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Current Methodologies for Membrane Permeability Assessment

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ABSTRACT – Orally administrated drugs permeate the biological membrane by various transport mechanisms. The oral absorption potential is closely related to the physicochemical properties of the drug and interaction with the physiological factors surrounding the site of absorption. Assessment of the drug membrane permeability is an integral part of the early stage drug developmental process. Appropriate selection of the permeability screening method at the right stage of drug development process is important in achieving successful developmental outcomes. This review aims at introducing currently available in vitro and *in vivo* screening methods for the membrane permeability assessment.

Key words – Membrane permeability, absorption, Caco-2, PAMPA, intestinal perfusion.

The drug discovery is an expensive and time consuming process with high-risk of failure (DiMasi et al., 1994; Kaitin and Healy, 2000; Kaitin et al., 2000). To become the successful drug candidate, good biological properties such as potency, selectivity, efficacy and oral bioavailability are required. Traditionally, drug discovery groups have focused primarily on the pharmacological aspects including potency and efficacy, without much attention given to the importance of the pharmacokinetic characterization in the early stage of drug development. The proportion of failure of investigational new drug (IND) applications has been estimated approximately 87% in Phase I, 60% in Phase II, and 20% in Phase III clinical studies, and only one compound is released to the market out of 9,000-10,000 newly synthesized compound (Kennedy, 1997). The primary reason for this high failure rate over the past 20 years was in part due to the pharmacokinetic inadequacy including low aqueous solubility, inadequate intestinal permeability, low chemical stability, intestinal or hepatic metabolism, and systemic clearance, although more attention is being given to the pharmacokinetic characterization in recent years and the failure rate is decreasing (Ganta et al., 2008). Owing to the combinatorial chemistry and automated high throughput screening, it is common to screen 100,000 compounds in a 1- to 6-month period (Broach and Thorner, 1996; Silverman et al., 1998), and this makes it possible to generate thousands of therapeutically active compounds in a short time period. However, the number of drugs released on the market has not been increased. Thus, in recent drug discovery, evaluation of the pharmacokinetic properties is being performed in the early stage of drug discovery. It provides useful information to design optimal pharmaceutical preparations without wasting valuable resource and time (Ganta et al., 2008).

Among various innovative drug delivery systems, the most preferred route of administration is still the oral route due to the ease of administration and high patient compliance. Oral bioavailability is the most crucial factor for molecules that are intended for oral administration. Oral bioavailability of a molecule is influenced by multiple factors, including solubility, permeability and intestinal and liver metabolism. There are various techniques used to assess the gastrointestinal absorption potential of new drug candidates. This article will focus on the permeability evaluation methods that are currently utilized in the drug development process.

Drug transfer through physiological membrane

The human small intestine is divided by duodenum, jejunum, and ileum and the approximate total length is 2-6 m. The major role of the small intestine is the barrier function to ingested foreign substances and absorption of nutrients and drugs. Approximately 90% of total absorption occurs in the small intestine (Balimene et al., 2000). The enterocytes, the major cells of the small intestine, act as the intestinal barrier.

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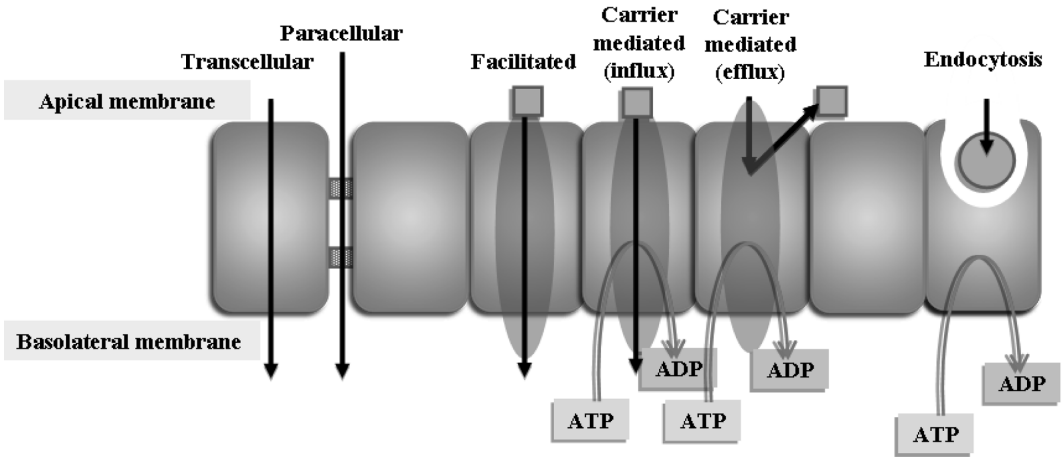

Figure 1. Routes of drug permeation in the gastrointestinal tract.

Other than enterocytes, the small intestine is consisted of various cells such as globlet cells, endrocrine cells, paneth cells, M cells, tuft and cup cells (Carr and Toner, 1984; Madara and Trier, 1987). The structure of the cellular membrane is composed of the phospholipid bilayer, with each enterocyte separated by negatively charged tight junction. The majority of lipophilic drugs are absorbed by passive diffusion through the cell membrane, which is called the transcellular transport (Camenisch et al., 1996). In contrast, the hydrophilic and small (MW <200) compound passes through the water-filled tight junction formed by fusion of adjacent cells (paracellular transport) (Conradi et al., 1996). If the drug is a substrate for intestinal transporters, its movement is significantly affected by these transporters. In active transport, drug molecules are transported against the concentration gradient by consuming energy (Hunter et al., 1993a). However, in facilitated transport, drug molecules are transported to the concentration gradient direction without consuming energy. Among various intestinal transports, P-glycoprotein (P-gp) is the most typical efflux transporter located in the apical side of membrane. (Thiebaud et al., 1987; Croop et al., 1989; Burton et al., 1993; Benet et al., 1996; Hidalgo and Li, 1996; Tsuji and Tamai, 1996; Sandstrom et al., 1998). High molecular weight compounds such as peptides and proteins tend to be transported by endocytosis (Hunter et al., 1993b). The various drug transport processes described above are summarized in Figure 1.

Physicochemical drug properties affecting intestinal permeability

The major physicochemical properties of a chemical that influence the passive intestinal permeability are the lipophilicity, molecular weight, hydrogen bonding, pKa, and solubility. A number of review articles have reported the relationship between the molecular properties and the intestinal permeability (Kramer, 1991; Clark and Pickett, 2000; Stenberg et al., 2000; Van de Waterbeemd, 2000; Clark, 2001; Van de Waterbeemd et al., 2001). The most widely used model describing the relationship between the molecular properties and the intestinal permeability was introduced by Lipinski et al. (1997) which is called the "rule of 5 model". This model states that a poor absorption or permeation is likely to occur when the calculated 1) hydrogen bond (H-bond) donors are more than five, 2) H-bond acceptors are more than ten, 3) the MW is over 500, and 4) log P is over five. When the physicochemical properties of a chemical meet more than two of these criteria, poor membrane permeability is to be anticipated. This model is simple and easy to interpret and fast to compute. However, this model is often of a limited scope as it does not take into account the physiological factors including the membrane transporters. Thus, substrates for the biological transporters become exception to the rule of five. The factor of the rule of five can be predicted by the computational method and these factors will be discussed below.

Lipophilicity

It has long been recognized that the lipophilicity is an important factor determining the membrane permeability (Leo et al., 1971; Hansch and Dunn, 1972; Hansch and Clayton, 1973). An adequate lipophilicity is essential for cell membrane permeation as it provides affinity between a drug molecule and the phospholipid membrane structure (Kramer, 1999). In the Fick's first law, the membrane permeability is proportionally increased as the membrane-water partition coefficient (K) increased. The cell membrane is composed of phospholipid bilayers that are both hydrophilic and lipophilic in nature. Thus, the relationship between lipophilicity and permeability is

nonlinear from low to high lipophilicity. These nonlinearities are due to a limited diffusion of less lipophilic molecules and an entrapping at the lipophilic portion of the phospholipid bilayer of high lipophilic molecule, preventing movement through the aqueous portion of the bilayer membrane (Hansch and Clayton, 1973; Martin, 1981; Gobas et al., 1988; Kararli, 1989; Dearden, 1990; Wils et al., 1994). Lipophilicity is conventionally expressed as the octanol/water partition coefficient (log P) or the distribution coefficient (log D) which is a pH dependent term of ionizable drugs. The log P and log D are determined by the shake flask method. In this method, the sample is dissolved in a flask containing *n*-octanol-water biphasic system and the flask is shaken until equilibrium is achieved between phases. Then, the phase is separated and the sample concentration and the pH of aqueous phase are measured. Finally, log P and log D are calculated by the following equations (Comer, 2003):

$$P = \frac{[\text{solute}]_{\text{octanol}}}{[\text{solute}]_{\text{watar}}}$$

$$D = \frac{[\text{unionized+ionized solute}]_{\text{octanol}}}{[\text{unionized+ionized solute}]_{\text{water}}}$$

The log P and log D can also be determined by computational approaches. These approaches allow the calculation of the log P values with incorporation data such as atomic contribution or molecular fragment or molecular properties including molecular lipophilicity potential (MLP) (Testa et al., 1996), molar volume, and hydrogen bonding (Ganta et al., 2008). These computational approaches are summarized in Table I (Ganta et al., 2008).

Hydrogen bonding (H-bonding)

Hydrogen bonding capacity is a major component of the lipophilicity along with the molecular size (Van de Waterbeem and Testa, 1987; Tayar et al., 1992). Various studies have reported a correlation between the hydrogen bonding capacity and the membrane permeability (Van de Waterbeemd et al., 1996; Dearden and Ghafourian, 1999; Raevsky and Schaper, 1998; Raevsky et al., 2000). Hydrogen bonding can be measured experimentally from the difference between octanol/water and alkane/water partitioning (Δlog p). However, the measurement of log p is a tedious experiment since octanol is miscible with alkane and the compound distribution of these two solvents cannot be measured simultaneously. As a more convenient measurement, the hydrogen bonding capacity may be calculated by counting the number of hydrogen bond donor atoms (hydrogen) attached to oxygen and nitrogen and hydrogen bond acceptor atoms (oxygen and nitrogen) (Lipinski et al., 1997). The hydrogen bond capacity may also be calculated by using the polar surface area (PSA) which measures the PSA of all N- and O-atoms (Van de Waterbeemd and Kansy., 1992). The relationship between the oral absorption and the polar surface area can be described by the following equation:

$$A\% = 100 / \left[1 + \left(\frac{PSA}{PSA_{50}} \right)^{\gamma} \right]$$

where A% represents the percentage of orally absorbed drug and PAS_{50} represents the PSA at 50% absorption level.

Commonly, poorly absorbed compounds have high polar surface areas (>140 $Å^2$) while a complete oral absorption may

Table I. *Computer Software Use in the Calculation of Log P*

Name	Software Package/Vendor	Web site
PrologP and Prolog P	PALLAS/CompuDrug Chemistry Ltd.	http://www.compudrug.com/
ACD/logP and logD	Verson 3.0/Advanced Chemistry Development Inc.	http://www.acdlabs.com/home/
CLOGP	Pcmodels/Daylight CIS, CLOGP/Biobyte OSIRIS Property Explorer/ Thomas Sander	http://www.daylight.com/ http://www.biobyte.com/ http://www.organic-chemistry.org/prog/peo/
HINT/logP	Edusoft	http://www.edusoft-lc.com/
KLOGP	Multicase Inc.	http://www.multicase.com/
LOGKOW	Syracuse Research Corp.	http://www.syrres.com/
SCILOGP	SCILOGP/Scivision	http://www.scivision.com/
TLOGP	TLOGP 1.0/Upstream Solutions	http://www.upstream.ch
XLOGP	XLOGP V 2.0/Insitute of Physical Chemistry, Peking University	http://www.ics.uci.edu/~dock/manuals/xlogp2.1/usage.html#subject_1
ALOGPS	ALOGPS 2.1/Virtual Computational Chemistry Laboratory	http://www.vcclab.org/

be anticipated when the polar surface area is less than 60 $Å^2$ (Kelder et al., 1997). Recently, a more accurate method for measuring the polar surface area has been introduced based on quantum mechanism and Hybot (Raevsky et al., 1992). In case the intestinal absorption is affected by active transporters and the gut wall metabolism, however, the relationship between the oral absorption and the polar surface area may be broken.

Molecular size

Molecular size is the component of lipophilicity and diffusion coefficient (log D) in physiological membrane permeation (Ganta et al., 2008). The transcellular diffusion in the physiological membrane is strongly affected by the molecular size of compound. According to the Lipinski's rule of 5 model, the upper limit of the molecular weight for oral absorption is 500 Da (Lipinski et al., 1997). The most commonly used descriptor for molecular size is the molecular weight. However, this may not be sufficient since the molecular weight contains no three-dimensional information.

In vitro Intestinal Permeability Test

Parallel artificial membrane permeability assay (PAMPA)

PAMPA was initially suggested by Mueller et al (1962). They reported that a small amount of phospholipids (2% w/v alkane solution) placed over a 0.5 mm small hole in a thin (10-20 μm) sheet of Teflon or polyethylene made a thin film at the center of hole. Such membrane has been shown to be a useful predictor of human drug absorption. However, this lipid membrane has weaknesses as it is fragile and hard to prepare. After this initial finding, various efforts have been made to overcome drawbacks of the artificial membrane permeability assay (Thompson et al., 1982; Cools and Janssen, 1983; Camenisch et al., 1998; Kansy et al., 2001; Avdeef, 2001a; Avdeef et al., 2001b; Faller and Wohnsland, 2001). Recently, PAMPA became a popular industry tool for high throughput permeability screening in early drug discovery due to its high efficacy, reproducibility, and low cost (Lipinski, 2002). PAMPA is run on a 96 microtiter well plate consisting of top and bottom parts. The top part contains a series of filter. One half of the top part is treated with a dispersed phospholipid on an organic solvent, which supposedly acts as the cell membrane and the other part is wet with methanol/buffer. The bottom part which matches with the well on top part is filled with buffer. The various PAMPA membrane compositions are summarized in Table II (Avdeef, 2003). The extent of drug permeation is determined by measuring the permeation rate of the test compound from the top to the bottom part. The membrane permeability of PAMPA is highly correlated with that of the Caco-2 system for passively diffused drugs (Camenisch et al., 1997). A good correlation is also found between PAMPA and the human absorption for selected passively transported compounds (Lipinski et al., 1997). Thus, PAMPA is highlighted as an alternative method to cell culture system for predicting passive permeability (Kansy et al., 1998). The limitation of this system is the lack of permeability prediction for the compound that is transported by paracellular pores and carrier proteins. Thus, PAMPA should be used with caution for human oral absorption screening, especially for the carrier-mediated drugs.

Immobilized artificial membrane (IAM) chromatographic assay

The IAM chromatographic assay utilizes the solute interaction with amphiphilic phospholipids covalently bonded to aminopropyl silica particles (Pidgeon et al., 1995; Ong et al., 1996; Taillardat et al., 2003; Yang et al., 1996; Caldwell et al., 1998; Bertschinger et al., 2003). The amphiphilic phospho-

Table II. *Membrane Compositions of Various PAMPA Models*

Model	Lipid Composition	References
Egg Lecithin PAMPA Model (Roche Model)	10% wt/vol dodecane solution of egg lecithin	Kansy et al., 1998 and 2001
Hexadecane PAMPA Model (Novartis Model)	Hexadecane without phospholipid	Faller and Wohnsland, 2001; Wohnsland and Faller, 2001
Brush-Border Lipid Membrane (BBLM) PAMPA Model (Chugai Model)	3% wt/vol lipid solution in 1,7-octadiene (lipid consisting of 33% wt/wt cholesterol, 27% PC, 27% PE, 7% PS, 7% PI)	Sugano et al., 2001a and 2001b
Hydrophilic Filter Membrane PAMPA Model (Aventis Model)	Egg lecithin, 1% wt/vol in dodecane	Zhu et al., 2002
Permeability–Retention–Gradient–Sink PAMPA Models (pION Models)	2% wt/vol DOPC in dodecane	Avdeef, 2001 and 2003; Avdeef and Testa, 2002

PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PS, phosphadityl serine; PI, phosphatidylinositol; DOPC, dioleoylphosphatidylcholine
Summarized form Avdeef, 2003

lipids on silica particles in column work as the cell membrane, and the membrane permeability is predicted from the chromatographic affinity as represented by the IAM capacity factor (K_{IAM}.).

$$K_{IAM} = \left[\frac{t_r - t_0}{t_0}\right]$$

where t_r and t_0 are the retention times of drug and hold up time of the column, respectively. The IAM chromatography is a simple and potentially useful method for screening a large number of compounds. Reasonable correlation have been reported between IAM capacity factor, K_{IAM} with log P (Yang et al., 1996), intestinal drug absorption in mice (Ong et al., 1996; Yang et al., 1996) and rats (Pidgeon et al., 1995; Yang et al., 1996; Genty et al., 2001), human skin permeation (Yang et al., 1996) and Caco-2 cell permeation (Pidgeon et al., 1995; Ong et al., 1996). Recently, Yoon et al. (2004) reported an improved IAM chromatographic application by using a modified IAM capacity factor corrected by the power of the compound molecular weight (K_{IAM} /MW^n). Nevertheless, the IAM capacity factor is governed mostly by the lipophilicity of the compound and, therefore, the IAM chromatographic assay have limitations in screening the permeability of compounds that are small and hydrophilic or transported by membrane transporters (Balimane et al., 2000).

Cell-based systems

Cell culture-based models are widely used to study the drug absorption potential and transporter or enzyme interactions. Currently available cell-based permeability models are summarized in Table III (Ganta et al., 2008; Ungell et al., 2003). Of these, Caco-2 and MDCK (Madin–Darby canine kidney) cell systems are most commonly used. These systems are well standardized and can reflect various absorption mechanisms occurring in the gastro-intestinal tract such as transporter and enzyme interactions. These cell-based models have been widely utilized in the early stage drug development processes and the carrier-mediated permeability assessments (Taylor et al., 1997; Stevenson et al., 1999).

Table III. *Cell Culture Models Commonly Used for Permeability Assessment*

Cells	Tissue Origin	Comments
Caco-2	Human colon adenocarcinoma	Well known and widely used; transporters and enzymes are available
MDCK	Dog kidney epithelial cells	Low level of P-gp
LLC-PK1	Pig kidney epithelial cells	Low level of transporters
HT-29	Human colon	Differentiated polarized monolayer with mucus-secreting and/or absorptive cells
TC-7	Subclone of Caco-2	Showing CYP 3A5 activity

Summarized form Ganta et al., 2008 and Ungell et al., 2003
Figure legend

Caco-2

The Caco-2 cell system is one of the most popular and well characterized tools used in drug absorption screening (Hidalgo et al., 1989; Artursson, 1991; Artursson et al., 1994; Borchardt, 1995; Artursson et al., 1996; Rubas et al., 1996). The Caco-2 cells originate from a human colon adenocarcinoma (Fogh et al., 1977), thus they exhibit various intestinal cell properties (Ganta et al., 2008). The major advantages of Caco-2 cells over other simplified artificial systems are in the similarity with the actual intestinal tract. Caco-2 cells have intercellular tight junctions and express numerous brush-border enzymes that can be found in the normal small intestine, including alkaline phosphatase, sucrase and amino peptidases (Pinto et al., 1983; Hauri et al., 1985; Chantret et al., 1988; Howell et al., 1992). In addition, cytochrome P450 isoenzymes (CYP450) and some phase II enzymes (e.g., glutathione-S-transferases, sulfotransferase and glucuronidase) have been identified (Baranczyk-Kuzma et al., 1991; Bjorge et al., 1991; Howell et al., 1992; Carrie're et al., 2001). Levels of the CYP expressions are, however, lower than those of the native intestinal cells (Pruesaritanont et al., 1996). Several influx and efflux transports have been characterized in the Caco-2 cell model (Hidalgo and Li, 1996). The group of OCT, OAT influx transports for glucose (Blais et al., 1987; Riley et al., 1991), amino acids (Hidalgo and Borchardt, 1990; Hu and Borchardt, 1992; Nicklin et al., 1992), dipeptides (Brandsch et al., 1994; Ganapathy et al., 1995; Thwaites et al., 1995), vitamins (Dix et al., 1990), and bile acids (Hidalgo and Borchardt, 1990; Wilson et al., 1990) as well as the efflux transports including MDR-1, P-glycoprotein (P-gp) (Hunter and Hirst, 1997), BCRP (Taipalensuu et al., 2001), and the multidrug resistance-related (associated) protein (MRPs) family (Kool et al., 1997; Stephens et al., 2001; Taipalensuu et al., 2001) have been identified in the Caco-2 cells. Due to the similarity with the actual intestinal tract, Caco-2 cells are considered the standard technique for predicting the oral absorption of drugs in humans (Camenisch et al., 1997; Hidalgo, 2001). Various studies have reported the correlation between the Caco-2 permeability and the fraction absorbed orally in humans (Artursson and Karlsson, 1991; Artursson et al., 1996; Yazdanian et al., 1997).

Caco-2 cells consist of a heterogeneous population of cells

(Artursson, 1991), thus these intestinal cell properties such as morphology, tight junction, enzymes and transporters activity can be altered by different culture conditions (Hidalgo et al., 1989; Walter and Kissel, 1995; Hidalgo and Li, 1996; Delie and Rubas, 1997; Hidalgo, 2001). The permeability of mannitol (paracellular hydrophilic marker) shows a 100-fold difference depending on the source of Caco-2 cells; this variability may be due to the difference in culture conditions and the composition of cell populations (Walter and Kissel, 1995). The cell culture protocol and correlations need to be regulated and validated in each laboratory by measuring TEER, ^{14}C-mannitol permeability and microscopic examination (Ungell and Karlsson, 2003).

The drug permeability may be expressed by the permeability coefficient (P_{eff}) through the cell monolayer as calculated by the following equation:

$$p_{eff} = \frac{V_A}{A(C_D - C_A)} \times \frac{dC_A}{dt}$$

where dC_A/dt is the change of drug concentration in basolateral side over time, A is the membrane surface area, V_A is the volume of solution in apical side, and C_D and C_A are the initial drug concentrations in basolateral and apical sides, respectively. Once the monolayer of Caco-2 membrane is prepared, the drug is added to the donor side, and the receiver side drug concentrations are monitored over time. Normally, the monolayers should be agitated during the experiment for more reproducible results as it may reduce the effect of aqueous boundary layers adjacent to the epithelial membrane that can results in an underestimation of the membrane permeability (Karlsson and Artursson, 1991).

There are limitations of the Caco-2 system that may lead to the mis-estimation of drug permeability. Due to the over-expression of tight intercellular junctions, the paracellular pathway may be limited, which may result in under-estimations in permeability for paracellularly transported compounds with MW <150 (Yee, 1997). Another limitation of this system is the under-expression of influx transporters such as peptide transporters (OCT, OAT). Thus, the substrates of these transporters including beta-lactam antibiotics and ACE inhibitors may be poorly permeated despite their high permeability property *in vivo* (Chong et al., 1997). The preparation of the monolayer membrane generally takes 21 days. However, the preparation time can be significantly reduced to less than 1-week by modifying both the coating material and the growth media (Chong et al., 1997). The recent trend of Caco-2 system is automation using a 24-well monolayer, LC/MS and robotics. A fully automated Caco-2 cell system can handle 500 to 2000 compounds per month. Despite some limitations, the Caco-2 system is the most popular cell culture model in laboratory and industry (Ungell and Karlsson, 2003).

Madin-Darby Canine Kidney (MDCK) cells

Whereas Coco-2 cells are originated from human colon adenocarcinoma, MDCK cells are derived from dog kidney cells. Owing to its different origin, expression levels of various transporters might be different from those of the human intestine. In the normal MDCK cell line, levels of efflux (e.g., P-gp) and influx (e.g., OCT) transporters are relatively low (Horio et al., 1989; Shu et al., 2001). Thus, this model may be sufficient for screening mainly passively transported drugs, while it may not be valid for screening actively transported drug. The major advantage of MDCK cells is the short culture time as the monolayer is formed within 3-7 days after starting the cell culture. An alternative cell model for MDCK is the MDR1-transfected LLC-PK1 cells (Adachi et al., 2001). However, the transfected cell line tends to form multilayers and not easily polarized (Hämmerle et al., 2000; Lentz et al., 2000).

Tissue-based models

The tissue-based models use the excised intestinal segment for measuring intestinal absorption. The advantage of the tissue-based model is to determine the membrane permeability using the real biological membrane and to easily screen different gastrointestinal segments for absorption potential. However, difficulty in maintaining the viability of the segments without blood supply is the main disadvantage of this model. Some examples of tissue-based models are described below.

Isolated intestinal cells

Isolated intestinal cell method was initially introduced in 1973 (Hopfer et al., 1973) and used extensively until recent years for characterizing intestinal drug transport (Murer & Kinne, 1980; Sinko et al., 1995; Waclawski & Sinko, 1996). The coarse membrane vesicles can be obtained by cell homogenates or intestinal scrapings and then purified by repeated precipitation-centrifugation procedures. The detailed procedures are introduced in a review paper by Hillgren et al (1995). The Isolated intestinal cells contain transporter proteins expressed on intestinal membrane, this allows for studying both passive and active transport pathways. However, one should keep in mind before using isolated intestinal cells that the prepared vesicles are not completely pure brush border cells but contain other types of cells and also transporter proteins and enzymes are prone to damage during the isolation process (Balimane et al., 2000). Compared with other tissue-

based models, the advantage to this method is that 1) a small quantity of drug is needed to perform the study, 2) a sufficient quantity of isolated cells can be acquired using a small number of animals, and 3) isolated cells can be cryopreserved and used for a long duration (Balimane et al., 2000). This method is rather simple and suitable to use in early stage of drug discovery where the available quantity of compounds may be limited.

Everted intestinal sacs

Everted intestinal sacs are used to evaluate drug permeability by using real intestinal fragments. A 2-4 cm section of the intestine is isolated from anesthetized animal, washed in ice-cold buffer to clear the segment. One end of the intestinal fragment is tied and everted by pushing a glass rod through the closed end. Then, the sac is filled with buffer, tied off at the other end, and placed in a flask with oxygenated (95% O_2/5% CO_2) buffer solution containing the compound. At the end of the experiment, the serosal fluid in the sac is collected and analyzed (Pento and Mousissian, 1988; Yamamoto et al., 1990). In this method, drug permeability is evaluated by using the real intestinal fragment, thus both the passive and active transports can be studied. Unlike isolated intestinal cells, the everted intestinal model can distinguish paracellular from transcellular pathways and can be used in the intestinal drug metabolism study (Bouer et al., 1999). The everted intestinal model has an additional advantage over other in vitro models because the absorption can be compared with different parts of the intestine while a small serosal volume is necessary.

A similar, everted intestinal ring method is also available. This method utilizes the everted intestinal ring instead of the everted intestinal sacs. This method has some drawbacks compared with everted intestinal sac method. Paracellular and transcellular pathways cannot be distinguished and the extra-absorption through exposed connective and muscle tissues at cutting side may result in overestimation of the permeability (Tukker, 2000).

Diffusion cells

Diffusion cells have been used to determine the drug transport in living cells. In 1951, Ussing and Zerahn first introduced the diffusion chamber so call Ussing cell (Ussing and Zerahn, 1951). In this method, appropriate size of the intestinal tissues is mounted between two chambers filled with oxygenated (95% O_2/5% CO_2) buffer including nutrients. The compound is added on donor chamber and the concentration in receptor chamber is measured as a function time. During the experiment, the potential difference between two cells is monitored and this potential is used to verify the viability of the mounted tissue during the experiment. The permeability of compound can be expressed as P_{eff} which is calculated by the similar equation used in the cell-based model.

$$p_{eff} = \frac{dc}{dt} \times \frac{V}{A \times C}$$

where V is the volume of the receiver chamber, A is the exposed tissue surface area, C is the initial drug concentration in the donor chamber, and dc/dt is the change in drug concentration in the receiver over time. The diffusion cell technique can easily adopt different segments of the excised tissues, thus this method is ideal for studying the various regional absorption factors (Ungell, et al., 1998).

In situ model

The intestinal perfusion is the most commonly used in situ model. Various intestinal perfusion techniques have been introduced such as single pass perfusion (Komiya et al., 1980; Amidon et al., 1981), recirculating perfusion (Van Rees et al., 1974; Tsuji et al., 1978), oscillating perfusion (Schurgers and DeBlaey, 1984), and the closed loop method (Doluisio et al., 1969). Of these methods, the single pass perfusion is most popular and well characterized (Lennernäs, 2000). The single pass perfusion is performed under anesthetic conditions. In most cases, animals are fasted overnight prior to experimentation. An intestinal segment is usually washed out and the proximal and distal segments of the intestine are cannulated, and the perfusion starts at a flow rate of 0.2 ml/min, a common perfusion rate in rats (Ho et al., 1983; Amidon et al., 1995; Fagerholm et al., 1996; Ganta et al., 2008).

In the single pass perfusion, net effective permeability (P_{eff}) is calculated by the following equation:

$$p_{eff} = \frac{-Q_{in} ln(C_{out}/C_{in})}{2\pi rL}$$

where C_{in} and C_{out} are the inlet and outlet concentrations at steady state after perfusion start. Q_{in} is the perfusion flow rate, and $2\pi RL$ is the applied intestinal surface area. The major advantage associated with this method is the presence of an intact blood, nerve and lymphatic supply in the experimental animals (Balimane et al., 2000; Ganta et al., 2008). Thus, this method has been used for studying passive and active transports.

Despite such advantages, use of the single pass perfusion method is limited because the permeability (P_{eff}) is calculated by the difference between inlet and outlet concentrations from the luminal side. The decrease in concentration on luminal side

does not, however, always represent the absorbed drug into the systemic circulation (Balimane et al., 2000). Another limitation is related to the amount of the test compound used. With this method, the steady-state condition for intestinal drug concentration should be achieved. To reach the steady state, a sufficient perfusion time and a large amount of compound are necessary. Yet, such a large amount of compound may not be easily obtainable in early stage of drug discovery (Balimane et al., 2000).

***In vivo* method**

Loc-I-Gut method is a single-pass perfusion experiment used to assess the regional permeability in conscious species. Loc-I-gut technique can be used from dog (Lipka et al., 1998) to human (Knutson et al., 1989; Lennernäs et al., 1992). This technique needs a specialized instrument consisting of sterile polyvinyl tube with six inner channels and two inflatable latex balloons placed 10 cm distant (Knutson et al., 1989; Lennernäs et al., 1992). The tube is inserted into the gut and the positioning is achieved by use of the fluoroscopic technique. After positioning, the latex balloons are inflated to separate proximal to distal balloon regions, and a single pass perfusion is performed in the fasted state at a 2-3 mL/min perfusion rate. The Loc-I-Gut method is a noninvasive method and thus it may be adapted in conscious animal and even in humans. This model can measure the regional drug absorption and drug dissolution at the absorption site. Another tool that can measure the *in vivo* regional drug absorption is the InteliSite capsule. The technique is rapid and totally noninvasive. In this technique, specially designed capsules release the drug by magnetic filed activation from outside of the body. The drug absorption can be measured by gamma scintigraphy (Pithavala et al., 1998).

Conclusion

Traditionally, the drug development strategy has focused primarily on the selection of appreciate target and in vitro and *in vivo* characterization of the pharmacological and toxicological profiles in the early stage developmental processes. However, a new paradigm for drug development has been shifted to screen the pharmacokinetic properties from the early stage of drug development. The oral absorption and membrane permeability are the key parameters of the pharmacokinetic properties. Thus, the need for more rapid, cost-effective, and highly predictive absorption screening methods has been increased. The drug absorption potential may be assessed by various in silico, in vitro, and *in vivo* methods. The lipophilicity, molecular weight, hydrogen bonding, pKa, and solubility are the physiochemical parameters that are closely related to the intestinal permeability. These parameters may be relatively easily determined by the in silico methods. However, these methods are not sufficient to explain the real absorption conditions. The in vitro and *in vivo* absorption screening methods, on the other hand, require more tedious and time consuming labors and complicate experiments even though they are close to real absorption conditions and can provide more useful information. In this review, various in silico, in vitro, and *in vivo* methods for screening the drug permeability are introduced. Each method has its own advantages and disadvantages. The screening methods need to be selected with consideration of the sample number, time, labor, and the stage of drug development. Appropriate selection of the permeability screening methods at the right stage of the development process will help bring successful drug developmental outcomes.

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