washing I out of the experimental system. The ability of the

Table 3.3.2 Effects of reversible inhibitors on kinetic constants

Type of inhibitor	*Effect*	*Rate law*	*Calculation of K_i from IC_{50}*
Competitive: I only binds to T	Raises $K_m V_{max}$ is unchanged	$V = \frac{V_{max}[S]}{K_m(1 + \frac{[I]}{K_i}) + [S]}$ (3.3.19)	$K_i = \frac{IC_{50}}{(1 + \frac{[S]}{K_m})}$ (3.3.22)
Uncompetitive: I only binds to TS	Lowers K_m and $V_{max}V_{max}/K_m$ is unchanged	$V = \frac{V_{max}[S]}{K_m + (1 + \frac{[I]}{K_i})[S]}$ (3.3.20)	$K_i = \frac{IC_{50}}{(1 + \frac{K_m}{[S]})}$ (3.3.23)
Non-competitive: I binds to T and TS equally	Lowers V_{max} and K_m remains unchanged	$V = \frac{V_{max}[S]}{K_m(1 + \frac{[I]}{K_i}) + (1 + \frac{[I]}{K'_i})[S]}$ (3.3.21)	$IC_{50} = \frac{K_m + [S]}{(\frac{K_m}{K_{i1}} + \frac{[S]}{K_{i2}})}$ (3.3.24)
I binds to T and TS unequally	Lowers V_{max} and raises or lower K_m		

compound to interact with T may, from the kinetic scheme in Equations 3.3.16 and 3.3.17 be described by Equation 3.18:

$$K_i = \frac{[T]\,[I]}{[TI]} \tag{3.3.18}$$

and the effect on the transport rate of S is given in Equation 3.3.19 (Table 3.3.2). K_i is a constant for the dissociation of I from the TI complex, and is called the inhibition constant. K_i is often termed 'affinity', and is an often used parameter in the evaluation of series of 'substrates' for transporters. However, from Equation 3.3.18 it is apparent that the K_i value is not easily determined because the concentration of both transporter and transporter–inhibitor complex should be known. However, the determination of the K_i value is facilitated by the use of radiolabelled standard compounds. The standard compounds are well characterised as substrates for specific transporters. Some of these standard compounds are available as radiolabelled isotopes, and this offers a natural starting point for investigation of the interaction between a transporter and novel compounds. An example of this is transport via PEPT1 (mentioned in Section 3.3.1.2 Symports), where the stabilised dipeptide Gly-Sar (either ^{3}H- or ^{14}C-labelled) has been characterised over a couple of decades, and is now widely used as a standard substrate for investigating transport via PEPT1. Novel compounds are typically characterised by their ability to inhibit the transport of Gly-Sar via PEPT1 in a concentration-dependent manner. Examples of this are seen in Figure 3.3.6.

The concentration of the new compound, which is able to inhibit 50% of the Gly-Sar uptake via PEPT1 is the concentration at 50% inhibition, i.e. the IC_{50} value. The lower this value, the higher the ability of the compound to inhibit the uptake of Gly-Sar, and the tighter the binding. The IC_{50} value can be used to calculate the K_i value of the competitive inhibitor (new compound) by determining the IC_{50} value along with the K_m value for the standard substrate (Equation 3.3.22). The concentration of standard substrate, [S], should be known; however since the radiolabelled standard compounds normally have a high specific activity, the assay concentration of S is normally much lower than its K_m value, and therefore the difference between its IC_{50} value and its K_i value is almost insignificant. IC_{50} values and K_i values are referred to as affinity values. Affinity values provide important information about transporter recognition and apparent binding strength of molecules. The affinity values are used to rank a lead series of new compounds with respect to their binding, and as such provide a basis for selecting molecules for further analysis. Affinity values are also used for molecular modelling purposes and for

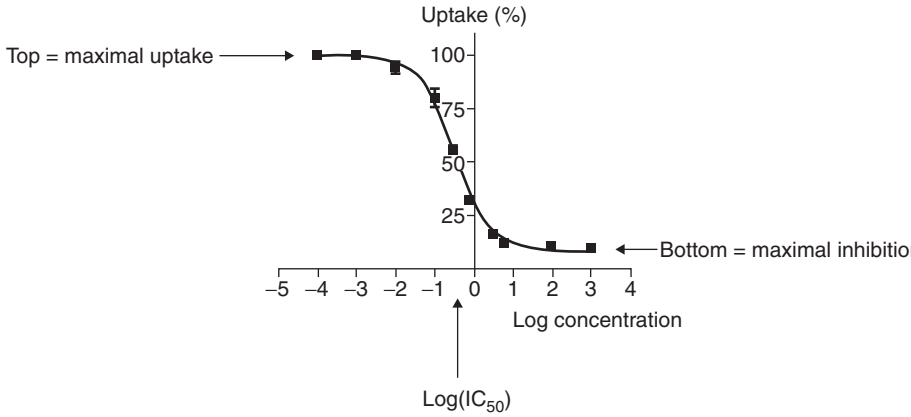

Figure 3.3.6 Illustration of the dependence of inhibitor concentration on uptake.

exploring binding sites of transporters. It is therefore very important to be able to compare results obtained by others. Therefore care should be taken when comparing IC_{50} values with the K_i values, when comparing results obtained with different standard substrates, and when using different protocols of for example pH in solution, the presence or absence of Na^+. Also affinity values using different tissues or species must be compared with care.

In the dynamic cellular system, effects related to the carrier protein rather than to the solute may be of importance. Functional upregulation of transport activity and/or capacity may be seen in response to cellular events. Biological processes increasing the available number of carriers will increase the V_{max}, and modulation of the carrier via second messengers may alter the K_m value of the carrier. In the field of molecular biology of carriers' point mutation or genetic variants, the study of altered transport capacity (V_{max}) or transport activity (K_m) is highly relevant.

3.3.2.5.3 Uncompetitive inhibitors

Uncompetitive inhibitors only bind to the transporter–substrate complex:

$$T+S \Leftrightarrow TS \Leftrightarrow T+S \tag{3.3.25}$$

$$TS+I \Leftrightarrow TI \tag{3.3.26}$$

The effect on kinetic parameters of such a substrate is shown in Table 3.3.2. It is important to remember that transporters are different from enzymes, and that the transporter–substrate complex exists in two,

probably different, conformations facing either the extracellular or intracellular side. The uncompetitive binding of an inhibitor or transportate may occur at both sides.

3.3.2.5.4 Non-competitive inhibitors

For non-competitive inhibition, binding of inhibitor to carrier binding sites of both the empty transporter and the transporter–substrate complex may occur:

$$T+S \Leftrightarrow TS \Leftrightarrow T+S \tag{3.3.27}$$

$$TS+I \Leftrightarrow TI \tag{3.3.28}$$

$$T+I \Leftrightarrow TI \tag{3.3.29}$$

In other words, the inhibitor may act by either decreasing the turnover rate of the carrier and/or decreasing the amount of carriers available for substrate binding. The effect on substrate transport rate of a non-competitive inhibitor is derived from enzyme kinetics, but since carriers and enzymes are distinct, the equation in Table 3.3.2 (Equation 3.3.21) is a point of reference more than an absolute guidance (Neame and Richards, 1972).

3.3.2.6 Mixed kinetics including multiple carriers and passive diffusion

The transport of a compound may occur via a single transporter as is seen for hydrophilic nutrients across a biological barrier. However, for compounds generated by the pharmaceutical industry or the academic researcher, the structural properties of the molecules may be such that the compound is a substrate for different types of carriers. Furthermore, alongside carrier-mediated transport, the compound may be able to permeate a membrane in a non-saturable manner.

3.3.2.6.1 Transport via one carrier along with passive diffusion

In a number of cases the study of carrier-mediated transport processes is complicated by parallel non-saturable transport processes. In an experimental system the compound may be able to passively cross the membrane in which the carrier is expressed. If, for example, we look at the

study of a carrier in an epithelial barrier, which could be the barrier of an enterocyte, the compound may diffuse into the cell. The total transport rate of compound from the extracellular medium into the cell is thus not sufficiently described by Equation 3.3.30:

$$V_o = \frac{V_{max}[S]}{K_m + [S]} \tag{3.3.30}$$

but must be combined with the expression of Fick's first law (see Section 3.2.1):

$$V = K_D \, \Delta[S] \tag{3.3.31}$$

In Equation 3.3.31, the uptake rate, V, has the unit of mol/time, and the concentration gradient, $\Delta[S]$, has the unit of mol/volume, which means that K_D has the unit of volume/time. Combining Equations 3.3.3 and 3.3.31 yields the equation for uptake of a compound by a carrier-mediated and a non-saturable transport process:

$$V_o = \frac{V_{max}[S]}{K_m + [S]} + K_D \, \Delta[S] \tag{3.3.32}$$

As already mentioned, the transport rate across a biological barrier is often normalised with respect to the transport area, thereby giving the flux of a compound. In a situation where a transport process is a combination of one carrier-mediated and a non-saturable transport, the following expression describes the flux:

$$J_o = \frac{J_{max}[S]}{K_m + [S]} + P_{app} \, \Delta[S] \tag{3.3.33}$$

where P_{app} is the apparent permeability coefficient. The origin of this constant is described in Chapter 3.2. Which of the transport processes involved in the transfer of compound from one compartment to the other is the main transport pathway in a given situation, is a result of the kinetic constants involved in relation to the concentration of compound.

3.3.2.6.2 Transport via two or multiple carriers

Transporters are generally relatively specific with respect to the substrates they accept; however some compounds may be substrates for more than one type of transporter. A substrate may also be transported by transporters belonging to the same transporter family, where different isoforms are expressed in the same cell or tissue. In these situations the description

of the rate of transport is a combination of the rate expression for the processes involved. For example if two transporters are transporting a substrate in one direction, the velocity of the process is defined by:

$$V_{\text{Total}} = \frac{V^1_{\max}[\text{S}]}{K^1_{\text{m}} + [\text{S}]} + \frac{V^2_{\max}[\text{S}]}{K^2_{\text{m}} + [\text{S}]} + \ldots\ldots + \frac{V^n_{\max}[\text{S}]}{K^n_{\text{m}} + [\text{S}]} \tag{3.3.34}$$

where 1 denotes the parameters for one transporter and 2 denotes the parameters for the other transporter, and n is the n'th transporter involved in the overall transport. If the transporters work in different directions, the velocity of the process is found by subtracting one rate equation from the other:

$$V_{\text{Total}} = \frac{V^1_{\max}[\text{S}]}{K^1_{\text{m}} + [\text{S}]} - \frac{V^2_{\max}[\text{S}]}{K^2_{\text{m}} + [\text{S}]} \tag{3.3.35}$$

If transporter 1 has the highest transport capacity at the given concentration, the transport direction is defined as positive. Equations 3.3.34 and 3.3.35 may be combined with an expression for non-saturable transport similarly to Equations 3.3.32 and 3.3.33 (Table 3.3.2) when appropriate for the description of the experimentally obtained data.

3.3.3 Methods for studying transport via carriers

Transport via carriers may be investigated in several different systems using several different techniques. In the following two sections a brief description of different systems for studying carriers and different techniques will be provided. The sections are meant as an overview, and the reader is advised to consult more specific literature for detailed information about the systems and techniques if further interested.

3.3.3.1 Systems for measuring transport via carriers

The choice of model system for investigating transport via carriers is highly dependent on the purpose of the study in question. There are various advantages and disadvantages associated with almost any system. It is therefore important to have a well-defined question before selecting a model. In general, a simple system allows for easier interpretation of the results. In general terms there are two purposes of investigating a carrier in biopharmaceutical work. One is to understand what the impact of a given transporter is in relation to a biological response. This could be the

impact on bioavailability of a drug, the response mediated by inhibiting the uptake of a neurotransmitter, or the effect of a carrier in renal reabsorption or excretion. For these purposes human pharmacokinetic or dynamic studies could be very relevant, although they are both laborious and expensive. Animals or perfused animal tissues may also be used, or excised tissues from animal. Tissue preparation could be used in the Ussing chamber or more simply as everted rings or sacs. Cell culture models, most notably the Caco-2 cell model, are also widely used for predicting the impact of transporters on intestinal transport of drug candidates. For investigating whether a compound is a substrate for a transporter or for probing the binding site or mode of action of a transporter, simpler systems could be used. These systems could be transient or stable transfection of a cell line with the cDNA for the transporter in question into mammalian cells (Hela, Cos7, HEK293, CHO or LLC-PK1). Heterologous expression of carriers is also widely carried out in yeast cells (*Pichia pastoris*) or *Xenopus laevis* oocytes.

3.3.3.2 Techniques for measuring transport via carriers

The study of carrier-mediated transport kinetics has benefited greatly from the development of advanced analytical methods along with many skilled biochemical and molecular biological techniques. In general, the techniques for studying transport via carrier fall into two categories: where the movement of substrate is measured directly or indirectly. Direct measurement of substrate is where the substrate is radiolabelled and the transport can be followed directly by counting the radioactivity present in different compartments. If radiolabelled molecules are not available, the researcher must depend on analytical methods (high-performance liquid chromatography (HPLC) or liquid chromatography/mass spectroscopy (LC-MS)-based methods or fluorescence assays) with a sufficiently low detection limit to be able to detect very small quantities of compound present inside the cells expressing the carrier of interest. The indirect methods are based on the function of the carrier in the system where it operates. For example for PEPT1, which is a H^+-co-transporter, the movement of substrate is coupled to the movement of protons. This movement of protons will affect both the intracellular pH and the membrane potential of the cell expressing PEPT1 (assuming that the substrate is neutral at the pH used in the experiment). By measuring the change in either intracellular pH or membrane potential it is possible to indirectly determine if a compound is a substrate for PEPT1. The intracellular pH

or membrane potential can be measured using fluorescent probes or with greater accuracy by using microelectrodes.

3.3.4 Conclusions

Carriers are fundamental in the movement of solutes across cell membranes. Furthermore, they have been shown to move drug substances across cell membranes. It is therefore important to have a basic knowledge about how carriers are classified and how they work. The function of transporters and the description of their kinetics have been discussed in this chapter.

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3.4

Classification of human transporters

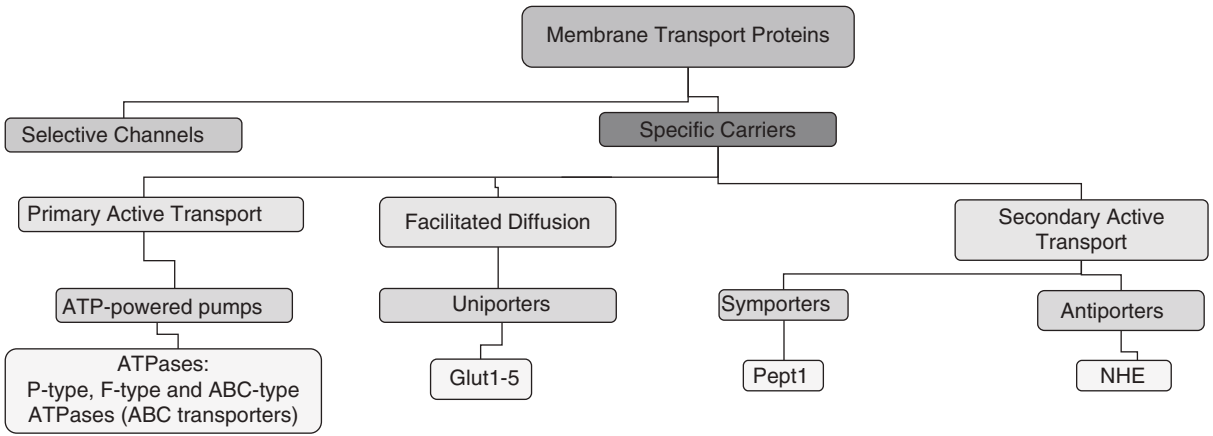
Pascale Anderlé

The plasma membrane represents a barrier between the cell and its environment. It is, however, essential that such separation is not complete. A large number of molecules must constantly move between the inside and outside of the cell, most frequently one at a time, but also in large groups. Hence, the plasma membrane functions as a selectively permeable membrane with well-defined selectivity with respect to which molecules cross and which direction they are allowed to travel.

Molecules can be transported either passively or actively across the membrane. Passive transport is driven by the kinetic energy of the molecules being transported (simple diffusion) or by membrane transporters that facilitate that diffusion (facilitated diffusion). They are always transported down their concentration gradient. In fact very few molecules enter or leave cells, or cross organelle membranes, without the help of proteins. Active transport, on the other hand, depends upon the production of cellular energy in the form of adenosine triphosphate (ATP) hydrolysis (Lodish *et al.*, 2000).

There are two classes of membrane transport proteins – carriers and channels (see Figure 3.4.1). Both form continuous protein pathways across the plasma membrane. Whereas transport by carriers can either be active or passive, solute flow through channel proteins is always passive (Alberts *et al.*, 2002).

Transport systems are essential to every living cell. They allow all essential nutrients to enter the cell and its compartments, regulate the cytoplasmic concentrations of metabolites by excretion mechanisms, control the concentration of ions inside the cell, which is very different from that outside the cell, export macromolecules such as complex carbohydrates, proteins, lipids, and DNA, catalyse export and uptake of signalling molecules that mediate intercellular communication, prevent toxic effects of drugs and toxins by mediating active efflux, and participate in biological warfare by exporting biologically active agents that



Primary active transport
Energy derived from hydrolysis of ATP to ADP liberating energy from high energy phosphate bond

Facilitated diffusion
Like any diffusion, transport from an area of higher concentration to lower concentration. Passive transport is powered by the potential energy of a concentration gradient and does not require the expenditure of metabolic energy

Secondary active transport
Use of energy from another source – another secondary diffusion gradient set up across the membrane using another ion. Because this secondary diffusion gradient initially established using an ion pump, as in primary active transport, the energy is ultimately derived from the same source – ATP hydrolysis.

Figure 3.4.1 Overview of mechanisms of transport according to Lodish *et al.* (2000).

insert into or permeate the membranes of target cells (Saier, 2000). Transport is an essential aspect of all life-endowing processes: metabolism, communication, biosynthesis, reproduction and both co-operative and antagonistic inter-organism behaviours.

Transporters also play an important role in diseases and drug therapies. Numerous Mendelian disorders caused by mutations in transporter and channel genes underscore their physiological relevance. Membrane transporters play a key role in drug entry and extrusion from cells endowed with efflux pumps such as the ABC (ATP-binding cassette) transporters. Moreover, electrochemical gradients across membranes are relevant to drug partitioning into and out of cells and cell organelles, such as mitochondria. Transporters are frequently responsible for drug–drug and nutrient–drug interactions (Anderlé *et al.*, 2004). In cancer therapy, these proteins can cause chemoresistance as the drugs are secreted out of the cells by efflux pumps. On the other hand, targeting transporters for drug delivery has also been shown to increase chemosensitivity (Huang *et al.*, 2004).

Membrane transporters, ion exchangers and ion channels are encoded by numerous gene families, comprising 4.1% of genes in the human genome (Venter *et al.*, 2001). The immense importance of these proteins and the increasing amount of data available on them mean that a systematic classification of transport systems is essential. Such a classification contributes to a comprehensive understanding of the basic functions of these proteins in any living cell (Saier *et al.*, 2006).

There are various ways to classify genes and proteins that function as transporters. In the following sections we will present the different major classification systems that currently exist and demonstrate the underlying ideas and structures of these systems. We will also discuss the problem of classifying such complex protein families and outline the different strategies for classification using two example transporters.

3.4.1 Classification according to transport mechanisms

A basic strategy to classify membrane transporters is undoubtedly one based on the mechanisms of transport (Lodish *et al.*, 2000; Alberts *et al.*, 2002). This system forms the basis for most of the current more detailed classification systems. In summary, there are three major classes of membrane transport proteins: carriers, channels and ATP-powered pumps. All are integral transmembrane proteins and exhibit a high degree of specificity for the substance transported. It has to be noted that the

nomenclature can vary. For instance, the term 'transporter' can also refer to the term 'carrier'. The rate of transport by the three types differs considerably due to the differences in their mechanism of action (see Figure 3.4.1). A solute can be transported by any of three distinct, but related processes: (1) facilitated diffusion; (2) primary active transport; and (3) secondary active transport (see Figure 3.4.1).

In general, two principal modes of facilitated diffusion can be distinguished in biological systems: channel type and carrier type. There are, however, significant differences between channels and carriers that function on the basis of facilitated diffusion. Therefore, these two transporter systems are considered to be distinct and, as a consequence, are classified differently. In the following sections we will discuss the various mechanisms of transport and the corresponding transporter systems.

3.4.1.1 Facilitated diffusion: channels

Channel proteins transport water or specific types of ions down their concentration or electric potential gradients, which is an energetically favourable reaction. In contrast to carrier proteins, channel proteins simply form open pores in the membrane, allowing small molecules of the appropriate size and charge to pass freely through the lipid bilayer. They form a protein-lined passageway across the membrane, through which multiple water molecules or ions move simultaneously, a relatively rapid rate – up to 10^8 molecules s^{-1}. The solute passes in a diffusion-limiting process from one side to the other via a channel or a pore that is lined by appropriately hydrophilic (for hydrophilic substrates), hydrophobic (for hydrophobic substrates), or amphipathic (for amphipathic compounds) amino acid residues of the constituent proteins. The plasma membrane of all animal cells contains potassium-specific channel proteins that are generally open and are critical to generating the normal, resting electric potential across the plasma membrane. Many other types of channel proteins are usually closed, and only open in response to specific signals.

One group of channel proteins is the porins, which allow the free passage of ions and small polar molecules through the outer membranes of bacteria. Channel proteins also permit the passage of molecules between cells connected at gap junctions. The plasma membranes of many cells also contain water channel proteins (aquaporins), through which water molecules are able to cross the membrane much more rapidly than they can diffuse through the phospholipid bilayer. The best-characterised channel proteins, however, are the ion channels, which mediate the

passage of ions across plasma membranes. Although ion channels are present in the membranes of all cells, they have been especially well studied in nerves and muscles, where their regulated opening and closing is responsible for the transmission of electric signals (Lodish *et al.*, 2000). Channels are also widely used as drug targets, but, according to Treherne (2006), are still largely under-exploited as drug targets.

3.4.1.2 Facilitated diffusion: uniporters

In contrast to uniporters, channel proteins form open pores through the membrane, allowing the free diffusion of any molecule of the appropriate size and charge. Uniporters, on the other hand, transport one molecule at a time down a concentration gradient. Unlike channels, they undergo conformational changes that allow the molecule to pass through the membrane and be released on the other side. This type of transporter, for example, moves small, hydrophilic substrates such as glucose or amino acids across the plasma membrane into mammalian cells. Similar to enzymes, uniporters accelerate a reaction that is already thermodynamically favoured, and the movement of a substance across a membrane down its concentration gradient will have the same negative ΔG value whether or not a protein transporter is involved (Lodish *et al.*, 2000). This type of movement is referred to as facilitated transport or facilitated diffusion. In contrast to passive diffusion, which follows Fick's law, facilitated diffusion is specific for a given compound and its transport rate is saturable. Moreover, its transport rate is significantly higher compared to passive diffusion, as the transported molecule is never in direct contact with the hydrophobic core of the membrane. In contrast to active transport systems, the direction of transport in facilitated diffusion is reversible, depending on the concentration gradient.

3.4.1.3 Secondary active transport: symporters and antiporters

Unlike uniporters, secondary active transporters are able to import or export ions and small molecules, such as glucose and amino acids, against a concentration gradient. They couple the movement of one type of ion or molecule against its concentration gradient to the movement of a different ion or molecule down its concentration gradient. In other words, they mediate coupled reactions in which an energetically unfavourable reaction is coupled to an energetically favourable reaction. As they have the

ability to transport two different solutes simultaneously, these carriers are also called co-transporters. When the transported molecule and co-transported ion move in the same direction, the process is called symport; when they move in opposite directions, the process is called antiport. As this transport mechanism involves catalysing an 'uphill' movement of certain molecules, these carriers are referred to as 'active transporters', but unlike the pumps, they do not hydrolyse ATP (or any other molecule) during transport. A very well-known example for a symporter is the dipeptide transporter PEPT1. The uptake of di- and tripeptides is associated with proton translocation in the same direction. Therefore, the transport rate is pH dependent (Daniel and Kottra, 2004). In contrast, the sodium/proton exchangers (NHE), which contribute to sodium and cytoplasmic pH homeostasis, are classical antiporters (Orlowski and Grinstein, 2004).

3.4.1.4 Primary active transport: pumps

Primary active transporters move ions or small molecules 'uphill' against a concentration gradient or electric potential across a membrane, which requires energy. In primary active transport, solute movement is coupled directly to an exergonic reaction (i.e. hydrolysis of ATP to ADP and inorganic phosphate (Pi), which releases energy). The overall reaction – ATP hydrolysis and the 'uphill' movement of ions or small molecules – is energetically favourable. Such pumps maintain the low calcium (Ca^{2+}) and sodium (Na^{+}) ion concentrations inside almost all animal cells relative to those in the medium, and generate the low pH inside animal-cell lysosomes, plant-cell vacuoles, and the lumen of the stomach. There are four principal classes. While the P, F, and V classes transport ions only, the ABC superfamily class transports small molecules as well as ions. The members of the ABC superfamily play a very important role in chemoresistance (Dean *et al.*, 2001a,b; Huang *et al.*, 2004).

3.4.2 Transporter classification system

In 1998, Milton Saier and his collaborators laid the basis for a comprehensive transporter classification system which was adopted in 2002 by the International Union of Biochemistry and Molecular Biology (IUBMB). The development of this classification system has been strongly influenced by the recent and fast progress in genomic sequencing and in computational biology. This transporter classification (TC) system provides a comprehensive overview of transport from structural, functional and evolutionary standpoints. The classification system is comparable to

the enzyme classification (EC) system approved by the Enzyme Commission. In contrast to the EC, the TC is not solely based on function, but 'additionally on phylogeny. Two different enzymes catalysing the exact same reaction sometimes exhibit completely different amino acid sequences and three-dimensional structures, function by entirely different mechanisms, and apparently evolved independently of each other. As the TC system takes into account the phylogenetic origins of transport systems it provides a reliable guide to protein structure, mechanism and function, although there may be some exceptions (Saier, 1999, 2000; Saier and Paulsen, 2001; Busch and Saier, 2002).

In the TC, the transporters are grouped on the basis of five criteria. Consequently, five different digits are assigned to each transporter system. The first digit corresponds to the transporter class, the second to the subclass, the third specifies the transporter family (or superfamily), the fourth the subfamily (family in a superfamily), and the fifth defines the substrate or range of substrates transported, as well as the polarity of transport (see Figure 3.4.2).

Any two transporters in the same subfamily that transport the same substrates using the same mechanisms are given the same TC number, regardless of whether they are orthologs or paralogs. Orthologs are genes that are evolutionary related, share a function, and have divergent speciation. Paralogs, on the other hand, have a common ancestor but have diverged by gene amplification and no longer have a common function. Basically, orthologs have the same function but occur in different species, while paralogs exist in the same genome but have different functions. The mode of regulation is not taken into account in the TC system. The primary level of classification (e.g. distinction of classes) is based on transport and energy-coupling source. Currently, there are seven classes recognised in the TC (see Figure 3.4.3).

The next level of classification is the families. While the classes and subclasses distinguish functionally different types of transporters, the families and subfamilies provide a phylogenetic basis for classification. In order for two proteins to belong to the same family, they must exhibit a region of a minimum of 60 residues in comparable portions of the two proteins that has a comparison score in excess of nine standard deviations (Saier, 2000). A minimum of 60 residues is arbitrarily selected because

Figure 3.4.2 Description of the TC number associated with each transporter.

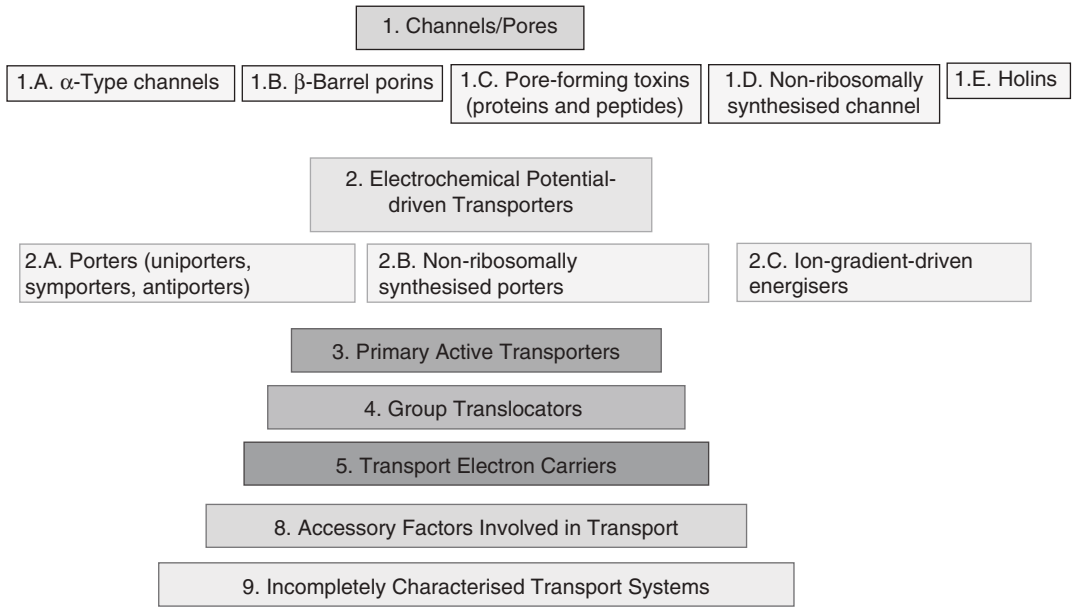


Figure 3.4.3 Classes and subclasses of the TC system. Classes 6 and 7 have not yet been assigned. These classes are reserved for novel types of transporters that do not fall into the other classes.

many protein domains in water-soluble protein are of about this size. More than 400 families are currently included in the TC system (Saier *et al*., 2006).

3.4.2.1 Transporter Classification Database (TCDB)

In parallel to the TC system, Milton Saier and collaborators established a relational, web-integrated database. The transporter classification database (TCDB; www.tcdb.org/tcdb/) contains sequence, classification, structural, functional and evolutionary information about transport systems for many different living organisms. All data are a summary of published information of over 10000 references. Currently, there are about 3000 distinct proteins organised in more than 400 families based on the TC system (Ren *et al*., 2004; Saier *et al*., 2006).

The classification system can be accessed through an intuitive web interface. The user can start at the top of the hierarchy and descend through the taxonomy. At the deepest level, individual protein information such as Swiss-Prot accession number, the primary sequence, source organism and the protein name, length, molecular weight and probable topology can be retrieved. Several links, such as links to the SwissPfam database (see below), the ExPASy server, the Swiss Institute of Bioinformatics BLAST Network service, and transmembrane segment (TMS) prediction are provided. A link to the FASTA formatted protein sequence, as well as a link to the hydropathy and amphipathicity plots for the protein, are also available. The TCDB can also be searched by entering the TC family name or TC number. Additionally, it is possible to search by keyword, disease name, and protein name. Also included is a section detailing human transporters that have been approved by the Human Genome Nomenclature Committee (HGNC), which will be discussed later (see Section 3.4.4). Importantly, each of these proteins has been cross-referenced with the TC system (Ren *et al*., 2004; Saier *et al*., 2006).

In conclusion, the TCDB is an essential tool in exploring the structures and functions of transporters and gaining a good understanding of how transporters are classified according to the TC system.

3.4.3 Gene ontology

The recent sequencing efforts and the resulting information about the genomes of different organisms have made it clear that a large fraction of the genes specifying the core biological functions are shared by all eukaryotes. The knowledge of the biological role of such shared proteins in one

organism can certainly illuminate its role in other organisms. As a consequence, in 2000 the Gene Ontology Consortium constructed a tool, the Gene Ontology (GO), to facilitate the biologically meaningful annotation of genes and their products in a wide variety of organisms (Ashburner *et al*., 2000; www.geneontology.org). The main aim is the possibility of an automated method of transferring biological annotation from experimentally well-studied model organisms to less well-known organisms, based on gene and protein sequence similarity. Ontologies are structured vocabularies in the form of directed acyclic graphs that represent a network in which each term may be a 'child' of one, or more than one, 'parent'.

The current GO system consists of three completely independent categories: (1) molecular function; (2) biological process; and (3) cellular component.

'Molecular function' GO terms describe activities at the molecular level. They represent activities rather than the entities (molecules or complexes) that perform the actions, and do not specify where or when, or in what context, the action takes place. Molecular functions generally correspond to actions that can be performed by individual gene products, but some activities are performed by assembled complexes of gene products. In contrast, a 'biological process' represents a series of events accomplished by one or more ordered assemblies of molecular functions. It has to be noted that a 'biological process' is not equivalent to a pathway. The GO system does not specifically focus on representing any of the dynamics or dependencies that would be required to describe a pathway. A 'cellular component' is a component of a cell with the possibility that it is part of some larger object, which may be an anatomical structure or a gene product group. Each annotation of a given gene is attributed to a source, which may be a literature reference, another database or a computational analysis. Such a GO evidence code provides a useful estimation of the annotation quality.

3.4.3.1 Human membrane transporters and GO

To date there are in total 27 894 human gene products annotated to the ontologies, of which a little fewer than 40% have not been electronically inferred (i.e. not purely based on sequence similarity, www.geneontology.org). Using GOA as a reference, we identified 2365 human gene products in Swiss-Prot that have been annotated as proteins with 'transporter activity' or with any daughter term – isoforms and subunits were counted independently.

The GO database is cross-referenced by the TC system (www.geneontology.org). In other words, each gene is associated with a TC code if possible. It has to be noted though that the hierarchical structure in the TC system does not correspond to the classification system in the GO database. One major difference is that in GO a transporter can be annotated with different GO terms, while in the TC a given protein gets only one TC code. Using two model transporters we will later discuss the differences between the two classification systems.

3.4.4 Human Genome Organization (HUGO) symbols

The Human Genome Organization (HUGO) was created in 1988. Some of the objectives of this association are to assist with the co-ordination of research on the human genome and to help with the exchange of data between scientists (Bodmer, 1991). One organisation, the Human Genome Nomenclature Committee (HGNC), is responsible for approving a unique gene name and gene symbol for each individual gene and storing this information in their HGNC database. Guidelines for the human gene nomenclature can be found on their web page (www.genenames.org/guidelines.html).

To date, the HGNC contains more than 40 transporter families of the so-called *SLC* (solute carrier) series. This family includes a major part of transporter-related genes. In the HUGO system, the remaining transporter genes are ATP-driven transporters, channels, ionotropic receptors, aquaporins, transporter and channel subunits, and auxiliary/regulatory transport proteins (www.genenames.org/guidelines.html) (Hediger *et al.*, 2004). The *SLC* families have been presented in detail in the prepared *SLC* mini-review series of *Pflügers Archiv* (Hediger, 2004). Hediger and his co-workers have prepared a database containing all *SLC* members (www.bioparadigms.org/slc/menu.asp). Similar to the *SLC* families, useful links to web pages which describe the various transporter families and provide some additional information on their members can be found on the HGNC web page. Overall, the HGNC requires that the nomenclature schemes apply a 'stem' (or 'root') symbol for members of a gene family or grouping, with a hierarchical numbering system to distinguish the individual members. However, the strategy on how genes are grouped into families is not very well documented.

Even though the HUGO symbols are cross-referenced in the TCDB, the way transporters are classified into the various families in the HGNC does not necessarily correspond to the classification in the TC.

3.4.5 Pfam

The Pfam-A database contains a collection of curated multiple sequence alignments for each family, as well as profile hidden Markov models (profile HMMs) for finding these domains in new sequences (Sonnhammer *et al.*, 1998; Bateman *et al.*, 2000). A multiple sequence alignment is an alignment of three or more sequences, with gaps inserted in the sequences such that residues with common structural positions and/or ancestral residues are aligned in the same column. Defining profile HMMs is a probabilistic approach to representing a motif in a sequence. Each family in Pfam is represented by two multiple sequence alignments and two profile HMMs. The Pfam families are grouped in so-called clans. A clan contains two or more Pfam families that have evolved from a single evolutionary origin. Proof of their evolutionary relationship is usually determined by similar tertiary structures, or, when structures are not available, by common sequence motifs. Clans have been introduced in Pfam as some protein families are highly divergent, thus making it very difficult to represent the family with a single HMM. These families are closely related, so sequences may significantly hit more than one member of the clan (www.sanger.ac.uk/Software/Pfam/browse/clans.shtml).

For all protein sequences that do not belong to any Pfam family, a new family is generated in the so-called Pfam-B database. As of release 19.0, Pfam contained 8183 Pfam families, which match 75% of protein sequences in Swiss-Prot and TrEMBL (and 53% of all residues) (http://pfam.sanger.ac.uk; Bateman *et al.*, 2000). Cross-references to the TC and GO databases (QuickGO) are integrated into the Pfam database.

3.4.6 Practical approach: *SLC15A1* and *ABCB1*

In the following we will use *SLC15A1* and *ABCB1*, two important drug targets, as examples of how these genes and their corresponding gene products can be classified by the different systems.

3.4.6.1 Classification based on transport mechanisms

PEPT1, the gene product of *SLC15A1*, has been shown to transport dipeptides and tripeptides and structurally related compounds. The uptake of these compounds into cells is directly coupled to a proton influx. As a consequence, increased proton efflux via the NHE exchanger can be observed (Daniel and Kottra, 2004). Taken together, as shown in Figure 3.4.1, PEPT is a typical secondary active transporter or

symporter. The term 'tertiary active transporter' has also been associated with *SLC15A1* (see Section 3.3.1.5 and Brandsch *et al.*, 2004). It has to be noted though that this concept has not yet been taken into account by the major classification systems.

On the other hand, it has been shown that the gene product of *ABCB1*, P-glycoprotein, acts as an ATP-dependent pump (Silverman, 1999). It is a typical ABC-type ATPase.

3.4.6.2 TC system

Searching the TC with the keyword 'SLC15A1', the TC number 2.A.17.4.1 can be retrieved. It is classified as a 'Peptide: H^+ symporter (transports cationic, neutral and anionic dipeptides; also transports β-lactam antibiotics, the antitumor agent, bestatin, and various protease inhibitors)'. Figure 3.4.4 outlines the detailed classification. The first digit indicates that SLC15A1 belongs to the class of carriers whose transport is driven by an electrochemical potential. The subclass (second digit) specifies that SLC15A1 is a porter, more specifically a symporter (see Figure 3.4.4). It is a member of the proton-dependent oligopeptide transporter (POT) family (third digit) and transports dipeptides, tripeptides and structurally related compounds (fifth digit).

As for ABCB1, the TC number '3.A.1.201.1' represents 'Broad specificity multidrug resistance (MDR) efflux pump (exports amphiphilic compounds of unrelated chemical structure); peptide efflux pump; phospholipid (e.g. phosphatidyl serine), cholesterol and sterol flippase (also called ABCB1 and p-gp))' (see Figure 3.4.4). ABCB1 forms part of the family 'ATP-binding cassette superfamily' (third digit). The mechanism of transport is driven by P–P-bond hydrolysis (second digit) and, thus, ABCB1 can be considered a typical primary active transporter (first digit.) In addition, both transporters can be found in the TCBD under the section 'Transporters from humans', listed with the official gene symbol according to HUGO.

3.4.6.3 GO

Figure 3.4.5 summarises the nodes in the GO tree where *SLC15A1* and *ABCB1* are annotated according to GOA (www.geneontology.org). In contrast to the TC system, in GO the two genes can be classified in different ways. On one hand, the transporters can be associated with different unique GO terms, on the other hand, the specific GO terms can be found in the different locations and hierarchical levels in the classification tree.

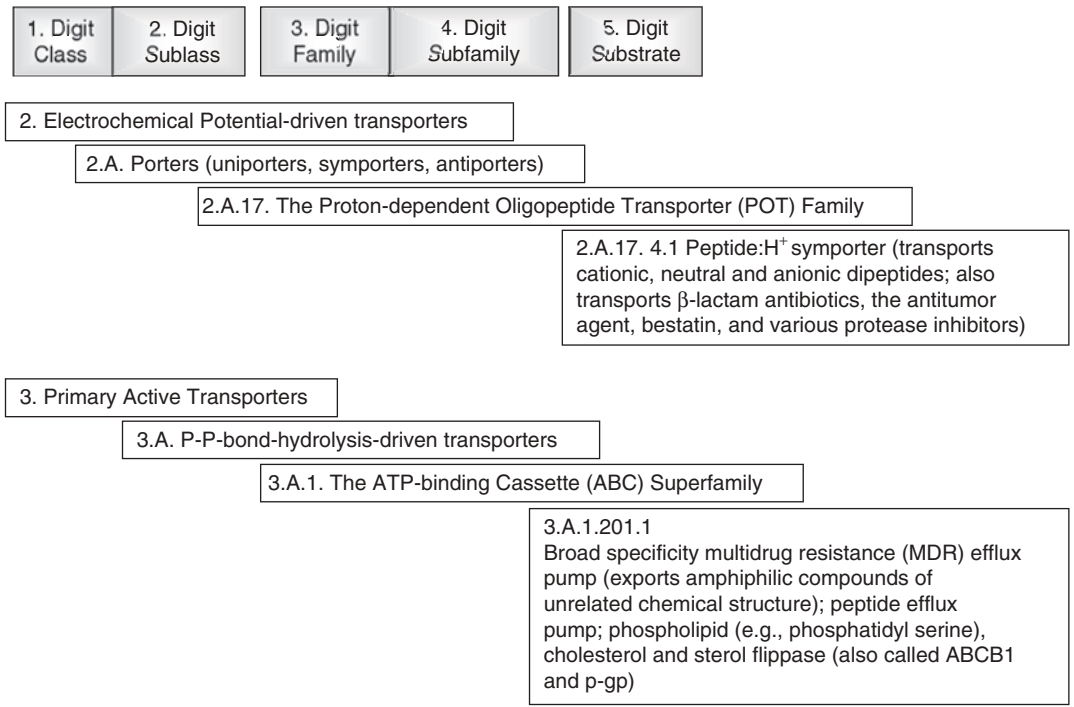


Figure 3.4.4 Classification of the gene products SLC15A1 and ABCB1 in the TC system.

SLC15A1

Function
oligopeptide transporter activity (IEA)
GO:0015198 : oligopeptide transporter activity (3)
peptide:hydrogen symporter activity (TAS)
GO:0005215 : transporter activity
GO:0005386 : carrier activity
GO:0015290 : electrochemical potential-driven transporter activity
GO:0015291 : porter activity
GO:0005427 : proton-dependent oligopeptide transporter activity
GO:0015333 : **peptide:hydrogen symporter activity**
GO:0015293 : symporter activity
GO:0015294 : solute:cation symporter activity
GO:0015295 : solute:hydrogen symporter activity
GO:0015333 : **peptide:hydrogen symporter activity**
GO:0015075 : ion transporter activity
GO:0008324 : cation transporter activity
GO:0015294 : solute:cation symporter activity
GO:0015295 : solute:hydrogen symporter activity
GO:0015333 : **peptide:hydrogen symporter activity**
GO:0015197 : peptide transporter activity
GO:0015198 : oligopeptide transporter activity
GO:0005427 : proton-dependent oligopeptide transporter activity
GO:0015333 : **peptide:hydrogen symporter activity**

GO:0015293 : **symporter activity** (4) (IEA)
GO:0005215 : **transporter activity** (1) (IEA)

Process
GO:0007586 : **digestion** (3) (TAS)
GO:0006857 : **oligopeptide transport** (5, 5, 6) (IEA)
GO:0015031 : **protein transport** (4,4,5,5,5) (IEA)

Component
GO:0008372 : **cellular component unknown** (1) (ND)
GO:0016021 : **integral to membrane** (4) (IEA)
GO:0005887 : **integral to plasma membrane** (5, 5, 5) (TAS)
GO:0016020 : **membrane** (2) (IEA)
GO:0005624 : **membrane fraction** (3) (TAS)

ABCB1

Function
ATP binding (TAS)
GO:0005488 : binding
GO:0000166 : nucleotide binding
GO:0017076 : purine nucleotide binding
GO:0030554 : adenyl nucleotide binding
GO:0005524 : ATP binding
ATPase activity (IEA)
GO:0003824 : catalytic activity (1)
GO:0016887 : ATPase activity (7)
ATPase activity, coupled to transmembrane movement of substances (TAS)
GO:0042626 : ATPase activity, coupled to transmembrane movement of substances (13)

GO:0000166 : **nucleotide binding** (2) (IEA)
GO:0005215 : **transporter activity** (1) (TAS)

Process
GO:0042493 : **response to drug** (4) TAS
GO:0006810 : **transport** (3, 2, 2) TAS

Component
GO:0009986 : **cell surface** (2) IEP
GO:0016021 : **integral to membrane** (4) TAS
GO:0016020 : **membrane** (2) IEA
GO:0005624 : **membrane fraction** (3) TAS

Figure 3.4.5 Classification of SLC15A1 and ABCB1 according to GO. The complete hierarchical tree is only shown for some selected GO terms. For the others, the hierarchical level is indicated in parentheses, defining 'molecular function', 'biological process' and 'cellular component' as 0. The evidence codes are indicated for each GO term: IEA = inferred from electronic annotation, TAS = traceable author statement, IEP = inferred from expression notation, ND = no biological data available.

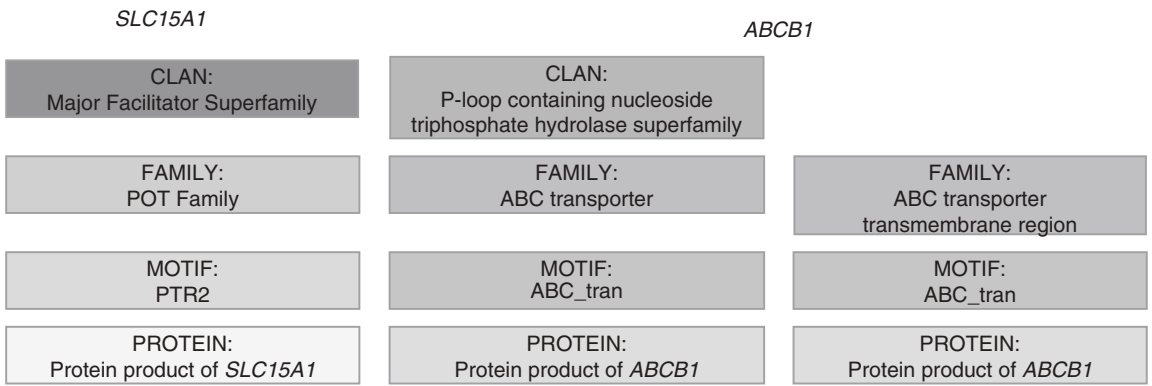


Figure 3.4.6 Classification of the gene products of *SLC15A1* and *ABCB1* in the Pfam-A database.

3.4.6.4 Pfam

Searching the Pfam database, which focuses on relevant protein motifs via the Sanger website (www.sanger.ac.uk/Software/Pfam/search.shtml), reveals that the protein product of *SLC15A1* has a so-called PTR2 domain which defines the *POT* family. In contrast to the TC system where the *POT* family and the major facilitator superfamily are two independent families, in the Pfam system the *POT* family is considered to make part of the clan 'the major facilitator superfamily'.

As for the protein product of *ABCB1*, two motifs can be found in the Pfam database: ABC_tran and ABC_membrane. The family 'ABC transporter transmembrane region' (ABC_membrane) represents a unit of six transmembrane helices. Many members of the other family, the 'transporter family' (ABC_tran), have two such regions (see Figure 3.4.6).

3.4.7 Conclusions

We have discussed the importance of transporters in living cells and their role in drug delivery. The increasing amount of data available on these proteins provokes the need for a systematic classification of transport systems. We have shown that classification strategies can actually contribute to a comprehensive understanding of the basic functions of these proteins in any living cell.

There are various ways to organise the vast amount of data available on transporters. Each of the systems presented has a different overall goal and uses different parameters to classify genes, or their products, respectively. Therefore, each system has limitations. Its advantages and disadvantages depend on the users' scientific questions. All the systems presented are integrated in publicly available databases. There is certainly a trend to converge these databases and to provide cross-references. However, a one-to-one comparison is very difficult as the various classifications systems have similar goals, but differ significantly in their structures.

Acknowledgment

I would like to thank Dr Viviane Praz for providing the latest statistics of the GO annotations.

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3.5

Absorptive transporters

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In Chapter 3.3 the various carrier types and their basic kinetics were described, and in Chapter 3.4 classification systems for human transporters were discussed. In this chapter, carriers, which are involved in transporting nutrients from the exterior to the interior of the body, will be described with respect to their relevance for the biopharmaceutical scholar. In this context the absorptive carrier is viewed as a carrier that transports nutrients from an exterior area, e.g. the intestine, the lung, or the cornea, into the epithelial cells and subsequently to the systemic circulation, i.e. the interior of the body. Carriers are integral players in the absorption, distribution, metabolism, and excretion (ADME) of many drug molecules. The challenge for the biopharmaceutical researcher is thus to sufficiently optimise ADME properties of drug candidates in order to reach drug compound(s) that have appropriate pharmacokinetic profiles for the dosage regimen and disease they are developed for. Alternatively, carriers themselves may also be pharmacological targets in which modulation of their activity is a part of the treatment of a disorder. This is especially seen for carriers in the CNS. The activity of L-glutamate transporters that belong to the *SLC1* gene family present in different CNS cell types is a pharmacological target for developing inhibitors for the treatment of CNS disorders or injury. Likewise transporters for γ-aminobutyric acid (GABA) (SLC32A1) are pharmacological targets in drug discovery programmes. The focus in the present chapter, however, will be primarily on nutrient carriers, which are relevant with regard to delivering drug candidates across epithelial barriers, and as such are important for the bioavailability of these compounds. In some cases it has retrospectively been shown that a transporter has influence on oral absorption of a drug compound and subsequently on its oral bioavailability. This has been seen for valaciclovir and β-lactam antibiotics, whose bioavailability partly depends on the transport

activity of the intestinal di/tripeptide transporter PEPT1. However, absorptive transporters are also relevant in drug-discovery and drug-development settings, since they offer a possibility for designing integral structural features in drug candidates by means of improving ADME properties, e.g. by designing analogues or prodrugs as substrates for absorptive transporters. Moreover, knowledge about molecular structural features of importance for interactivity with transporters may add to rational pharmaceutical formulation development since some pharmaceutical excipients may have molecular features that may interact with transporters as well. Alternatively, some pharmaceutical excipients may alter transporter function, by inhibiting or activating its regulation on both a short- and long-term basis, or by having influence on its co-substrate, e.g. Na^+ or H^+. Thus, if that is the case, it seems possible, by using recipients that increase (or decrease if desirable) the driving force of the transporter, to increase transport capacity and subsequently the absorption fraction of the drug candidate substrate. Such preclinical studies may as well add to knowledge with regard to possible drug–drug interactions and/or drug–food interactions.

3.5.1 Searching for absorptive transporters

In this chapter, some absorptive transporters of relevance for pharmaceutical exploitation will be presented. The chapter will also focus on transporter properties that are advantageous for the biopharmaceutical scientist to consider, as well as strategies to utilise transporters for increasing intestinal permeation. The field of transporters is a rapidly evolving research area, and the present chapter is based on the current available knowledge. However, it is advisable to consult the HUGO Nomenclature Committee database for ongoing updates. At present the database includes more than 40 transporter families of the so-called *SLC* (solute carrier) gene series. The *SLC* families represent a major portion of the transporter-related genes but additional *SLC* transporters are constantly being identified. The *SLC* tables were originally prepared by the authors of the *SLC* mini-review series of *Pflügers Archiv* (Heidiger *et al.*, 2004). Apart from latest updates of the *SLC* families and their members, the database also links to relevant gene databases and reviews in the literature. This database is thus a gateway to a wealth of information, whereas the following only describes a selection of known absorptive transporters. Chapter 3.4 discusses the databases available for information, search and classification of transporters.

3.5.1.1 Absorptive transporters present in the intestine and kidney

Table 3.5.1 presents selected absorptive transporters that are mainly expressed in the intestinal and renal epithelial cells. The human intestine has transport systems for the diverse group of hydrophilic 'nutrients, e.g. vitamins, minerals, amino acids, peptides and hexoses, which are transported across the lipophilic cell membranes of the epithelial enterocytes via carriers. Likewise, transporters expressed in the kidney are responsible for reabsorption of nutrients present in the ultrafiltrate. In Table 3.5.1 the carriers have been organised according to their transport mode. The gene name of the carrier is given along with its protein name. The general types of natural substrates are listed and the major tissue distribution and cellular localisation is provided. As an example the proton-dependent symports consist of transporters for amino acids, di- and tripeptides and minerals. Within the group of proton-dependent peptide transporters two different genes, *SLC15A1* and *SLC15A2*, have gene products that are peptide transporters. These proteins are called PEPT1 and PEPT2, respectively. The natural substrates of PEPT1 and PEPT2 are dipeptides (A_1A_2) and tripeptides ($A_1A_2A_3$). The cellular localisation of PEPT1 and PEPT2 is in the apical membrane, which is the side of the enterocytes facing the exterior (luminal) side of the intestinal and kidney tissues.

3.5.1.1.1 Amino acid transporters

In the intestinal fluid, amino acids are the results of protein hydrolysis. Amino acids form a group of molecules with large physicochemical differences; they may be small neutral molecules like glycine, or large hydrophobic ones like tryptophan. Amino acids may be neutral, cationic or anionic, which may depend on pH, but the battery of transporters responsible for cellular absorption and vectorial transport in the intestine and kidney is relatively specialised. The organisation of amino acid transporters is rather confusing, since historically the transport systems have been characterised in tissue preparation and named according to their function and thus named by transport system names. However, with the advances in molecular biology the cDNAs encoding membrane transporter proteins have been cloned and named by gene and protein names. Thus, transporters are referred to by transport system names arising from functional studies in tissues, as well as by transporter names arising from cDNA and protein studies. These names are mixed in the literature; however, in this chapter we aim to use gene (*SLC*) and protein names.

Table 3.5.1 Selected absorptive transporters for nutrients, vitamins, and minerals in epithelial cells

	Gene name	*Protein*	*Substrates*	*Tissue*	*Cellular location*
Proton-dependent symports					
Peptides	*SLC15A1*	PEPT1	A_1A_2, $A_1A_2A_3$	Intestine, kidney	Apical
	SLC15A2	PEPT2	A_1A_2, $A_1A_2A_3$	Kidney, lung	Apical
Amino acids	*SLC36A1*	PAT1	Gly, Ala, Pro	Intestine, kidney, lung	Apical
	SLC36A	PAT2	Gly, Ala, Pro	Kidney, lung	Apical
Minerals	*SLC11A2*	DMT1	Fe^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}	Intestine, kidney, lung	Apical
Sodium-dependent symports					
Amino acids	*SLC6A19*	B^0AT1	Neutral amino acids	Intestine, kidney	Apical
Bile acids	*SLC10A2*	ASBT	Bile acids	Ileum, kidney, biliary tract	Apical
Vitamins	*SLC23A1*	SVCT1	Ascorbic acid	Kidney, intestines	Apical
Nucleosides	*SLC28A1*	CNT1	Pyrimidine nucleosides	Small intestine, kidney	Apical
	SLC28A2	CNT2	Purine nucleosides	Kidney, intestine	Apical
	SLC28A3	CNT3	Purine and pyrimidine nucleosides	Intestine, lung	Apical
Hexoses	*SLC5A1*	SGLT1	Glucose	Small intestine, kidney	Apical
Sodium-chloride-dependent symports					
Amino acids	*SLC6A14*	ATB(0+)	Neutral, cationic amino acid	Colon, lung	Apical

Uniports					
Nucleosides	*SLC29A1*	ENT1	Purine pyrimidine nucleosides	Ubiquitous	Plasma membrane (basolateral in polarised renal epithelial cells) and perinuclear membranes
	SLC29A2	ENT2	Purine pyrimidine nucleosides nucleobases	Ubiquitous, plasma membrane	Basolateral in polarised renal epithelial cells. Particularly abundant in skeletal muscle
Hexoses	*SLC2A5*	GLUT5	Fructose	Small intestine, kidney	Apical
	SLC2A2	GLUT2	Glucose, galactose, fructose, mannose, glucosamine	Intestine, kidney	Basolateral

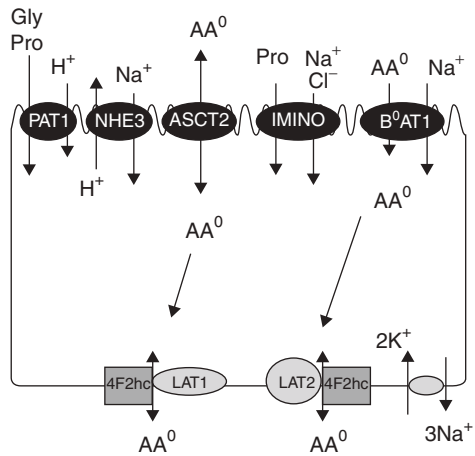

Figure 3.5.1 Transport of neutral amino acids (AA^0) in the small intestine and the kidney. PAT1 is the proton-dependent amino acid transporter, ASCT2 is the neutral amino acid exchanger; IMINO is the sodium- and chloride-dependent proline transporter; B^0AT1 is the neutral amino acid transporter; NHE3 is the apical sodium proton exchanger 3; LAT is a glycoprotein-associated amino acid transporter (gpaAT) of L-type1; LAT1 (SLC7A5) or 2; LAT2 (SLC7A8); 4F2hc (SLC3A2) is a heavy chain amino acid transporter protein.

In Figure 3.5.1 the transport of neutral amino acid in the intestine and the proximal tubule is illustrated. At least four different amino acid transporters transport neutral amino acids (AA^0) across the apical membrane of intestinal and renal epithelial cells, with overlapping substrate specificity. The PAT1 (SLC36A1) transporter primarily transports proline and glycine. Proline is also transported by the IMINO/SIT (SLC6A20) transporter; however, the transporters differ in the driving forces, proton versus sodium, and in the K_m values, 0.13 mM versus 2.8 mM, for PAT1 and IMINO, respectively (Broer *et al.*, 2005). The B^0AT1 (SLC6A19) transporter transports 'large' aliphatic amino acids such as leucine, valine, isoleucine and methionine, with the highest-affinity K_m values of approximately 1 mM, whereas phenylalanine, glutamine and alanine have affinities that are three-fold higher (Broer *et al.*, 2005). The ASCT2 (SLC1A5) transporter is an exchanger, which exchanges alanine, serine, cysteine, asparagine, threonine and glutamine for each other. ASCT2 is an obligatory antiporter and it is therefore not mediating a net absorption of amino acid. In the colon, $ATB^{0,+}$ (SLC6A14) is another amino acid transporter for neutral amino acids.

Charged amino acids are transported by heteromeric amino acid transporters (HATs), which are composed of a light and a heavy subunit

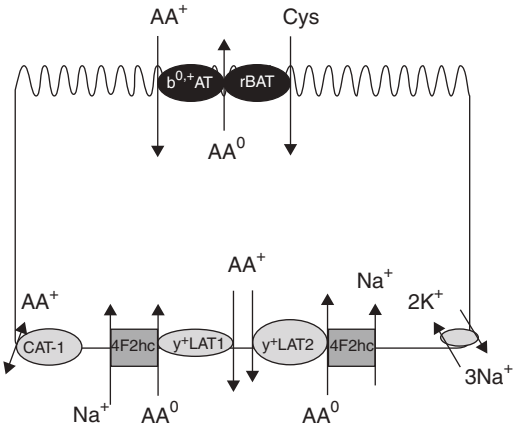

Figure 3.5.2 Transport of charged amino acids (AA^+) in the small intestine and the kidney. $b^{0,+}AT$ is a sodium-independent amino acid transporter for neutral and cationic amino acids (exchanger); rBAT is a protein, which associates with $b^{0,+}AT$ to form a transport system; y^+LAT1 (SLC7A7) is a glycoprotein-associated amino acid transporter (gpaAT) of L-type1 or 2; y^+LAT2 (SLC7A6); 4F2hc (SLC3A2) is a heavy chain amino acid transporter protein.

linked by a disulphide bridge. The heavy subunits are SLC3 members, namely, rBAT (SLC3A1) and 4F2hc (SLC3A2). These proteins are type II membrane glycoproteins, which means that they have one single transmembrane domain and the C-terminus is located outside the cell. rBAT heterodimerises with the light chain protein $b^{0,+}AT$ (SLC7A9), to constitute the transport system, which exchanges cysteine and positively charged (dibasic) amino acids with AA^0 (Palacin and Kanai, 2004; Verrey *et al*., 2004; see Figure 3.5.2).

As seen in Figures 3.5.1 and 3.5.2, the amino acids moving into the epithelial cells are transported or exchanged across the basolateral membrane by several different transport systems composed of a light- and a heavy-chain protein. These systems are present to maintain cellular homeostasis and aid in vectorial absorption or re-absorption.

3.5.1.1.2 Di/tripeptide transporters

Small peptides consisting of 2–3 amino acid residues are an important nutritional source of amino acids. These di- and tripeptides are transported across biological membranes via the transport capacity of two peptide transporters (see Table 3.5.1 and Figure 3.5.3), which belong to the SLC15 family. As discussed in Chapter 3.4, peptide transporters are proton-dependent symports. In the small intestine, PEPT1 is responsible

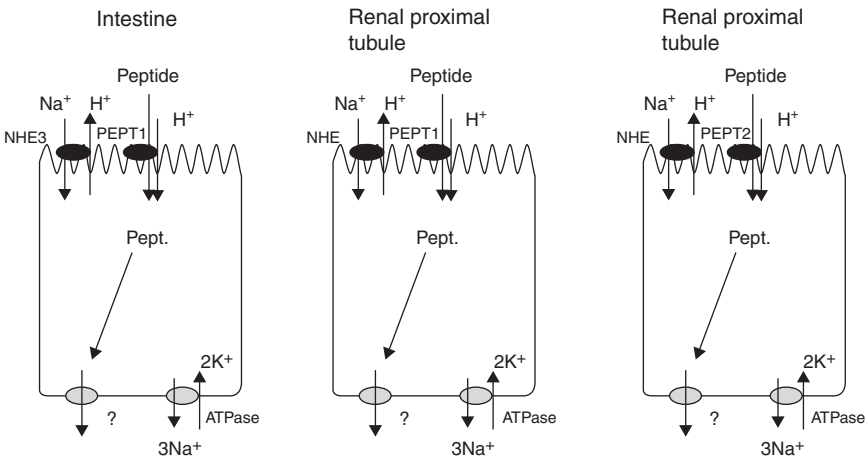

Figure 3.5.3 Transport of small peptides (2–3 amino acid residues) in the small intestine and the kidney. In this context a peptide consists of either 2 or 3 amino acid residues. The '?' indicates that the basolateral transporter for peptide substrates transported into the epithelial cell via PEPT1 or PEPT2 has not been identified. NHE3 is the apical sodium proton exchanger 3.

for a large fraction of the amino acid uptake in the form of dipeptides or tripeptides. The di- or tripeptides are present in the intestinal lumen and are a result of dietary breakdown of protein coming from the ingested food. In the kidneys, di- or tripeptides arising from metabolism of hormones, neuropeptides or cytokines are reabsorbed by PEPT1 and PEPT2. In the kidney, both peptide transporters are expressed in a sequential manner in order to ensure an efficient reabsorption of amino acid nitrogen. In both the small intestine and the kidney, the apical uptake is mediated via well-characterised PEPT. Most of the substrates transported into the cell via PEPTs are rapidly metabolised inside the cell to the constituent amino acids, but some peptidomimetics are not metabolised. Therefore, for transepithelial transport to occur, these peptidomimetics must exit the cell via basolateral efflux. The efflux of peptidomimetics is mediated via a transport protein, which has been partly characterised with respect to pH dependency and substrate specificity. However, the molecular nature of the transporter is at present unknown, since a basolateral peptide transporter so far has not been cloned. It is unknown if the carrier-mediated transport process, which has been functionally characterised, is due to one or more transporter(s). It is important to consider the apical as well as the basolateral transport step in the overall evaluation of transepithelial transport pathways of both standard nutrients and novel drug

candidates, and therefore knowledge about the transporter responsible for the basolateral efflux step for PEPT substrates is eagerly anticipated.

3.5.1.1.3 Hexose transporters

Transport of glucose and related hexoses across biological membranes is mediated by members of two different SLC families, the sodium-dependent SLC5 family and the sodium-independent SLC2 family. The SLC5 family consists of 11 members where their functions, based on expression studies, have been ascribed to 9 out of the 11. SGLT1 is the primary apical sodium-dependent transporter for glucose; however the SLC5 family also has a vitamin transporter, SLC5A6, where the gene product, SMVT, transports biotin and pantothenate. The SLC2 family comprises 13 members, the glucose transporters (GLUTs) 1–12 and a H^+-*myo*-inositol (HMIT). GLUTs function as simple uniport carriers with the transport direction defined by the electrochemical gradient. Glucose has a key role in providing metabolic energy and being a building block for biosynthesis of biomolecules, and glucose transporters are expressed in every cell of the body. The different isoforms are expressed in different tissues, which is illustrated in Figure 3.5.4.

In Figure 3.5.4 the focus is on the GLUT expression in the intestine and the kidney. In the intestine, transepithelial hexose transport is mediated via an apical influx of glucose and galactose via SGLT1, and fructose via GLUT5. For transepithelial transport of hexoses to occur,

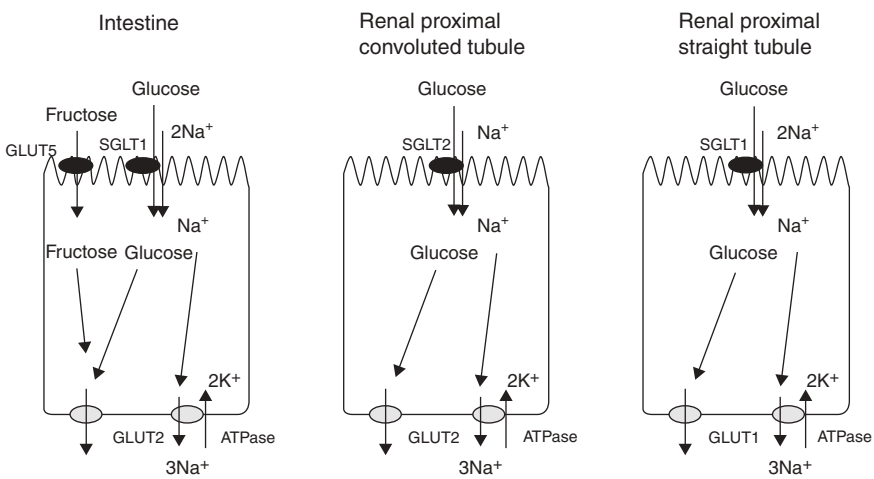

Figure 3.5.4 Transport of hexoses in epithelial cells of the intestine and the kidney via members of the SLC2 and SLC5 families.

the transport into the cell must be followed by exit from the cell via basolateral efflux. This efflux of hexoses is mediated via GLUT2. Since SGLT1 is sodium dependent, sodium is exchanged for potassium across the basolateral membrane.

3.5.1.1.4 Nucleoside transporters

Purine and pyrimidine nucleosides and their metabolic products are important precursors for the biosynthesis of DNA and RNA. Moreover, nucleosides participate in numerous other biological processes. Nucleosides are, like a number of other nutrients, relatively hydrophilic molecules, and their ability to be absorbed and distributed in the body is highly dependent on transporters. Members of the SLC families 28 and 29 are responsible for the transport of nucleosides. In epithelial cells, transport via members of the SLC28 family is dependent on the sodium gradient across the cell membrane, and these transporters are expressed in the apical membrane (see Figure 3.5.5 and Table 3.5.1). The transporters of the SLC29 family are independent of sodium in the transport process, and are thus facilitative or equilibrative transporters. In epithelial

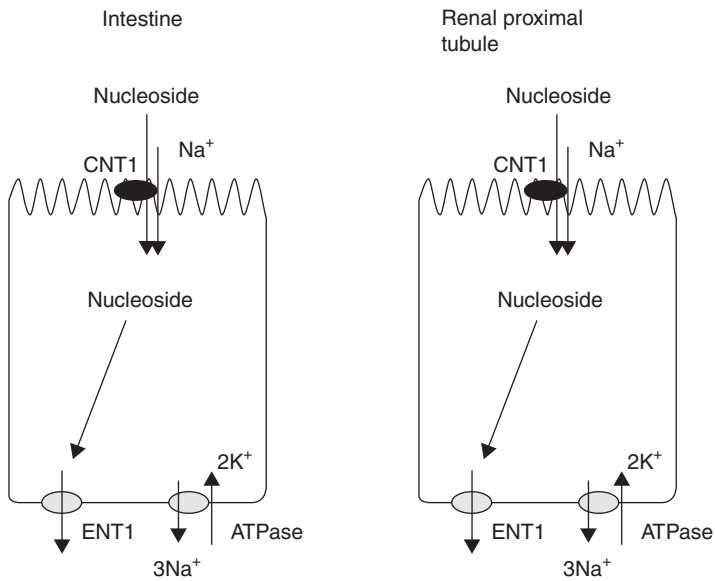

Figure 3.5.5 Transport of nucleosides in epithelial cells of the intestine and kidney. CNT1, Na^+-dependent concentrative nucleoside transporter type 1; ENT1: Na^+-independent equilibrative nucleoside transporter type 1.

cells they are expressed in the basolateral membrane. The concentrative nucleoside transporters CNT1–3 transport uridine and certain uridine analogues. In general, hCNT1 is selective for pyrimidines, except a modest transport of adenosine by hCNT1, and hCNT2 is selective for purines. hCNT3 is non-selective for both purine and pyrimidine nucleosides. The equilibrative nucleoside transporters hENT1 and hENT2 transport both purine and pyrimidine nucleosides, although the substrate affinities are lower than for the CNTs. For transepithelial transport of nucleosides, the CNTs and ENTs function in series to bring a nucleoside from the surface of the epithelial barrier into the systemic circulation.

3.5.1.1.5 Vitamin transporters

Vitamins are a group of structurally diverse compounds, which need to be obtained from the diet. They are involved in a wealth of biological processes, where, among other things, they act as cofactors for metabolic processes. Since they are essential to the body, it is important to consider their oral absorption. Vitamins are generally divided into two groups based on their solubility: vitamins A, D, E and K are lipid soluble and the group of B vitamins and vitamin C are water soluble. Overall, the vitamins A, D, E, and K have log*P* values >5, and are therefore highly lipophilic, and so are absorbed from the intestinal fluid by passive non-saturable diffusion. The more hydrophilic vitamins of the group of B vitamins and vitamin C rely on carriers in order to be both absorbed from the intestinal fluid and re-absorbed in the kidney. L-Ascorbic acid, the reduced form of vitamin C, is an effective antioxidant and scavenger of free radicals, and moreover an important cofactor in several enzymatic reactions. Two sodium-dependent carriers for L-ascorbic acid, SVCT1 and SVCT2, have been cloned, and these are the products of the *SLC23A1* and *SLC23A2* genes, respectively. SVCT1 is widely expressed in epithelial barriers such as the intestine, kidney, and lung, whereas SVCT2 is expressed in the tissues of the brain, eye and placenta (see Table 3.5.1 and Figure 3.5.6). Several other transporters have been demonstrated to be involved in the intestinal absorption and renal re-absorption of the water-soluble B vitamins: these include the thiamine transporters 1 (ThT1) and ThT2, the reduced-folate transporter-1 (RFT-1), and the sodium-coupled multivitamin transporter (SMVT) for biotin, pantothenate and lipoate. In Figure 3.5.6, the intestinal transport of a selected B vitamin has been illustrated. Nicotinic acid (niacin, B_3) has been suggested to be absorbed in the intestine by the monocarboxylate transporter (SLC16), MCT1; however it is possible that the sodium-coupled

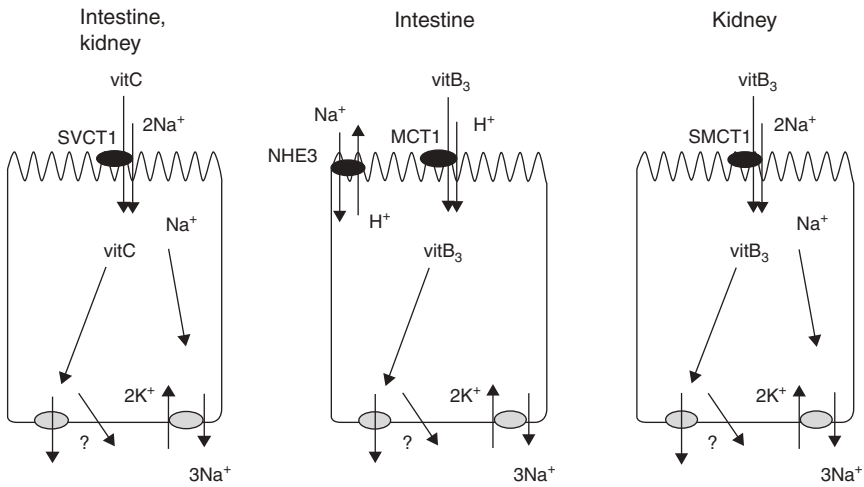

Figure 3.5.6 Transport of vitamins in epithelial cells of the intestine and kidney. vitC, vitamin C; SVCT 1, sodium-coupled vitamin C transporter 1; MCT 1, monocarboxylate transporter 1; SMCT 1, sodium-coupled monocarboxylate transporter 1; vitB$_3$, vitamin B$_3$.

monocarboxylate transporter (SLC5A8), SMCT1, also participates in the absorption of vitamin B$_3$ in segments such as the ileum and colon. In the kidney it has been proposed that the reabsorption of B$_3$ is mediated by SMCT1 (Gopal *et al.*, 2005).

Besides the carriers described above, other mechanisms also participate in vitamin absorption and re-absorption. In the kidney proximal tubules, endocytosis of folate bound to folate-binding protein (FBP) occurs via endocytosis. In the intestine, receptor-mediated endocytosis is observed for vitamin B$_{12}$ in complex with the transport protein intrinsic factor (IF), following binding to the receptor protein cubilin.

3.5.1.2 Important absorptive transporters in drug delivery and biopharmaceutics

In Section 3.5.1.1, some of the absorptive transporters present in the intestine and kidney were described. These transporters perform an important function in relation to normal growth and function of human and animals. Why are they relevant for the student of pharmaceutics? Medicine is made to act in a biological system. The biological system may be the human body or an animal. It has been estimated that 40% of the drug molecules on the market interact with transporters. Several of these 40% are intended to act on a transporter in the CNS. However, as the knowledge on transporters increases, the classical view of enzymes and

receptors as the main drug targets will be altered to include transporters in the drug discovery phase, and must then be integrated in the development of pharmaceutics in the drug development phase. The plasma–time profile of a drug or drug candidate may depend to various degrees on the transport capacity and tissue expression of transporter. Factors such as substrate specificity, transporter capacity, expression profiles, and genetic variation will determine to what extent the transporters will be relevant for a given individual. The development of a pharmaceutical formulation must be able to appropriately accommodate the knowledge about capacity and expression of absorptive transporters in order to maximise the fraction of administered dose absorbed. Knowledge about transporters and their function and expression may also be useful in describing abnormal pharmacokinetic profiles of a drug molecule in the discovery or development phases. Some examples of drugs interacting with transporters are given in Table 3.5.2. Thus, inclusion of the increasing knowledge about transporter interactions with drug candidates already in the drug discovery phase and throughout the pharmaceutical development phase makes transporters an integral factor to be considered in drug development and may increase the success rate of rational drug development.

3.5.1.3 Impact of transporters on intestinal absorption/ intestinal drug delivery

Besides the intrinsic kinetic and regulatory characteristics of a given transporter, a number of other parameters should be taken into consideration when utilising transporters for drug delivery or when dealing with unusual or unexpected pharmacokinetics. One of these elements is the *expression of the transporter(s) in tissue segments*. Knowledge regarding intestinal expression of a relevant transporter for a drug candidate may influence the design of its pharmaceutical formulation. For example, since hPEPT1 is expressed in the small intestine, one may choose an instant-release formulation of a possible drug candidate substrate for hPEPT1. In contrast, since the amino acid transporter $ATB^{0,+}$ is expressed in the colon, then subsequently a colon-specific drug-release formulation will be relevant for a drug candidate substrate to $ATB^{0,+}$ in order to maximise absorption from the relevant tissue segment. The likelihood of drug–drug and drug–food interactions should be considered in relation to the expression of transporter. The effect of segmental pH variations may also influence not only the driving force regarding transport for proton-dependent transporters, but also the ionisation state of the substrates. A transporter may recognise a substrate differently

Table 3.5.2 Selected absorptive transporters relevant in drug delivery and biopharmaceutics; some typical drug molecules transported via the transporters have been listed

Natural substrate	*Gene name*	*Protein*	*Drug molecules*	*References*
Proton-dependent symports				
Peptides	*SLC15A1*	PEPT1	β-lactam antibiotics, valaciclovir, angiotensin-converting enzyme (ACE)-inhibitors, bestatin, various amino acid or dipeptidomimetic prodrugs	Dantzig, 1998; Brodin *et al*., 2002; Nielsen *et al*., 2002
	SLC15A2	PEPT2		
Amino acids	*SLC36A1*	PAT1	GABA, GABA anologues, vigabatrin, D-cycloserine, D-serine	Thwaites and Anderson, 2007
	SLC36A	PAT2		
Sodium-dependent symports				
Nucleosides	*SLC28A1*	CNT1	Zidovudine (3′-azido-3′-deoxythymidine, AZT), lamivudine (2′,3′-dideoxy-3′-thiacytidine, 3TC), and zalcitabine (2′,3′-dideoxycytidine, ddC), cytarabine [1-(b-d-arabinofuranosyl)cytosine, AraC], gemcitabine (20,20-difluorodeoxycytidine, dFdC)	Gray *et al*., 2004
	SLC28A2	CNT2	Didanosine (2′,3′-dideoxyinosine, ddI), ribavirin	Gray et al., 2004
	SLC28A3	CNT3	Cladrabine, gemcitabine, FdU, 5-fluorouridine, fludarabine and zebularine	Gray et al., 2004
Sodium-chloride-dependent symports				
Amino acids	*SLC6A14*	$ATB^{0,+}$	NOS inhibitors, D-serine, amino acid prodrugs of aciclovir	Ganapathy and Ganapathy, 2005

depending on its ionisation state as well as its stereochemistry. This indicates the importance of considering *substrate specificity* of the ADME-relevant transporter(s).

It is a highly relevant, but also difficult, task to evaluate the *kinetic parameters*, K_m *and* V_{max} *with regard to administered dose. In vitro* studies may give information about the binding of a drug candidate to a transporter, or even yield data on the translocation. However, what are the likely consequences of drug candidate substrate binding/translocation on its intestinal absorption and ultimately its oral bioavailibility? Let us say we are developing a prodrug for the peptide transporter, hPEPT1. From the literature we know that the natural substrates for hPEPT1 have affinities in the range of 0.1–3 mM (Nielsen *et al.*, 2002). Furthermore, we know for one distinctive class of substrates, the β-lactam antibiotic, that the likely cut-off value for where the affinity of a compound is too low to translate into a relevant bioavailability is approximately 15 mM (Bretschneider *et al.*, 1999). This indicates that the prodrug we are developing should have affinities in the lower millimolar range, i.e. 0.1–5 mM, unless it has a structural resemblance to β-lactam antibiotics. The concentration of the prodrug in the intestine would then determine the transport rate. The concentration in the intestine is rarely measured, but may for convenience be estimated as described in the Biopharmaceutics Classification System (BCS) (see Chapter 4.3) as the concentration yielded by the dose divided by 250 ml water. Because the transport of prodrug, measured as its flux, is approximately linear in the concentration range from 0 to the K_m value, then an increase in its dose would give a proportional increase in its flux across the intestine. Increasing the dose, to give concentrations higher than the K_m value, will not give a proportional increase in the flux. The flux will be saturated by increasing the concentration of prodrug if its absorption is specifically transporter mediated. The involvement of transporters in the absorption step may therefore be one cause of non-linear pharmacokinetics. Apart from relating the K_m values to dose, it may also be relevant to relate V_{max} or J_{max} to dose, since V_{max}/J_{max} determines the maximal transport capacity of the transport system. However, V_{max} values are mainly obtained from *in vitro* systems, and it is difficult to estimate the *in vivo* V_{max} of a transport system.

In some cases, *variations in the transporter genes* may have effects on the pharmacokinetic profiles of a drug compounds. Differences can be ascribed to processing and stability of the transcribed mRNA, to processing, stability, and sorting of the resulting proteins, or to changes in the kinetic parameters (K_m and V_{max}) of the proteins. Single nucleotide polymorphisms (SNPs) present in the coding region of a gene may thus

lead to altered drug responses or even to other phenotypic differences, such as diseases. For a large number of transporters, the most updated information on genetic variations may be found in the following two databases: www.ncbi.nlm.nih.gov/Entrez/ and www.pharmgkb.org/.

3.5.1.4 Influx transporters present in the liver and kidney

Besides the absorptive transporters present in the intestine and re-absorptive transporters in the kidney, the basolateral influx transporters in the kidney and liver are very important for overall pharmacokinetics. Some of the re-absorptive transporters in the kidney have been described in the previous sections. In this section, other influx carriers will be discussed with regard to their expression in the kidney and liver. Absorptive transporters in tissues other than the intestine and the kidney are not yet well investigated; however, in order for tissues to be relevant for transporter-mediated drug delivery to the systemic circulation, via a given epithelial barrier, then the transporter must be present in large tissue organs such as the lung. Futhermore, its expression in the tissue must be sufficiently high in order to deliver the relevant dose for treating the disease in question. In the lung a number of transporters such as peptide and amino acid transporters have been identified. Future studies may shed light on delivery of drug compounds via transporters expressed in the lung.

3.5.1.4.1 Organic anion-transporting polypeptides

Organic anion-transporting polypeptides (OATPs) are membrane transport proteins that mediate sodium-independent transport of substrates from the blood into hepatocytes. The substrates are a wide range of amiphipathic organic molecules such as some bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides and some drug compounds, e.g. some xenobiotics (Hagenbuch and Meier, 2004). The nomenclature of OATPs has been rather confusing, and for further information on the history and nomenclature of these the reader is referred to the review by Hagenbuch and Meier (2004). We will use the new SLCO-nomenclature and the new protein names as given in the review.

In the liver, protein-bound drug compounds are extracted from the circulation. The free drug is taken up by the hepatocytes for further hepatic handling such as metabolism and export (see Chapter 3.6). In the uptake process from the blood into hepatocytes, OATPs play an important role as outlined in Figure 3.5.7. The transport mechanism of OATPs seems to be an anion exchange, in which the cellular uptake of

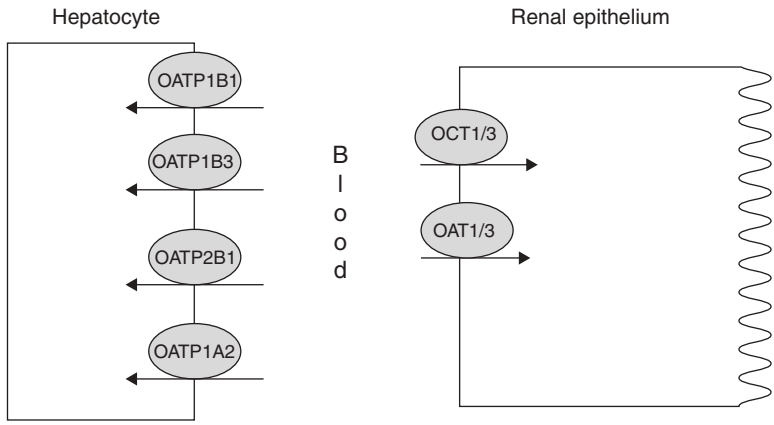

Figure 3.5.7 Organic anion-transporting polypeptides (OATPs), organic cation transporters (OCTs), and organic anionic transporters (OATs) in the liver and the kidney.

organic compounds is coupled to an efflux of cellular bicarbonate, glutathione and/or glutathione-*S*-conjugates (Hagenbuch and Meier, 2004). The transport direction is thus dependent on these gradients, which are driving forces for the transport process (see Chapter 3.6).

OATP1B1 (SLCO1B1) transports bile salts and organic anions. Specifically, OAPT1B1 transports conjugated and unconjugated bilirubin, steroid conjugates, thyroid hormones T_4 and T_3, and drugs such as pravastatin, rifampicin and methotrexate. The carrier is exclusively expressed in the liver. OATP1B3 (SLCO1B3) has similar substrates to OATP1B1, and thus transports bile salts and organic anions. Moreover, OATP1B3 transports digoxin and cholecystokinin (CCK-8) and the opioid peptide deltorphin II. The carrier is expressed in the liver and in some cancer cells. OATP2B1 (SLCO2B1) transports oestrone-3-sulphate and dehydroepiandrosterone. The carrier is most strongly expressed in the liver and, to a lesser extent, in the lung, kidney, brain, intestine and placenta. OATP1A2 (SLCO1A2) transports bile salts, organic anions and organic cations. More specifically, the carrier has been shown to transport the thyroid hormones T_4 and T_3, prostaglandin E_2, and ouabain. The carrier is expressed in the endothelial cells of the brain, and in the kidney and liver.

3.5.1.4.2 Organic anion and cation transporters (SLC22)

Transporters of the SLC22 family are polyspecific, which means that they transport multiple different substrates. The family can be divided into

various subgroups according to the substrates and transport mechanism. One of the subgroups comprises the organic cations transporter OCT subgroup. The OCT transporters transport organic cations, including weak bases (Koepsell and Endou, 2004). The transport process is electrogenic and independent of Na^+. A second subgroup of transporters is the organic anion transporters, OATs, which transport anions. Since transporters of the SLC22 family are expressed in tissues such as the intestine, liver and kidney, they are important in drug absorption and excretion (see Figure 3.5.7).

OAT1 (SLC22A6) is strongly expressed in the kidney. It is localised to the basolateral membrane in renal proximal tubule epithelial cells. The substrates are organic anions which are exchanged for α-ketoglutarate. Drug molecules such as antibiotics, diuretics, antineoplastic drugs, and anti-inflammatory drugs are recognised as substrates (Koepsell and Endou, 2004). Moreover, endogenous compounds such as cyclic nucleotides, prostaglandins and uric acid are substrates. OAT3 (SLC22A8) is strongly expressed in the liver, with weaker expression in the kidney (Koepsell and Endou, 2004). It is localised to the basolateral membrane in renal proximal tubule epithelial cells. The substrates are organic anions, which may be exchanged for dicarboxylates. Drug molecules such as cimetidine and ochratoxine A are recognised as substrates (Koepsell and Endou, 2004). OCT1 (SLC22A1) is mainly expressed in liver tissue. It is localised to the sinusoidal membrane, and the basolateral membrane in enterocytes and renal epithelial cells. The substrates are organic cations and some weak bases; however, some anions are also transported. Drug compounds such as desipramine, aciclovir, ganciclovir and metformin are recognised as substrates. Moreover, endogenous compounds such as serotonin and prostaglandins E_2 and $F_{2\alpha}$ are substrates. OCT3 (SLC22A3) is expressed in the liver, placenta and kidney. The substrates are organic cations and some weak bases; however, some anions are also transported.

3.5.2 Conclusions

In this chapter a number of carriers, likely to be relevant in the overall pharmacokinetics of many drugs in the human body, have been described. Knowledge about these transporters is important in describing ADME properties of new chemical entities and drug compounds. It may thus be useful in describing unusual kinetics, or in order to predict the impact of interaction with transporters on absorption, clearance, excretion or

metabolism. Knowledge about the carriers as described in this chapter may also be applied in the drug discovery phase to rational design of prodrugs and analogues as substrates for absorptive carriers in the intestine, in order to increase intestinal permeation properties and thus oral bioavailability.

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3.6

Efflux transporters

Carsten Uhd Nielsen, Birger Brodin and Bente Steffansen

In the previous chapter the absorptive transporters in the intestine and the kidney were discussed. In this chapter, the focus is on efflux transporters – membrane proteins that transport compounds from the cell to the exterior. In 1976 Juliano and Ling discovered that the over-expression of a membrane protein in colchicine-resistant Chinese hamster ovary (CHO) cells conferred resistance to a wide range of anti-cancer drugs (Juliano and Ling, 1976). The protein was termed P-glycoprotein 170 (P-gp) and the phenomenon was termed multidrug resistance (MDR). The consequence of expression of this glycoprotein was cellular export of structurally unrelated compounds. This caused a reduced retention and increased excretion of anti-cancer compounds from the cell. P-gp turned out to be the first member of a large superfamily of ATP-dependent efflux transporters. These are now termed ABC transporters, for ATP-binding cassette transporters. It has been found that ABC transporters are part of MDR, which is a cause of therapeutic failure in chemotherapy. ABC transporters are present in the normal human cell, where they are a natural part of the body's defence against xenobiotic compounds, i.e. compounds that are foreign to the body. Since ABC transporters act by pumping their substrates out of the cell by utilising ATP, they are often referred to as efflux transporters. From a pharmaceutical point of view efflux transporters affecting the ADME properties of drug candidates are of special interest in the present context. For the absorption part, it is relevant to consider the expression of efflux transporters in the intestine. The distribution aspect of efflux transporters is relevant for drugs that are intended to act in the CNS, since efflux mechanisms have been shown to prevent some drugs, such as ivermectin and digoxin, from accessing the brain. In terms of metabolism, efflux transporters in the intestinal epithelia and hepatocytes have been shown to work together with metabolising enzymes such as CYP3A4 and glutathione-*S*-transferases to decrease intracellular accumulation of various compounds. Efflux transporters are

also relevant in the excretion of drugs from the systemic circulation via intestinal or renal efflux. It is evident from this that efflux transporters must be taken into consideration when dealing with the ADME properties during drug discovery and in pharmaceutical and preclinical development (see Chapter 3.7). In the present chapter we will describe the expression and function of efflux transporters in tissues that are relevant for the biopharmaceutical scientist.

3.6.1 ATP-binding cassette (ABC) transport proteins in the intestine

The ABC drug-transporting proteins, often referred to as efflux transporters, are in general from either the multidrug resistance (MDR) or the multidrug-resistance-associated protein (MRP) type. The transporters are listed in the Table 3.6.1 (Chan *et al.*, 2004), and in the following section the focus will be on the efflux transporters that are most relevant in the drug discovery and preclinical setting.

3.6.1.1 MDR transporters

The most studied efflux transporter is P-gp (MDR1, ABCB1). P-gp belongs to the MDR/TAP subfamily within the ABC superfamily. In the intestine, the expression of P-gp is confined to the apical membrane of the enterocyte. The expression increases from the crypt to the tip of the villus, and on the segmental level the expression increases from the proximal to the distal part of the small intestine (Chan *et al.*, 2004). The mechanism behind transport via P-gp is highly complex, and not yet well understood. However, P-gp acts by pumping substrate out of the cell or the cell membrane by utilising ATP. The 'hydrophobic vacuum cleaner' hypothesis predicts that P-gp pumps substrates either from the outer membrane of the lipid bilayer, or from the inner membrane of the bilayer into the extracellular space (Gottesman and Pastan, 1993). This implicates that P-gp recognises its substrate after the substrate has partitioned into the cell membrane. Substrate may thus enter the cell membrane from either the extracellular or intracellular side.

P-gp has an extremely broad substrate specificity, with a preference for lipophilic and cationic compounds; however, these general structural features are by no means restrictive. This is illustrated by the diversity in the substrates identified for P-gp, including anti-cancer drug substances such as vinblastine, doxorubicin, etoposide and paclitaxel, cardiac drug

Table 3.6.1 Efflux transporters that are relevant in biopharmaceutics and drug disposition

Transporter	*Gene*	*Location*	*Expression*	*Substrate*
MDR1/P-gp	*ABCB1*	Apical	Intestine, liver, kidney, blood–brain barrier	Anti-cancer drugs, cardiac drugs, endogenous compounds, HIV drugs, fluoroquinolones, immunosuppressive drugs (Chan *et al.*, 2004)
MDR3	*ABCB4*	Apical	Liver	Phospholipids, digoxin, paclitaxel, vinblastine
BSEP	*ABCB11*	Apical	Liver	Bile acids
MRP1	*ABCC1*	Basolateral	Intestine, brain, kidney, lung, liver	Glutathione and glutathione conjugates
MRP2	*ABCC2*	Apical	Intestine, liver, kidney	Glutathione and glutathione conjugates as well as non-conjugated anionic molecules
MRP3	*ABCC3*	Basolateral	Intestine, liver, kidney	Bile acids, drugs
MRP4	*ABCC4*	Apical	Intestine, kidney, brain, liver	Bile acids, drugs
MRP5	*ABCC5*	Basolateral	Colon, liver, kidney, brain	Glutathione, adefovir, 6-mercaptopurine
MRP6	*ABCC6*	Basolateral	Kidney, liver	Some anti-cancer drugs, BQ123
MRP7	*ABCC10*			
MRP8	*ABCC11*			
MRP9	*ABCC12*			
BCRP	*ABCG2*	Apical	Intestine, liver	

Notes: For further detail consult a current review (suggested references: Seelig and Gerebtzoff, 2006; Gottesman and Ling, 2006; Huang and Sadee, 2006; Kerb, 2006; Teodori *et al.*, 2006; Varma *et al.*, 2006).

substances such as digoxin and some β-blokers, endogenous compounds such as steroid hormones and bile salts, HIV drug substances such as indinavir and saquinavir, fluoroquinolones such as sparfloxacin, and immunosuppressive drug substances such as ciclosporin A and tacrolimus (Chan *et al.*, 2004). In general, P-gp substrates are thus amphipathic compounds with a molecular weight ranging from approximately 300 to 2000 Da. It has been suggested that P-gp binds substrates through an 'induced-fit mechanism', where the shape and size of the substrate changes the packing of the transmembrane segments in order to accommodate the substrate (Loo and Clarke, 1999; Loo *et al.*, 2003).

Thus, in addition to the function of P-gp in regulating absorption of several compounds, a further dimension is added, giving evidence for the notion that P-gp functions in conjunction with metabolising enzymes. P-gp may thus play a dual role in limiting the oral bioavailabilities of drug substrates, i.e. by reducing their absorption and by delivering them to metabolic enzymes. In the liver and intestine the P-gp function is present in metabolically active cells. Phase I enzymes such as CYP P450s and phase II enzymes such as glutathione-*S*-transferases are key factors in limiting drug bioavailability. There is also some degree of overlapping substrate specificity between CYP3A4 and P-gp (Wacher *et al.*, 1995, 1998; Benet *et al.*, 1999). It is therefore important to consider both the efflux properties and the metabolic properties of drug candidates. This is shown in Figure 3.6.1, where the action of both P-gp and metabolising enzymes is illustrated.

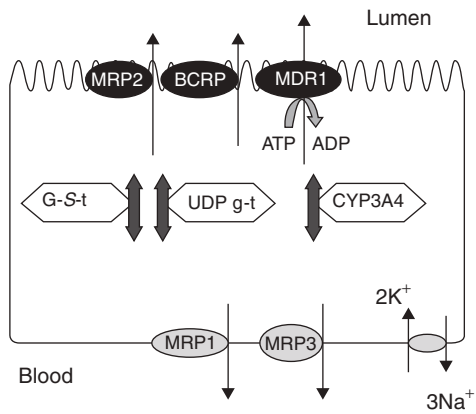

Figure 3.6.1 Principal efflux transporters and intracellular enzymes in the intestinal epithelium. MDR1, the multidrug-resistance protein alias P-gp; G-*S*-t, glutathione-*S*-transferase; UDP g-t, UDP glucuronosyl transferase; MRPs, multidrug-resistance-associated transport proteins; BCRP, breast cancer resistance protein.

3.6.1.2 MRP transporters

The MRP transporters are active transporters belonging to the MRP/CFTR subfamily within the ABC superfamily.

MRP1 expression is widespread within the body. MRP1 is expressed in the basolateral membrane of polarised epithelial cells, and in the intestine, brain, kidney, lung and liver. MRP1 exports conjugated metabolites such as glutathione- and glucuronide-containing compounds from the cell. Furthermore, MRP1 and P-gp have overlapping substrate affinities Examples are vincristine, paclitaxel and etoposide.

MRP 2 is expressed in the apical membrane of polarised epithelial cells of the intestine and kidney as well as in the liver. The expression increases from the crypt to the tip of the villus, and on the segmental level the transporter expression decreases from the proximal to the distal part of the small intestine (Chan *et al.*, 2004). The substrates are both conjugated and unconjugated anionic compounds such as glucuronides and glutathiones, as well as anti-cancer compounds such as vincristine and doxorubicin. There exist overlapping expression patterns with metabolising enzymes such as glutathione-*S*-transferases, which are phase II metabolising enzymes that catalyse the conjugation of a compound with glutathione. Furthermore, UDP-glucuronosyltransferases are membrane-bound phase II metabolising enzymes that are relevant in detoxicification and subsequent metabolism and elimination of drug compounds. This is illustrated in Figure 3.6.1.

MRP3 is expressed in the basolateral membrane of polarised epithelial cells of the intestine and kidney. It is also expressed in the liver, kidney and lung. The expression increases from the crypt to the tip of the villus, and on the segmental level the transporter expression increases from the proximal to the distal part of the small intestine (Chan *et al.*, 2004). The substrates are conjugated compounds and bile acids. MRP3 transports some of the same substrates as recognised by MRP2 and the bile salt export pump (BSEP).

3.6.1.3 BCRP transporter

BCRP, breast cancer-resistance protein (ABCG2), belongs to the White subfamily (Chan *et al.*, 2004). BCRP is expressed in the intestine and liver. In the small intestine and the colon, it is expressed in the apical membrane, and in the liver it is expressed in the hepatocyte canalicular membrane (see Figures 3.6.1 and 3.6.2). BCRP has overlapping substrate

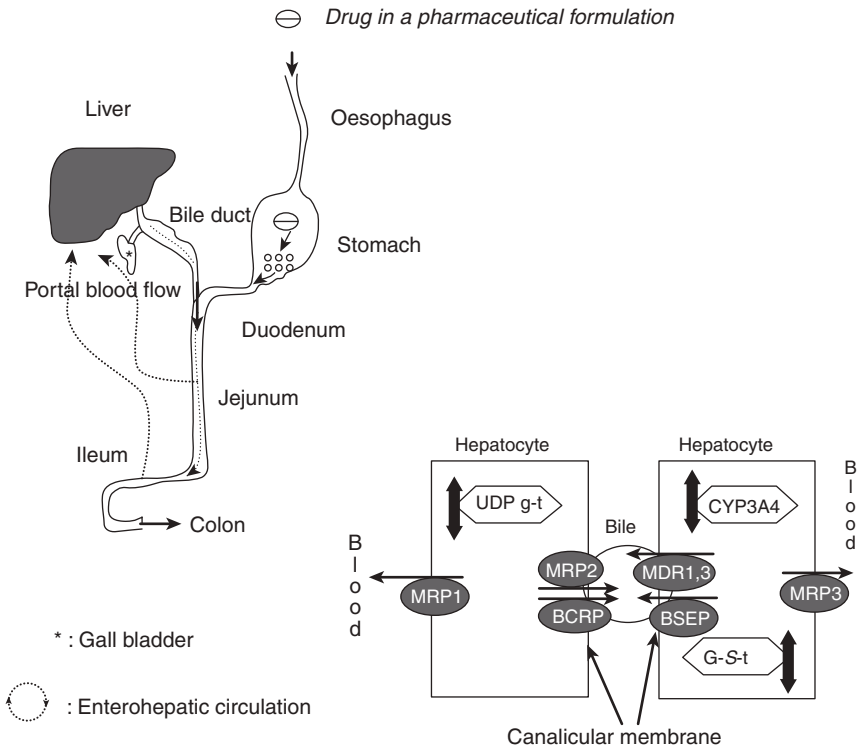

Figure 3.6.2 Principal efflux transporters and intracellular enzymes in the liver. MDR1, multidrug-resistance protein alias P-gp; G-*S*-t, glutathione-*S*-transferase; UDP g-t, UDP glucuronosyl transferase; MRPs, multidrug-resistance-associated transport proteins.

specificity with P-gp, and it is therefore likely that it plays a similar role to P-gp in influencing drug absorption and disposition.

3.6.2 Efflux transporters in the liver

In Figure 3.6.2 the intake of a solid pharmaceutical formulation is illustrated. After ingestion, the drug formulation starts to disintegrate in the stomach and dissolution of the drug substance is initiated. After emptying of the stomach into the intestine, the solute drug substance is possibly absorbed across the intestinal epithelium. At the intestinal barrier, efflux transporters may – as illustrated in Figure 3.6.1 – limit the absorption of drug substrates and/or, after absorption, take part in its elimination. The

absorbed drug substance is circulated via the portal blood flow to the liver. The liver is a major metabolising and detoxifying organ. Here efflux transporters play a major role, together with metabolising enzymes, in the overall pharmacokinetics of the absorbed drug substrate(s). The intact drug substrate or its metabolites may pass into the bile, which is subsequently emptied into the duodenum. The formation of bile is a critical function of the liver, since it is the route for excretion of bilirubin, hormones, cholesterol and xenobiotics such as drugs. The drug or its metabolites may then either be excreted into the faeces or be available for a new absorption cycle. In the liver, some of the above-mentioned transporters are expressed. In the following section other efflux transporters in the liver are described.

3.6.2.1 The bile salt export pump (BSEP)

In the canalicular membrane of human hepatocytes, the bile salt efflux pump, BSEP (ABCB11) causes a vectorial transport of bile salts from the blood to the bile. The BSEP is almost exclusively expressed in the liver. It belongs to the subfamily B (MDR/TAP). BSEP substrates include glycocholate, taurochenodeoxycholate, and tauroursodeoxycholate. In addition to bile salts, the transporter is also able to transport certain anticancer compounds such as vinblastine and taxol. Mutations in BSEP are the causes of some hereditary and acquired cholestatic disorders.

3.6.2.2 MDR transporters

In the liver, another MDR transporter is expressed. MDR3 (ABCB4) is a canalicular phospholipid translocator. Besides transporting phospholipids, MDR3 may transport compounds such as digoxin, vinblastine and paclitaxel. The impact of this transporter in drug disposition and overall pharmacokinetics of lipophilic drug substances is not yet well investigated.

3.6.3 Efflux transporters in the kidney

The kidney is the principal organ for elimination of a number of xenobiotics and metabolic waste products but, as discussed in Chapter 3.5, several re-absorption processes also take place rescuing nutrients from renal elimination. In the kidney, plasma is filtered through the glomerular capillaries into the renal tubules. This is known as glomerular filtration.

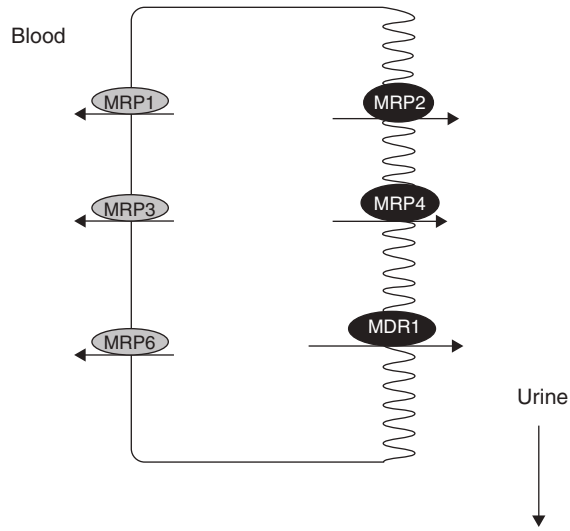

Figure 3.6.3 Principal efflux transporters in the renal epithelium. MDR1, multidrug-resistance protein alias P-gp; MRPs, multidrug resistance-associated transport proteins.

The filtrate passes down the tubules, where nutrients, salts and most of the water are reabsorbed. This increases the circulation time of drug substances or nutrients, which are substrates for absorptive transporters, and for a drug substance this may increase the overall bioavailability by reducing elimination. At the same time efflux transporters mediate the excretion of either intact drugs or their metabolites into the urine. This is illustrated in Figure 3.6.3 for efflux transporters present in the kidney. The expression of both influx and efflux transporters may give rise to various drug–drug interactions in treatments with multiple-drug regimens. The likelihood of drug–drug interactions depends on a variety of factors such as the transport capacity of the transporters, the relative binding of the substrate, the presence of inhibiting drugs, and expression patterns of the relevant transporters, as well as individual genotypes.

3.6.3.1 MRP transporters

The multidrug-resistance-associated transport protein MRP4 (ABCC4) is present in the renal epithelium. MRP4 is furthermore expressed in the intestine and at the blood–brain barrier. The transport protein is expressed at the apical membrane of the renal proximal tubules as illustrated in Figure 3.6.3. MRP4 substrates include methotrexate,

glucuronides and bile acids. MRP6 (ABCC6) is highly expressed in the basolateral membrane of the renal epithelium. MRP6 substrates include anti-cancer drugs such as etoposide, doxorubicin and cisplatin; the substrates are thus quite similar to those of MRP3.

3.6.4 Efflux transporters in the brain

For the majority of the previous chapter and the present one, the focus has been on transporters that are present in epithelial tissues. In this section we will look at the presence of efflux transporters in endothelial cells. Endothelial cells form the capillaries of the blood circulation system. The capillaries differ significantly between the periphery and central system as illustrated in Figure 3.6.4. In the periphery the capillaries are fenestrated, which means that the diffusion barrier between the individual cells is very low. In the brain, however, the capillaries form tight junctions, which means that the paracellular diffusion barrier is very high (with the exception of the capillaries in the choroid plexus). This implies that there will be little or no transport of hydrophilic compounds

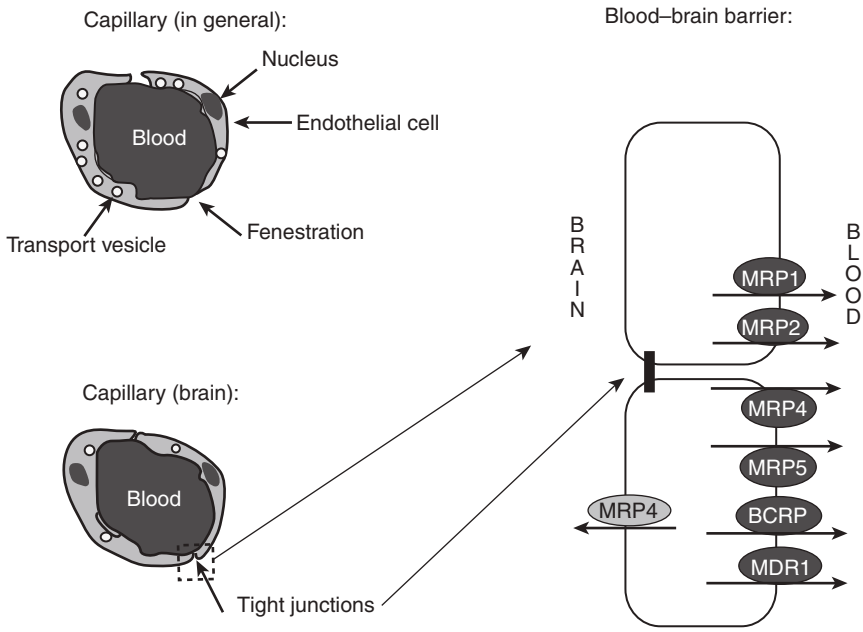

Figure 3.6.4 Principal efflux transporters in the blood–brain barrier. MDR1, multidrug-resistance protein alias P-gp; MRPs, multidrug-resistance-associated transport proteins; BCRP, breast cancer-resistance protein.

across the barrier. As in all transporting epithelia, the cells therefore have an asymmetric distribution of transport proteins, which gives rise to a polarised transport. As illustrated in Figure 3.6.4, endothelial cell efflux transporters are present both in the membrane facing the blood side and in the membrane facing the brain side. The consequence of the tight junction is that drugs intended to work in the CNS must permeate the endothelial cell via the transcellular route, and this will favour lipophilic compounds. However, these types of compounds may be substrates for efflux transporters, thus rendering transepithelial transport low or absent. Furthermore, intracellular metabolism followed by cellular efflux will maintain a low accessibility to the cells of the brain.

3.6.5 Conclusions

In this chapter, the efflux transporters expressed in the intestine, kidney, liver and brain have been described. It is important to remember that the chapter is an overview with respect to the currently available literature. For expert knowledge regarding specific transporters, overlapping substrate specificity between metabolising enzymes and transporters, genetic polymorphisms of transporters, drug–drug interactions etc, readers are encouraged to consult the current available literature, as the field is developing rapidly. The presence of efflux transporters is a natural part of the body's defence against xenobiotics. In the drug discovery and preclinical development processes, transporters are highly relevant to consider, in order to get new drugs through registration. Thus the US FDA has published guidelines for evaluation of drug–drug interactions with focus on transporters and metabolising enzymes (FDA, 2006). The following Chapter 3.7 'Preclinical evaluation of drug transport' will describe examples of how knowledge regarding transporters' influence on *in vitro* ADME properties of drug candidates may influence the preclinical process.

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3.7

Preclinical evaluation of drug transport

Anna-Lena Ungell

Transport of drugs across biological membranes is characterised by the membrane permeability. This characteristic of membranes is an important factor for the disposition of drugs because of the control it exerts over both the bioavailability and transport into and out of tissues and organs of the body such as the intestine, liver, kidney and central nervous system (CNS). Membrane permeability as well as other biological parameters (e.g. potency, metabolism and solubility in biological media) of a drug molecule can change dramatically with the molecular structure. All these biological properties essentially follow different rules of the quantitative structure–property relationship (QSPR). Parts of the molecule may be changed to optimise each single property separately, but in most cases the biological properties may be connected with the same region of the molecule, and thus optimisation of one property may dramatically attenuate the others. Therefore, screening of compounds using multiple techniques in parallel has become the rationale during preclinical evaluation of drugs.

Drug transport is a multifactorial process determined by several rate-limiting steps, i.e. the events and processes occurring before and during the transport across the membrane. Conceptually, one may divide all the factors influencing the potential for a drug to be transported into three proposed groups (Ungell, 2005). First are those factors associated with the chemical structure and physicochemical properties of the drug molecule, such as water solubility, lipophilicity (log$D$, log$P$), acid/base properties (pK_a), and molecular weight/volume. Second are factors within the biological system – the intestinal lumen, plasma or intraluminal milieu – e.g. pH, ion composition, bile acids and soluble enzymes. The third group consists of factors that represent the anatomy and physiology of the membrane, e.g. surface area, transit times, blood flow, enzymes and transporters (Ungell, 1997, 2005; Ungell and Abrahamsson, 1997). In addition to this complexity of drug transport, there are also differences in

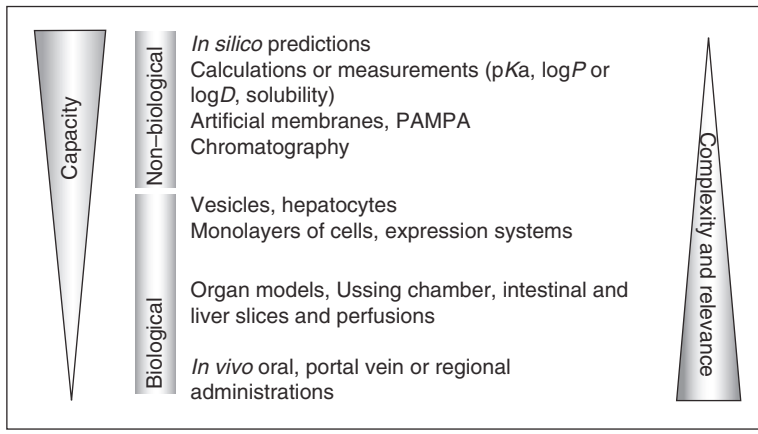

Figure 3.7.1 Schematic drawing illustrating an overview of models for the determination of drug transport in relation to complexity and relevance *in vivo* in humans. PAMPA, parallel intraluminal membrane permeability approach.

in vitro and *in vivo* methodologies, since an *in vitro* system is a static system with well-defined conditions and may very well lack the proper milieu, dynamics and changes that can occur *in vivo*. This may be one explanation why *in vitro–in vivo* correlations might fail.

In vivo animal kinetic studies can provide a good basis for prediction of drug transport kinetics in humans. However, *in vivo* kinetic studies will be too complex to guide the chemist to change a specific property of the molecule, and the capacity is low. On the other hand, no single *in vitro* method may provide all the important information needed around key factors influencing drug transport for the huge diversity of drug molecules to be tested during the preclinical phases. Therefore, the majority of preclinical methods are flexible *in vitro* techniques offering solutions to different problems at different preclinical stages, and also permitting rapid feedback to medicinal chemists. In addition, due to the small amount of compound synthesised during early drug discovery, *in vitro* techniques offer a special advantage over *in vivo* ones (see Figure 3.7.1).

3.7.1 Mechanisms of drug transport across membranes

Drugs permeate cellular membranes not only by passive diffusion, but also by multiple and parallel processes (see Figure 3.7.2). In the gastrointestinal (GI) tract, passive diffusion can occur transcellularly

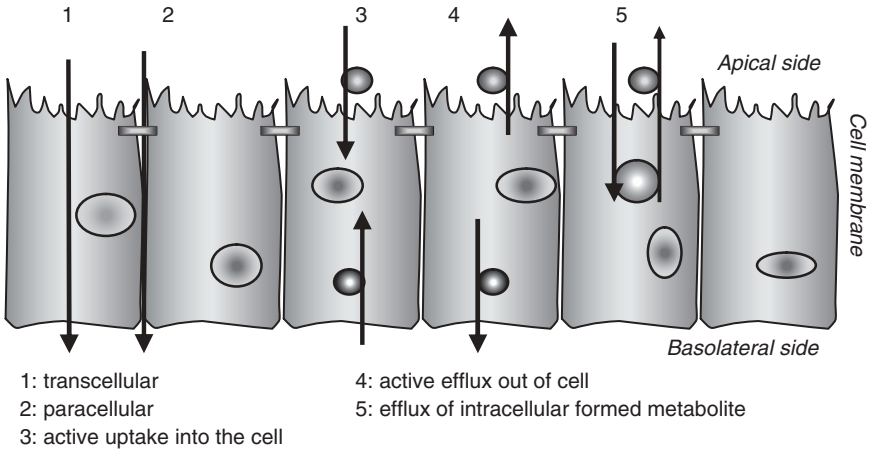

Figure 3.7.2 Mechanisms of drug transport across an epithelial membrane.

across the lipid membrane or paracellularly between the epithelial cells through water-filled pores in the tight junctional complex. Usually, lipophilic drugs permeate more easily by a transcellular route and small polar and ionisable solutes are restricted to the paracellular route. The transcellular route can also be transversed via carrier-mediated processes for drugs that are substrates for transporter proteins, favouring uptake into or efflux out of the epithelial cell (Tsuji and Tamai, 1996; Hunter and Hirst, 1997). In other organs, e.g. the liver and kidneys, transporters are thought to regulate intracellular concentrations of drug compounds and thus may influence both intracellular metabolism and toxicity. In these organs, drug clearance (CL) can be a mixture of both enzymatic reactions and transporter interaction (Hinter and Hirst, 1997). The relative contributions of active and passive transport across membranes are variable between compounds, species and methods, as well as being influenced by the concentration and ion gradient applied (Ungell and Karlsson, 2003). For example, at low concentrations within the intestinal lumen, i.e. for low-solubility drugs with very high potencies, the involvement of efflux transport processes has a greater impact on the effective transport than it does for highly soluble compounds administered at high doses. In addition, in the systemic circulation, compounds may be protein bound and the fraction that is unbound might be small. This will reduce the effective concentration at the transporter protein. Similarly, a compound given at high doses can saturate processes at the intestinal level, but in the systemic circulation and at other tissue barriers, the concentration might be low and the risk for drug–transporter interactions increases.

The models used for the preclinical evaluation of compounds are often focused on investigating specific processes that are important for drug transport (see Figures 3.7.1 and 3.7.2). This is because the processes the methods describe are quite different, since molecular properties resulting in transport via carrier proteins are completely different from those favouring simple passive diffusion (see Figure 3.7.2) (Ungell, 2004). Firstly, passive transcellular diffusion is often guided by partitioning into and out of a lipid bilayer membrane characterised by well-known properties such as lipophilicity, polar surface area (PSA) etc. (Artursson *et al*., 2001; Lipinski *et al*., 2001; Stenberg *et al*., 2001; Van de Waterbeemd *et al*., 2001; Egan and Lauri, 2002; see also Section 2.1.1).

Secondly, the aqueous pathway of the paracellular route is dependent on molecular weight/volume, flexibility (number of rotable bonds) and charge (Artursson *et al*., 1993; Veber *et al*., 2002). This pathway is considered less in the screening since most compounds are lipophilic and of higher molecular weight. However, the literature describes this pathway as being undervalued since it can contribute to the absorption of small ionised molecules (Matsson *et al*., 2005). Thirdly, structural requirements for carrier-mediated processes depend on binding of the compound to the carrier (affinity) and translocation; thus they are driven by both the size and structure of the transported molecule (Vig *et al*., 2006) (see Section 3.3.2.5.1). Thus, using only passive transcellular estimates (e.g. log*D*) will not predict net permeability correctly if transporters are involved.

In view of this, it is important to understand the different factors influencing drug transport and potential transporter interaction when interpreting *in vitro* and *in vivo* data. Thus, the method to be used should be selected on the basis of the mechanisms of transport to be studied (Figures 3.7.1 and 3.7.2).

3.7.2 Tools to assess drug transport during phases of drug discovery

The preclinical phase of drug discovery is divided into different phases, from target identification (TI), through lead generation (LG) and lead optimisation (LO) to the selection of a candidate drug suitable for development (see Figure 3.7.3). These phases are named differently among industries and can generally be divided into early discovery phase and late discovery phase. Early discovery starts after the high-throughput screening (HTS) of large combinatorial libraries on a specific target, through generation of potential lead series of new entities, and ends by

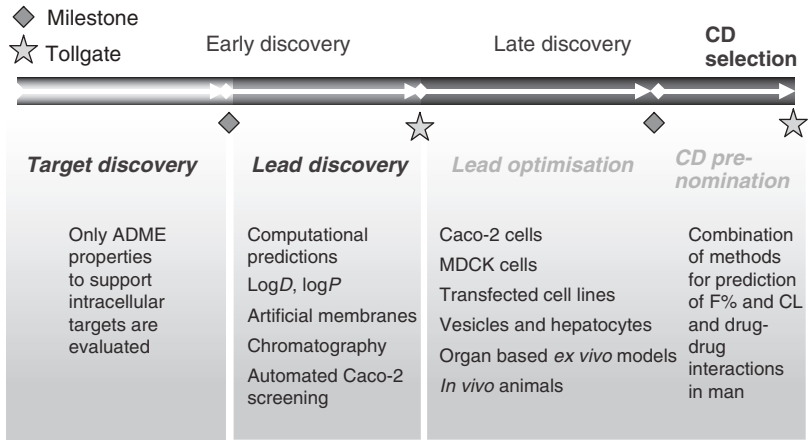

Figure 3.7.3 Schematic drawing of the main drug discovery phases. From target identification (TI), via lead generation (LG) towards lead optimisation (LO) and pre-candidate selection (pre CD) and candidate drug selection (CD). After the CD selection, the development phase starts and the clinical testing in humans proceeds. MDCK, Madin–Derby canine kidney cells.

delivering series of compounds into the late discovery phase where the optimisation of a few series occurs. The late discovery phase ends with the selection of precandidates that are usually submitted to short toxicological programmes before a drug can be selected as a candidate.

Generally, in the early discovery, ADME properties with problems are identified and series of compounds are designed to build a structure–activity relationship (SAR). In the late discovery phase these problems are solved by stepwise optimisation of the different properties. A short description of some *in vitro* tools used in the different preclinical phases to optimise drug entities with respect to drug transport is given next. A summary of the tools is presented at the end of each section and in Figure 3.7.3.

3.7.2.1 Early discovery phase

The ideal *in vitro* technique for evaluation of drug transport at this stage needs to expedite a wide spectrum of molecules in order to range large and diverse data sets for ADME property information. Simple techniques addressing solubility, passive transcellular diffusion across a membrane, and metabolic stability, using rat or human liver microsomes, are commonly used in parallel with computational and physicochemical or preformulation approaches such as lipophilicity, log*D* or log*P* and aqueous solubility measurements (see also Part 2).

Nowadays, computer-based prediction models are valuable tools to estimate permeability properties of molecules with unfavourable biopharmaceutical properties, and require no synthesis as well as being almost unlimited in capacity (Ungell, 2004). First, a compound set is selected that is appropriate for the model to be built (active or passive processes). The next step is to both obtain experimental data and select the appropriate molecular descriptors. Multiple molecular properties of different complexity can be generated via computation using commercially available programs, for example, GRID (www.moldiscovery.com), HYBOT (www.timtec.net/software/hybot-plus.htm), VolSurF (www.moldiscovery.com), Molconn-Z (www.tripos.com), Cerius2 (www.accelrys.com/) (Ungell, 2004). The interrelationship between the most important generated descriptors is then mathematically described in a series of equations to obtain a calculated estimate of the apparent permeability coefficient, P_{app}, by, for example, the use of statistical multivariate analysis, such as principal component analysis (PCA) and partial least square (PLS) (Simca Umetrics AB; www.umetrics.com).

Correlation between calculated and experimentally observed permeability values is then performed to obtain the final QSPR model. The last part of the building is the validation of the model. An external test set of compounds can provide an insight into how good the model is and ensure proper evaluation of predictions. Multiple *in silico* permeability models of varying complexity and success have been presented in the literature and many predict Caco-2 (human colon carcinoma cells) permeability or interaction with a certain transporter (Artursson *et al.*, 1996; Stenberg *et al.*, 2001; Ungell, 2004; Vig *et al.*, 2006). It should be mentioned that models generated from Caco-2 data can only be used for drug permeability in the intestine and do not generally predict permeability or disposition in all other tissues of the body, since tissues contain differences in expression of transporters and lipid composition of the membranes.

Experimental physicochemical measurements are often performed at this early stage of drug discovery. Capillary electrophoresis and chromatographic measurements using columns with different packing materials (immobilised phospholipids onto a silica surface (IAMS), liposomes and reversed phase C18, as well as measurement of log*P* and log*D* (Hartmann and Schmitt, 2004) are well-suited high-throughput methods for early predictions of permeability or lipophilicity (k') (Camenisch *et al.*, 1997; Hartmann and Schmitt, 2004; Örnskov *et al.*, 2005). Recently, artificial membranes (parallel intraluminal permeability approach, PAMPA) made of mixtures of lecithin or membrane phospholipids and inert organic solvents on a permeable support have also been developed to describe the

transcellular passive membrane permeation process (Camenisch *et al.*, 1997; Kansy *et al.*, 1998, 2004; Ottiger and Wunderli-Allenspach, 1999). However, using passive transcellular permeability as a single tool for optimising compounds can under- or over-predict permeability for compounds that are mainly restricted to either paracellular or carrier-mediated transport, respectively (Ungell, 2004). Thus, if the methods are used without knowledge of drug transport mechanisms, data can provide misleading information for prediction of drug transport in humans.

Evaluation of drug transport can also be made in this early phase using biological tools such as automated cell monolayer permeability methods, e.g. Caco-2 cells (see below), with high throughput, and both absorptive and secretory transport may be studied if needed. In most cases, at this stage of drug discovery, ranking compounds or series of compounds into levels or banding of permeability (low/moderate/high) (Ungell and Karlsson, 2003) may be enough to fill the purpose. The Caco-2 data can be used for validation of the physicochemical or computational predictions or for a 'quick and dirty' indication of carrier-mediated transport. For instance, if the passive permeability indicates a highly permeable compound, but the Caco-2 cell data show a low level of permeability, efflux transporters might be involved, attenuating drug transport. However, the exact identity of which transporter is involved is usually of no concern at this stage.

3.7.2.1.1 Summary of models used in the early discovery phase

- Computation of surface descriptors for permeability; building of QSPR models, and computation of physicochemical properties
- Physicochemical properties, experimentally, e.g. log*D* using chromatography or PAMPA
- Automated Caco-2 cell screening in recommended pH gradient of 6.5/7.4, one or few time points
- Bidirectional Caco-2 permeability for transporter involvement.

3.7.2.2 Late discovery phase

In this stage of drug discovery, in-depth mechanistic evaluation of drug transport is performed to optimise compound structures in order to avoid poor permeability properties or drug–drug interactions in the clinic. Information from the models is used to solve problems by a stepwise optimisation of the molecules. Numerous techniques such as cell cultures,

membrane vesicles, hepatocytes, transfected cells, intestinal slices or sacs, Ussing chamber technique, *in vitro* and *in situ* intestinal and liver perfusions, *in vivo* cannulated animals, and *in vivo* gavaged animals (see Figure 3.7.1; Borchardt, 1996; Barthe *et al*., 1999; Irvine *et al*., 1999; Balimane *et al*., 2000; Salphati *et al*., 2001; Ungell, 2002, 2005; Miret *et al*., 2004; Balimane and Chong, 2005; Li, 2005; Sahi, 2005) can be used in parallel to complement physicochemical knowledge and obtain a better understanding of drug transport during preclinical screening. A short description of some models used follows next.

3.7.3 Cell cultures

Caco-2 cells serve as an easy screen of drug permeation and for prediction of human intestinal permeability and fraction of the oral dose absorbed (f_a) in man (Ungell and Karlsson, 2003; Ungell, 2004). The cells are easy to culture and show good experimental reproducibility and robustness, and much is described in the literature about their performance. The heterogeneous properties of the Caco-2 cells may be one explanation for the differences in morphology, paracellular permeability and expression of enzymes and transporters that have been reported from different research groups (Chong *et al*., 1996; Chen *et al*., 2002; Ungell, 2003). The culturing conditions, e.g. the age of the cells and the passaging process as well as nutritional conditions, can dramatically alter the biological characteristics and transport properties of the Caco-2 cell monolayers (Anderlé *et al*., 1998; Li *et al*., 2003; Ungell, 2003; Seithel *et al*., 2006). The cell culture protocol therefore has to be standardised for screening, and experiments should only be performed within a limited and well-defined number of passages (Ungell and Karlsson, 2003; Seithel *et al*., 2006). Since the cells are of colonic origin, their predictability to f_a has been debated (Balimane and Chong, 2005), and active transport of some compounds in these cells shows weak predictability for humans (Chong *et al*., 1996; Lennernas *et al*., 1996; Salphati *et al*., 2001).

Experiments using the Caco-2 model and also other cell culture models are easy to perform. After seeding on microporous filters for a specified number of days (normally 14–21), the cultivation media is exchanged to transport buffer solution. Thereafter, the substance is added on one side, either apically or basolaterally, and samples are withdrawn from the opposite side at predefined time points. All experiments should be performed under stirring conditions and at 37 °C. Permeability of the cell monolayer (P_{app}; apparent permeability coefficient, cm s^{-1}) is calculated from the determination of the amount of drug transported to the

receiver side at the different time points and is also related to both the surface area of the monolayers and the donor concentration (Ungell and Karlsson, 2003).

A set of carefully selected compounds, based on available clinical f_a and Caco-2 permeability data forms the basic prediction curve (Artursson *et al.*, 2001; Ungell and Karlsson, 2003; Artursson and Tavelin, 2003) used for settings of levels of absorption or a ranking between compounds, and for classification of permeability according to a Biopharmaceutics Classification System (BCS) (see Section 3.7.8 and Chapter 4.2). An example of such levels can be obtained in the literature (Ungell and Karlsson, 2003).

It should be noted that the Caco-2 cells are not passive membranes. A multiplicity of uptake and efflux transporters contributing to enhancing or attenuating transport of compounds, as well as enzymes, have been identified in these cells (Ungell, 2004).

The MDCK cell line is also frequently used by several pharmaceutical industries to measure intestinal drug transport despite its origin from dog kidney (Horio *et al.*, 1989; Irvine *et al.*, 1999; Artursson and Tavelin, 2003; Balimane and Chong, 2005), and the cells are generally thought to serve more as a passive membrane model than Caco-2 cells do (Ungell, 2004). MDCK cells have also replaced Caco-2 in many research organisations because the cell cultivation is only 3–7 days compared with 14–21 days for Caco-2 cells (Irvine *et al.*, 1999; Ungell, 2004). However, even though a good correlation between MDCK and Caco-2 cells has been obtained for many compounds (Irvine *et al.*, 1999), and studies indicate their usefulness, MDCK cells are not of human origin. Thus, the MDCK monolayers may be sufficient for estimating passive epithelial transport, but not for mechanistic studies or for predicting active uptake or efflux across the intestinal epithelium, since the influence of background expression or dog transporters may obscure data (Goh *et al.*, 2002).

Stably transfected cell lines are used nowadays to screen for transporter-mediated permeability, i.e. as either specific uptake, efflux or inhibitory effect on permeability (see also Chapters 3.3, 3.4, 3.5 and 3.6). MDCK-MDR1 is one of the stable transfected cell lines that are frequently used as a tool for investigating the influence of human P-glycoprotein (P-gp) on transepithelial transport (Horio *et al.*, 1989; Keogh and Kunta, 2002; for assay see later).

Other cell lines forming monolayers, such as the human colon goblet cell line HT-29 (and subclones), Caco-2 cell subclone TC7, the rat small intestinal cell line IEC-18, microvillus-expressing human colon carcinoma cells LS180, and the rat duodenal cell line 2/4/A1, have all been developed to investigate mainly intestinal drug transport (Artursson

and Tavelin, 2003; Ungell and Karlsson, 2003; Brandon *et al*., 2006). Most of these other cell lines can provide data for mechanistic information, but are too demanding of resources and sensitive to cell culture variables to be used routinely in drug screening in the industry. Cell lines for hepatic drug uptake and metabolism have also been developed to serve as a tool for biotransformation studies in conjunction with drug transport, e.g. HepG2 and IGROV-1 cells (Brandon *et al*., 2006).

3.7.3.0.1 Summary of cell culture-based models in late discovery

- Caco-2 cells for overall net transport in the intestine
- MDCK wild-type for passive permeability with low involvement of transporters
- Transfected cells for transporter-mediated permeation in all organs
- Other cell lines for identifying different transport routes, transporter involved and biotransformation.

3.7.3.1 Organ-based *ex vivo* models for drug transport

Although cell monolayers and transporter-transfected cell lines are offered as rapid tools to determine drug transport, the optimisation of pharmacokinetic properties in the later stages of drug discovery should be related to the transport mechanism(s) during disposition *in vivo*. Several more complex methods such as *in vitro* organ-based or *ex vivo* techniques have therefore been developed, e.g. vesicles, hepatocytes, sandwich cultures, intestinal or liver slices, intestinal sacs, Ussing chamber technique, intestinal or liver perfusions (Olinga *et al*., 1997, 2001; Akhteruzzaman *et al*., 1999; Balimane *et al*., 2000; Salphati *et al*., 2001; Ungell, 2002; Hoffmaster *et al*., 2004; Li, 2005; Sahi, 2005; Shitara *et al*., 2005; Zamek-Gliszcynski *et al*., 2006).

Vesicles can be isolated from both brushborder and basolateral membranes in the kidney tubular epithelial cells, and from intestinal enterocytes. Furthermore, vesicles can be obtained from the sinusoidal and bile canicular membranes in the liver isolated both from humans and preclinical species such as the rat and dog (Sahi, 2005; Shitara *et al*., 2005; Zamek-Gliszcynski *et al*., 2006). Vesicles are used for an in-depth evaluation of transporter interactions in general without the confounding influence of drug metabolism. Nowadays, vesicles can also be obtained as overexpressed transfected membranes (both mammalian and baculovirus-infected insect cells (sf9) with a specific transporter (www.Solvo.com). Usually, the uptake of a compound into the vesicles is studied at certain

concentrations and incubation times to obtain kinetic parameters (K_m and V_{max}) or, as in competition experiments, to determine an inhibitory effect on transport of a specific substrate. When efflux via transporters is being studied, ATP is added to the incubation media and transport into the inside-out vesicles can be studied (Sahi, 2005).

One disadvantage of vesicles is the day-to-day variation in vesicle preparation and a leakage of drugs from the vesicles during washing and filtration, which can affect drug accumulation. A relatively high level of expression of transporters in the material is needed to secure low variability in data (Sahi, 2005). In addition, transporter interaction for highly permeable compounds is hard to study due to the high passive (intrinsic) permeability (i.e background permeability). Despite these drawbacks, vesicles can be used in the late discovery stage for mechanistic studies of drug transport to identify the involvement of a specific transporter (Sahi, 2005; Shitara *et al*., 2005; Zamek-Gliszcynski *et al*., 2006).

Isolated and cultured hepatocytes have been used for a long time as an *in vitro* model of the liver (Shitara *et al*., 2005). Hepatocytes are isolated by liver perfusion of either an animal or human liver, followed by harvesting of live cells in the perfusate (Ishigami *et al*., 1995; Akhteruzzaman *et al*., 1999). Several studies indicate that both fresh and cryopreserved hepatocytes retain at least part of their viability and active transport and metabolism, as well as being inducible (Shitara *et al*., 2005). An effect on transporter expression of using different types of extracellular matrix and dexamethasone during isolation has also been reported (Luttringer *et al*., 2002). Uptake of compounds into the hepatocytes can be extrapolated to obtain *in vivo* clearance assuming a well-stirred model (Akhteruzzaman *et al*., 1999). However, since only the uptake of compounds, but not the efflux, can be studied using this method, data for CL predictions are not complete.

Instead of freshly harvested hepatocytes, cryopreserved hepatocytes can be used and these are also available commercially (www.bdbioscience.com). Hepatocytes can also be cultivated to form a multilayer structure called a sandwich culture, offering a better organ-like model for prediction of hepatobiliary disposition than fresh hepatocytes (Hoffmaster *et al*., 2004). This model is, however, very complex and requires very careful handling and is therefore not used in routine screening in the industry. The models representing the hepatic CL of drugs – hepatocytes, sandwich cultures, liver slices and liver perfusions – have also been proven to generally predict *in vivo* disposition of drugs and to explain drug–drug interactions in humans (Sahi, 2005; Zamek-Gliszcynski *et al*., 2006). However, care should be taken with

respect to species differences in the capacity and expression of transporters in these models that can affect predictions of actively transported compounds (Zamek-Gliszcynski *et al.*, 2006).

The intestinal sac method is a simple method experimentally, and is based on the preparation of a 2–3-cm-long tube of the intestinal part of interest, which is tied off at the ends after evertion on a glass rod (Barthe *et al.*, 1999; Ungell, 2002). Samples of fluid are taken from the buffer solution in the flask and also from the inner parts of the sac fluid. Several modifications have been made with this method since it was introduced in the 1950s, especially with regard to increasing the viability of the tissue (Barthe *et al.*, 1999). A clear advantage of this method is that in contrast to the Ussing chamber and cell culture models, it needs no specialised equipment and can easily be learned by the experimentalist. Even though this method clearly has a lot of practical advantages, the general usage in an industrial setting is low.

The main part of data presented using tissue slices concerns metabolism, and only a limited number of reports exist dealing with uptake and/or accumulation in slices of liver, kidney and/or intestine (Olinga *et al.*, 1997, 2001; De Kanter *et al.*, 2002; Sahi, 2005; van de Kerkhof *et al.*, 2006). The slice method is based on rapid excision of the tissue and cutting it into thin slices ($<100\,\mu m$), which are then incubated with the drug solution. A variant of the tissue slice technique, i.e. precision-cut slices, has also been reported in the literature, offering better viability of the tissue slices and, thus, more relevant metabolic activities (Olinga *et al.*, 1997, 2001; De Kanter *et al.*, 2002; van de Kerkhof *et al.*, 2006).

The Ussing chamber technique has been used for drug transport studies using excised intestinal tissues from different animals and humans and different regions of the GI tract (Ungell *et al.*, 1997; Polentarutti *et al.*, 1999; Wu-Pong *et al.*, 1999; Sjöström *et al.*, 2000; Ungell, 2002). The intestinal segments are quickly cut open into planar sheets, which may be stripped of the serosa and the muscle layers and then mounted between two diffusion half-cells (Polentarutti *et al.*, 1999). The integrity and viability of the tissue must be verified simultaneously when using this technique, because it will strongly impair transport of the drug molecules (Polentarutti *et al.*, 1999), and electrical values and markers for integrity have been suggested to ensure good predictive data (Ungell, 2002). Rat intestinal segments in Ussing chambers have been reported to correlate well to permeability coefficients of human jejunum *in vivo* (Ungell, 2002). Correctly used as a mechanistic low-capacity screening tool, data from the Ussing chamber technique

are well suited to predict the human fraction absorbed (Wu-Pong *et al*., 1999; Sjöström *et al*., 2000; Ungell, 2002).

Both isolated *ex vivo* intestine and *in situ* intestinal perfusions are known from the literature (Fagerholm *et al*., 1996; Lindahl *et al*., 1998; Balimane *et al*., 2000; Augustijns and Mols, 2004; Sahi, 2005). A small part of the intestine is cannulated at both ends and perfused with a buffer solution at a flow rate of approx 0.2 ml min^{-1} (Fagerholm *et al*., 1996). The blood side can also be cannulated and perfused with a separate perfusion system, or sampling from the blood side can be performed directly from a mesenteric vein. The permeability coefficient is calculated by the difference in concentration between given and collected fluid, and correction for intestinal fluid flow is made using polyethylene glycol (PEG) 4000 as a non-absorbable marker. Intestinal perfusion data from the rat correlate well with human permeability for passively transported compounds (Fagerholm *et al*., 1996). *In situ* perfusions using anaesthetised animals have also successfully been used for in-depth mechanistic studies of the efflux of drugs (Fagerholm *et al*., 1996; Lindahl *et al*., 1998; Augustijns and Mols, 2004).

The liver perfusion setup is similar to the intestinal one (Geng *et al*., 1995; Ishigami *et al*., 1995). The animal is anaesthetised and catheters are inserted in the portal vein for inflow and hepatic vein as outflow. The media perfusing the liver are variable in the literature and consist of an oxygenated Krebs–Henseleit solution buffered to pH 7.4 complemented with 20% washed red blood cells (RBCs), 1–4 % albumin, and 5–17 mmol/l of D-glucose (Geng *et al*., 1995). Usually, the liver is perfused in a single-pass fashion without recirculating the solution. The difference in drug concentration between inflow and outflow is used for calculation of drug transport (Geng *et al*., 1995).

The main disadvantage of all perfusion methods is the use of anaesthesia, which has been reported to affect drug transport (Ungell, 2002). The perfusion methods could have a better position in the industry since they reflect the complexity of organs of the body, but they are too time- and animal-consuming to fulfil the purpose of screening.

3.7.3.1.1 Summary of organ-based and ex vivo models

- Vesicles from different membranes and recombinantly expressed transporters in vesicles
- Hepatocytes, primary, fresh and cryopreserved
- Sandwich cultures

- Tissue slices and precision-cut slices
- Ussing chambers
- Intestinal and liver perfusions.

3.7.4 Optimising experimental conditions

Care should be taken with respect to suboptimal conditions for studying drug transport using any of the experimental models available. Several factors, such as pH and media compositions, will affect the outcome of data and, thus, influence interpretation especially with respect to intestinal absorption. A pH gradient of 6–6.5 on the apical side and pH 7.4 on the basolateral side has been recommended for intestinal absorption studies to obtain a more *in vivo*-like permeability value (Fallingborg *et al*., 1989; Yamashita *et al*., 2000; Neuhoff *et al*., 2003, 2005a). If the secretion (efflux) of a compound is studied, then a non-pH gradient system should be used to discard false predictions of efflux of weak bases (Neuhoff *et al*., 2003), and if active uptake is evaluated, e.g. for weak acids or for compounds taken up by proton-dependent mechanisms, then two different pH systems, one without and one with a pH gradient, should be used to obtain maximal information on passive and active drug transport (Neuhoff *et al*., 2005a). For liver or hepatocyte uptake studies, a non-pH gradient should be used for all studies mimicking drug transport in the systemic circulation (Geng *et al*., 1995). The measurement of plasma protein binding is important for full interpretation of drug transport *in vivo* (Neuhoff *et al*., 2005b). It has been recommended to use BSA/HSA (bovine serum albumin/human serum albumin) to increase the sink condition in *in vitro* systems and, thus, mimic the *in vivo* blood sink (Geng *et al*., 1995; Neuhoff *et al*., 2005b). Indeed, highly protein-bound compounds will be affected by the addition of protein on the basolateral side, hence, both transport in the absorptive direction metabolism and the efflux of compounds may change (Neuhoff *et al*., 2005b).

Many compounds being researched today are sparingly soluble. This affects not only the data quality and recovery (mass balance) using *in vitro* methods, but also the *in vitro* and *in vivo* correlation (Yamashita *et al*., 2000; Krishna *et al*., 2001; Ungell and Karlsson, 2003). Different *in vitro* models have been used for evaluation of experimental solvents in *in vitro* experiments, and several systems, e.g. surfactants or biorelevant media, have been proposed (Ingels and Augustijns, 2003; Ungell, 2005; Ingels *et al*., 2006). Solvents used for intestinal studies have been more evaluated than the solvents for hepatic models. General caution should be

taken with respect to the possible effect of solvent systems on intracellular enzymes and on transporter function before using them during screening for drug–drug transport (e.g. inhibition of P-gp; Nerurkar *et al.*, 1996; Bogman *et al.*, 2005).

Due to the increased number of highly lipophilic compounds emerging from early drug discovery and difficulties with adsorption to plastics, a mass balance or recovery calculation has to be performed to ensure good and consistent data quality. An example of such an approach can be found in Ingels *et al.* (2004).

3.7.4.0.1 Summary of optimised conditions

- A pH 6.5/7.4 system is recommended for absorptive studies
- A pH 7.4/7.4 revealing active transport that is not pH dependent
- Combination of the above pH conditions for active drug uptake or secretion which is pH dependent
- Use BSA on the blood plasma side to mimic *in vivo* conditions for highly protein-bound compounds
- Use solvents to increase solubility with care
- Always check for recovery.

3.7.5 Screening for transporter interaction

Transporter proteins may potentially mediate drug–drug interactions in the clinic, and are therefore regularly evaluated during drug discovery, e.g. using MDCK-MDR1 and other transfected cell lines, Caco-2 bidirectional transport assays, uptake into vesicles with recombinantly expressed transporters, etc. One of the most frequently used models for active transport in the preclinical setting is bidirectional transport across a monolayer, which provides information around asymmetry of drug transport at a given concentration, and an efflux ratio (ER) ($B \rightarrow A/A \rightarrow B$) or uptake ratio (UR)($A \rightarrow /B \rightarrow A$) is calculated (see Figure 3.7.4). An asymmetry over the membrane of approx $ER > 2$ indicates that the compound is secreted into the apical compartment by the cells (Polli *et al.*, 2001) and similarily, a $UR < 0.5$ indicates uptake. These ratios can therefore give valuable information for interpretation of compound pharmacokinetics *in vivo*. However, the ratios (ER or UR) are concentration dependent and also dependent on the test system used, and should also be evaluated in relation to passive transport of the compound (Hochman *et al.*, 2002). Troutman and co-workers (Troutman and Thakker, 2003a) have suggested calculating a ratio between the net efflux component (difference between $A \rightarrow B$ and $B \rightarrow A$ normalised to $A \rightarrow B$) called

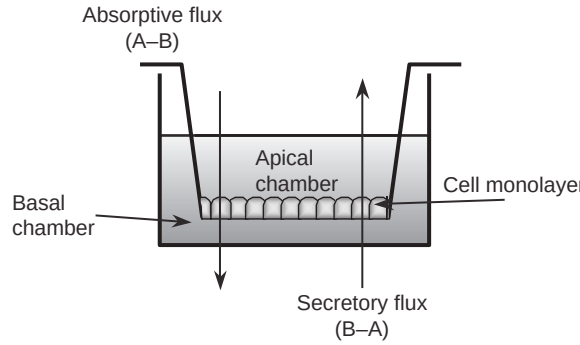

Figure 3.7.4 An experimental setup using monolayers of cells. Cells are cultivated on filters in the plastic inserts. During the experiment, transport buffer solution is added to both the apical and basolateral side and drug to be tested on one side. The sampling of the solution on the receiver side (opposite the side where the drug is added) is made at predetermined time intervals, and the permeability coefficient, P_{app}, can be calculated.

absorption quotient (AQ), or secretion quotient (SQ), depending on which of the two transport directions are exceeding the other (Equations 3.7.1 and 3.7.2).

$$AQ = (A \rightarrow B) - (B \rightarrow A)/(A \rightarrow B) \quad (3.7.1)$$

$$SQ = (B \rightarrow A) - (A \rightarrow B)/(B \rightarrow A) \quad (3.7.2)$$

These parameters can be enough for a screening mode and will guide the chemist to an overall change in the molecular property of the drug candidate with respect to passive and active components, whereas the exact identity of the transporter/s involved is not always needed.

In some industries, the involvement of P-gp (*MDR1* gene product) is evaluated relatively early in drug discovery. The reason for the interest in P-gp is that in numerous clinical investigations possible interaction between drugs and P-gp seems to influence the kinetics of these drugs (Keogh and Kunta, 2006). The method used is bidirectional studies in MDCK-MDR1, and asymmetry is calculated as ER (see above). This asymmetry in permeability using MDCK-MDR1 cells (see Figure 3.7.4) can be corrected using wild-type (wt) MDCK cells (lacking human P-gp), e.g. $ER_{corr} = ER_{MDR1}/ER_{wt}$, to obtain a more clear human P-gp interaction value. Important knowledge using this correction is that the expression of transporters in a non-transfected cell line of non-human origin used as background cell (i.e. MDCKwt) might be different from the transformed cell, resulting in substrate-specificity differences and a risk of misinterpretation (Goh *et al.*, 2002). Bidirectional transport in cell

lines can also be studied in combination with the use of inhibitors to specific transporters, thus providing a first and rough insight in which a transporter/family of transporters is/are responsible for the unidirectional or bi-directional transport and drug–drug interactions (Troutman and Thakker, 2003b; Keogh and Kunta, 2006; Rautio *et al.*, 2006). However, since many inhibitors and substrates to transporters show overlapping specificities, the interpretation of the data is complex. The relative contribution of each transporter to the overall transport of the compound is also complex and remains difficult to quantify. Therefore, data obtained from such *in vitro* studies are mainly used as rough guidance in designing new chemical entities in drug discovery.

Decision trees for identifying P-gp substrates can be found in the literature (Zhang *et al.*, 2006), and an example of a decision tree is shown in Figure 3.7.5 for a general screening for evaluation of the involvement of transporters and metabolism using several different techniques.

Transporter inhibition data (P-gp) are currently also evaluated during preclinical evaluation due to an increased number of reports showing raised and potentially toxic levels of compounds after concomitant administration with other therapeutic agents (Fromm *et al.*, 1999;

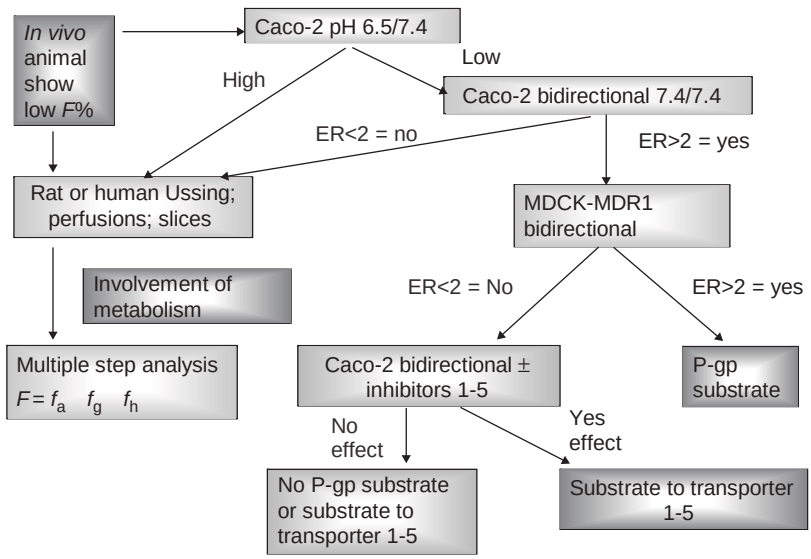

Figure 3.7.5 Decision tree for evaluation of drug transport across GI membranes using several preclinical models. After each method is used, yes or no indicates the new direction and a new model is suggested. Such a decision tree can be made for each evaluation. *F*, bioavailability; f_a, fraction absorbed; f_g, fraction escaping gut metabolism; f_h, fraction escaping metabolism/extraction in the liver.

Sadeque *et al*., 2000; Englund *et al*., 2004). A preclinical technique that can be used for evaluation of P-gp inhibition is the taxol or digoxin transport inhibition assay (Gao *et al*., 2001; Keogh and Kunta, 2006; Rautio *et al*., 2006). A similar decision tree for evaluation of potential inhibitors to P-gp to the one for identifying P-gp substrates has also been proposed recently (Zhang *et al*., 2006).

3.7.5.0.1 Summary of screening using tools

- Caco-2 bidirectional studies and calculation of ER and UR and/or AQ and SQ
- MDCK-MDR1 bidirectional studies to identify P-gp substrates
- Caco-2 bidirectional studies in the absence and presence of inhibitors to identify involvement of a transporter or transporter family
- Caco-2 or MDCK-MDR1 cells for estimating P-gp inhibition to aid design of drug–drug interaction studies in the clinic.

3.7.6 Influence of metabolism during transport

Cytochrome P450 isoforms, such as CYP3A4, CYP2D6, 2C19 and 2C9, are involved in the biotransformation of lipophilic compounds of both endogenous and exogenous origin in the intestine and liver (Paine *et al*., 2006; Daly, 2006). The main CYP450 isoforms in the intestine can vary between animal species and the intestinal region. In addition, phase II enzymes, the conjugating enzymes uridine diphosphate glucuronosyl transferases (UGTs) and glutathione-*S*-transferases (GSTs) and sulphotransferases (SULTs) are also relevant for inactivation of drugs and either may influence the data from the different *in vitro* techniques or should be taken into account for *in vivo* interpretation (Zamek-Gliszcynski *et al*., 2006). The clinical relevance of intestinal metabolism during absorption of drugs has often been debated (Groothuis, 2005), and the interest in relevant models for studying the influence of metabolism during drug transport has increased.

Using the Ussing chamber system with rat and human intestinal segments, the use of either radiolabelled or cold testosterone resulted in different permeability values (Ungell, 2005). When radiolabelled testosterone was used, high apparent permeability values (P_{app}) were obtained. However, when unlabelled testosterone and ultraviolet (UV) detection were used to analyse the parent compound, the values of permeability were less than one-tenth of that obtained as total transport in both rat and human intestine (Ungell, 2005). Testosterone is assumed to be completely absorbed in humans. However, since the intestine significantly

contributes to extraction of the compound before it enters the liver, the number of intact testosterone molecules reaching the systemic circulation is probably less than complete. Therefore, care should be taken to extrapolate transport values to humans if the compound is metabolically unstable. Metabolic activity in the liver hepatocytes may also obscure total transport similarly to the results obtained in the Ussing chamber. Indeed, there are now several publications showing direct interaction between metabolism and efflux of metabolites via several transporters (Zamek-Gliszcynski *et al.*, 2006; Shitara *et al.*, 2006; Kusuhara and Sugiyama, 2002; Suzuki and Sugiyama, 2000; Benet *et al.*, 2004; Jeong *et al.*, 2005). This means that careful monitoring of the content of both parent compound and metabolites in different compartments of the *in vitro* methodology is needed for correct interpretation of data.

CYP450 3A4, which is the most important CYP isoform in the human intestine, is absent or at a low level in the parent clone of the Caco-2 cell (Ungell and Karlsson, 2003). This can explain the low predictability of permeability obtained by this model for drugs that are substrates to this enzyme family and may, thus, overestimate the extent of absorption *in vivo*. Caco-2 cell monolayers have also been used to evaluate the importance of ester hydrolysis of prodrugs in parallel with transport (Narawane *et al.*, 1993; Augustijns *et al.*, 1998). However, carboxyesterase activity within the Caco-2 cell line seems to be more liver than intestine specific (Imai *et al.*, 2005). This indicates that degradation of ester bonds during transport studies using the Caco-2 cell model can overestimate the involvement of intestinal esterases, especially when studying ester link-based prodrug activation.

3.7.6.0.1 Summary of influence of metabolism on drug transport

- The presence of enzymes will affect data interpretation
- Species differences in enzymes and transporters exist
- Model differences in presence of enzymes and transporters exist
- Metabolites can be substrates to transporters.

3.7.7 Use of preclinical models for prediction of drug transport in humans

Regionally, within the gastrointestinal tract, both the properties of the intestinal membrane, i.e. lipid composition, surface area, protein content of transporters and enzymes, as well as the components and pH of the

intestinal lumenal fluid change (Neuhoff *et al*., 1989; Artursson *et al*., 1993; Narawane *et al*., 1993; Erickson *et al*., 1995; Homsy *et al*., 1995; Ungell *et al*., 1997; Makhey *et al*., 1998; Ungell, 2002; Seithel *et al*., 2006; Zimmermann *et al*., 2005). Data on functional studies for CL in isolated perfused livers have also suggested zone-dependent metabolism, especially phase II conjugation favouring the peripostal region of the liver Zamek-Gliszcynski *et al*., 2006). Hence, the transport (and metabolism) characteristics of a drug may change by region, a process that is very difficult to mimic in a static *in vitro* system (Homsy *et al*., 1995). This illustrates the importance of having more complex organ-based models for better prediction of drug transport *in vivo*.

Selection of a drug candidate for development (see Figure 3.7.1) is usually based on both *in vivo* and *in vitro* ADME data in relation to the *in vivo* efficacy of the drug. In general, the most frequently used data for prediction of bioavailability (F) in man is the *in vivo* hepatic clearance (CL_h) data from animals (Rowland and Tozer, 1980; Poggesi, 2004) (see Figure 3.7.6).

F in humans is estimated from allometrically scaled CL_h and hepatic blood flow (Q_h) from *in vivo* studies using two to three animals and assuming complete absorption (i.e. $f_a = 1$) and no intestinal loss of compound (e.g. $f_g = 1$) (according to Equations 3.7.3–3.7.5 (Rowland and Tozer, 1980; Poggesi, 2004):

$$F = f_a \times f_g \times f_h \tag{3.7.3}$$

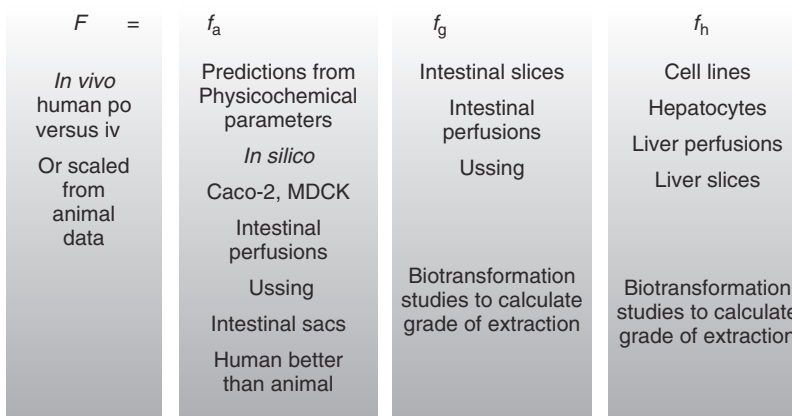

Figure 3.7.6 Models for prediction of bioavailability in humans. po, oral; iv, intravenous; F, bioavailability; f_a, fraction absorbed; f_g, fraction escaping gut metabolism; f_h, fraction escaping metabolism/extraction in the liver.

or:

$$F = f_a \times (1 - CL_h/Q_h) \quad (3.7.4)$$

or simply:

$$F = (1 - CL_h/Q_h) \quad (3.7.5)$$

However, an accurate prediction of human drug absorption based only on animal *in vivo* data might not be successful, since a variation in expression of transporters and enzymes between animal species exists. An alternative approach has been discussed (Ungell, 2005) and proposes to use predicted f_a obtained from Caco-2 cells and/or human intestinal segments (or other reliable and validated human *ex vivo* tools) combined with the allometrically scaled human CL (obtained from *in vivo* animal studies or *ex vivo* human hepatic models) according to Equation 3.7.4, for a better estimate of human *F* value. This suggestion assumes that $f_g = 1$, but that f_a can influence the estimate from *in vivo* scaled *F*. Thus, the use of predicted f_a values from human-relevant *in vitro* assays to support *in vivo* animal data would by this rationale probably give better confidence in the selection of clinical candidates (Li, 2005) (see Figure 3.7.1). However, if gut extraction is assumed to be substantial, none of the above models will correctly predict *F* in humans, and corrections for intestinal extraction need to be performed.

At this stage of drug discovery, the development of potential clinical formulations also starts and the predicted values for human drug transport will be used as information to guide the formulation development. To aid the development of a specific formulation, drug permeability (and solubility) data can also be used for prediction of human plasma profiles using commercially available tools such as GastroPlus™ (Parrott and Lave, 2002; Kuentz *et al.*, 2006).

3.7.7.0.1 Summary of prediction of drug transport in humans

- *In vitro* data from humanised drug transport systems to be used to support *in vivo* animal data to obtain better predictions of human drug transport and bioavailability.

3.7.8 The Biopharmaceutics Classification System

The Biopharmaceutics Classification System (BCS) was developed to aid interpretation of solubility and permeability data for formulation development (Amidon *et al.*, 1995; FDA, Center for Drug Evaluation and

Research (2000); Yu *et al.*, 2002). BCS classifications are generally used to bridge between different versions of clinical formulations of a marketed product or clinical material in phase III, to reduce the need for bioequivalence studies (Yu *et al.*, 2002; Polli *et al.*, 2004). So far this guidance is helping the biowaiver to identify the class I drugs used in immediate-release formulations.

Four different classes of drugs are used: high permeability/high solubility (class I), high permeability/low solubility (class II), low permeability/high solubility (class III) and low permeability/low solubility (class IV) (see Chapter 4.2) (Ungell and Abrahamsson, 2001; Yu *et al.*, 2002; Polli *et al.*, 2004). Since the introduction of this classification system, its validity and applicability have been the subject of extensive research and debate (Yu *et al.*, 2002; Polli *et al.*, 2004). Opinions have been forwarded concerning the boundaries and criteria to be fulfilled for the different classes as well as suggested standard compounds. Two variants of this BCS system have been proposed in the literature; a six-class system (Bergström *et al.*, 2003), based on molecular surface properties of drugs, and a Biopharmaceutics Drug Disposition Classification System (BDDCS) that takes into account both metabolism and transporters (Wu and Benet, 2005).

Classification of drug compounds following these guidelines requires accurate and thoroughly validated models to assess permeability and solubility. If the model is not good enough, or if the validation of the technique is not properly made, the classification of new chemical entities can be inaccurate and misleading. The original guidance proposes several types of methods to be used: pharmacokinetics studies using human subjects for evaluation of fraction absorbed using mass balance, absolute bioavailability or intestinal perfusion approaches; or intestinal perfusion models using the rat or even cell models such as the Caco-2 model. If an *in vitro* system will be used, each laboratory is recommended to use a number of reference molecules with known clinical f_a for validation of the permeability properties of their model, to set boundaries of low and high permeability, and to show the presence of active transport. When the *in vitro* model is carefully validated, it can also be used earlier in the screening as a prediction curve for f_a in humans (Ungell and Karlsson, 2003).

3.7.8.0.1 Summary of BCS classification

- BCS needs a thorough validation of the *in vitro* technique
- A set of 10–20 reference compounds with known f_a in humans is needed to set boundaries between classes.

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