Membrane-based drug assays

Permeability assays and oral absorption modeling can make the difference between drugs and dregs.

BY JEFF RUELL

Pharmaceutical companies spend hundreds of millions of dollars developing drugs to be administered orally, which is why it is unacceptable for a compound to demonstrate low oral absorption in clinical trials. To prevent this problem, drug candidates are screened for their oral-absorption potential early in the discovery and development phase, when investment in a compound is low, as a filter to remove poor performers and identify candidates that need to be modified.

Evolving screens

In the 1990s, the development of these screens was rapid because of the increased throughput of chemical synthesis and biological screening. Initially, in silico methods were favored. The analysis of commercial drugs illustrated that there was a relationship between a compound's molecular properties and its oral absorption. For example, molecular weight, hydrogen bonding, and lipophilicity (log P) have all been associated with a compound having high oral absorption (the Lipinski rule of 5). Medicinal chemists traditionally use partitioning coefficients to explain permeation and in vivo oral absorption differences. Within chemical series, this is often true, as values of log P or log D (the distribution coefficient) often correlate well with permeability.

But despite repeated efforts, computational models based on molecular properties typically fail when large sets of diverse compounds are analyzed. This is particularly a problem when conformationally flexible compounds are used. Log P is also expensive to measure experimentally, and most techniques depend on estimates obtained with varying degrees of accuracy. To date, in silico methods work well within limited series but not when examining diverse sets of compounds.

The failure of physiochemistry-based predictions led to the development of simple assays to evaluate compound absorption. Because most drugs are absorbed through the intestines without using cellular pumps, passive permeability models became the focus. Different permeability techniques have been described in the literature, but most of them require too much material for analysis or cannot be implemented in a high-throughput environment. As a result, only two permeability assays have become prevalent in the past five years: the Caco-2 cell permeability assay and the parallel artificial membrane permeability assay (PAMPA). These assays have risen to play important roles in industry, and most companies perform at least one of them in their research programs.

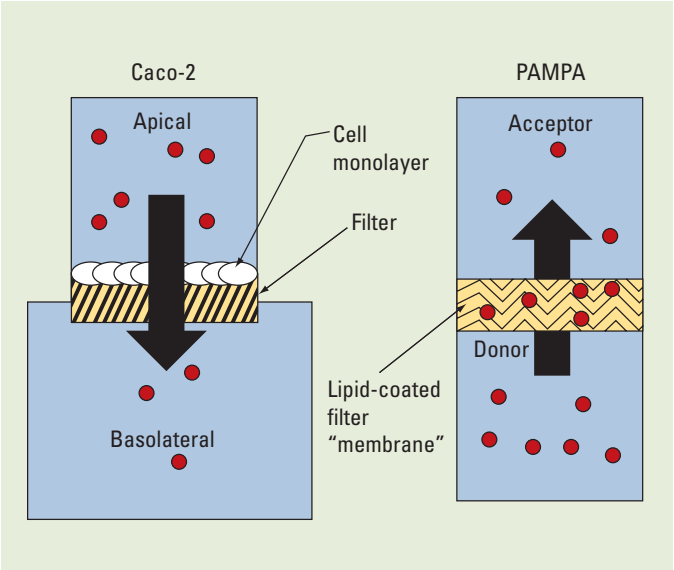

Figure 1. Comparing permeability assays. Caco-2 uses compartments of different volumes separated by a monolayer of cells grown on a filter. PAMPA, however, uses chambers of the same size, separated by a filter coated with lipid in organic solvent.

The implementation of these assays, however, has not stopped critics, and there remains lingering resistance to measuring permeability. Some people mistakenly assume that permeability can be directly related to partitioning coefficients, but membrane permeability experiments described in the literature illustrate why this is not so (*1*). For example, permeation often involves non-steady-state conditions. Solutes sometimes have different rates of adsorption to, and desorption from, membranes. Solutes can also aggregate in solution or within the membranes, affecting permeability. And membranes often have charged surfaces that introduce solute–membrane attraction or repulsion effects.

Membrane retention also is an important phenomenon. Two compounds can have the same permeability but different membrane retention characteristics. This retention is often misunderstood or neglected, which leads to incorrect permeability estimates and calculations.

Caco-2

First described in the early 1990s, cell permeability studies came to industry from academia, where several groups worked to develop cell-based assays that mimicked the passage of drugs through the intestinal mucosa (*2, 3*). The Caco-2 assay was the result.

In typical experiments, a monolayer of cells is grown on a filter separating two stacked microwell plates. The permeability of compounds through the cells is determined after the introduction of a drug on one side of the filter. The entire process has been automated, and when used in conjunction with LC-MS detection, it enables any compound's permeability to be deter-

IMAGE REDRAWN FROM *JCIN*

mined. It is recognized by the FDA as one of the few means to measure permeability as part of the bioequivalence waiver process. Since its introduction, Caco-2 has been championed by many as a standard for measuring permeability, but it is not without some shortcomings.

Caco-2 experiments require up to 20 days for the preparation of stable monolayers, and the cells must be maintained in protective environments, free from contamination, and examined for tight-junction formation prior to use. The method requires careful sample analysis to calculate permeability correctly. Interlaboratory variation is a problem because of differences in cell line strains. Caco-2 cells also contain endogenous transporter and efflux systems, the latter of which work against the permeability process and can complicate data interpretation for some compounds. In addition, test compound solubility appears to be a problem in Caco-2 assays because of the assay conditions.

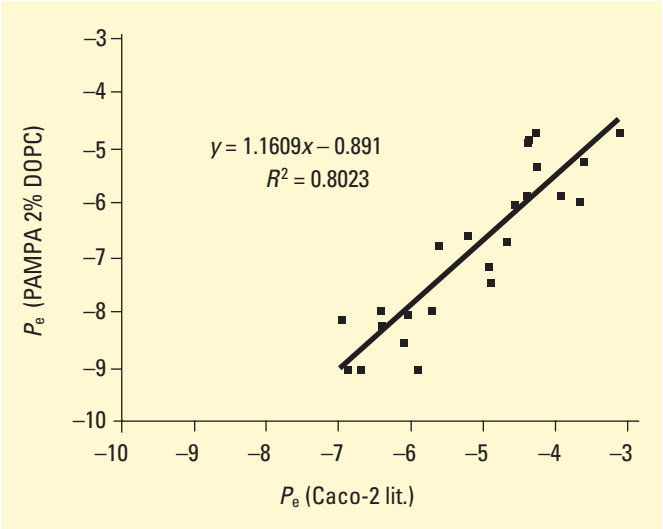

Figure 2. Comparing two permeability measurements. Caco-2 data are based on values from the Artursson research group (Uppsala, Sweden), while the PAMPA data are measured at pH 7.4

PAMPA

As a less expensive alternative to Caco-2, Manfred Kansy of F. Hoffmann-La Roche developed PAMPA (*4*). A "PAMPA sandwich" is prepared from two plates that are similar to those used for traditional Caco-2 experiments (Figure 1). One plate contains a porous filter disk at the bottom of each well. The other one is a reservoir plate that is precisely molded to sit under the filter plate so that contact between the two occurs at the filter. The filter is coated with a solution of lipid material in inert organic solvent to prepare the artificial membrane. The wells of one plate are then filled with donor solution (i.e., drug), and the other with acceptor solution (i.e., buffer); the plates are then stacked to create the sandwich and are incubated. The drug concentration in the donor and acceptor wells is then determined by UV or LC-MS methods, and permeability is calculated (*5*). The whole PAMPA process is easily automated and commercially available.

The lipid choice is flexible and often varies by research group. The original PAMPA model used phosphocholine isolated from egg yolk dissolved in dodecane to create membranes (*4*). Since then, different lipids have been used, ranging from mixtures that reflect the lipid composition of mammalian cell membranes to simple synthetic phospholipids (*6*). Some even forgo the lipid altogether and use nonpolar organic solvents (*7*). Lipid extracts from tissues can be used as long as they completely dissolve in an inert solvent.

With PAMPA, the emphasis is on simplicity. Because the membrane has no transporters or efflux systems, only passive permeability is observed. And because there is no growth period, the analysis can be set up quickly from standard stock supplies without concern about contamination. This allows PAMPA to be run without restructuring laboratories to create sterile environments, making PAMPA experiments easier, faster, and much less expensive to run than Caco-2 assays.

Oral-absorption models

For most models, a binning approach is favored over linear correlations. Both Caco-2 and PAMPA produce results that bin compounds into high- and low-permeability classes fairly well when passive permeability is the mechanism of transfer. These binning results agree with human in vivo jejunal permeability experiments (*8*).

PAMPA permeability results depend on the lipid used to create the membranes and the pH of the buffer. The buffer pH controls the amount of un-ionized material in solution. Because only a neutral species permeates, any pH favoring the neutral species will give the highest-permeability results. The choice of buffer is also limited by the underlying physiology of the process. The gastrointestinal tract has a pH gradient, increasing in pH with distance from the stomach. Therefore, single measurements at pH 7.4 may be misleading, and a range of pHs should be studied. Most groups have found that multiple measurements between pH 5.0 and 7.4 are necessary for agreement with in vivo results (*4, 6, 7*).

Caco-2 sometimes shows better linear correlation with oral absorption than PAMPA, but the two methods are comparable (Figure 2). This difference with respect to oral absorption is often the result of examining compounds when passive permeability is not the rate-limiting absorption factor, and for most drugs, this assumption is not true. Other issues affecting permeability include efflux, active transport, and solubility.

Industry now looks at compound solubility at the discovery level differently than it did 10 years ago. At Pfizer, Christopher Lipinski estimates that approximately one-third of the compounds assayed by in-house solubility analysis have poor solubility (< 20 µg/mL). For more than half of these compounds, predictive methods do not indicate poor solubility, and measurements must be made (*9*). Because of the growing ease of measuring solubility, such assays should be included in oral-absorption models. The best models should have mini-

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mum solubility limits for compounds to be analyzed or for the data to have meaning.

Outlook

PAMPA also allows chemists to pursue targets other than intestinal absorption. Researchers at Wyeth-Ayerst recently described PAMPA assays to predict central nervous system penetration that are high-throughput and accurate but require less than 0.5 mg of material for testing (*10*). This is a huge improvement over current hit-or-miss in silico methods. The researchers achieved this result using brain extracts as the lipid. The importance of an experimental model for blood–brain barrier (BBB) permeation cannot be understated, because there is "nothing ideal" yet in cell permeability models for high-throughput screening, according to Joan Abbott, director of neurosciences at King's College in London. Caco-2 assays appear to generate BBB results that are too optimistic for use.

Biological Caco-2 and artificial PAMPA assays have risen to dominate permeability screening. The use of Caco-2 continues, but the future of its role as a primary screen is coming into question. The high cost per assay, slow turnaround time, and mixed mechanism are starting to take their toll. This change in attitude is apparent at scientific meetings. As recently stated by Lipinski at the Society for Biomolecular Screening ADMET conference in September 2002 (*9*): "Caco-2 screening is going away and being replaced [in part] by PAMPA (for the purely passive component of permeability)."

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