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Evidence-based approach to assess passive diffusion and carrier-mediated drug transport

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Evidence supporting the action of passive diffusion and carrier-mediated (CM) transport in drug bioavailability and disposition is discussed to refute the recently proposed theory that drug transport is CM-only and that new transporters will be discovered that possess transport characteristics ascribed to passive diffusion. Misconceptions and faulty speculations are addressed to provide reliable guidance on choosing appropriate tools for drug design and optimization.

Introduction

The roles of carrier-mediated (CM) processes and passive diffusion in drug bioavailability and disposition were discussed extensively by Dobson and Kell [1] and Sugano *et al.* [2]. These two recent publications expressed completely different points of view on the topic. Dobson and Kell [1] claimed that CM cellular uptake is the rule rather than the exception for pharmaceutical drugs. In the recent paper [3], they claimed that ''drug transport is essentially CM only''. By contrast, Sugano *et al.* [2] supported the view that passive diffusion and CM processes coexist in drug transport.

This is important to researchers in drug discovery and development because it impacts drug design and development significantly. Structural molecular properties that optimize for passive diffusion differ greatly from those that optimize for CM transport. Similarly, screening assays and tools designed to identify these attributes differ. Thus, researchers rely on sound guidance on permeation processes for drug design and assay implementation. Misunderstanding the relative importance of different transport mechanisms can mislead drug discovery researchers, cause confusion and result in lost opportunities to discover much needed drugs or to avoid clinically problematic drug–drug interactions.

Sugano *et al.* explained that passive diffusion and CM processes have crucial roles in cellular transport and the relative importance of each process is dependent on the specific drugs under specific *in vitro* and *in vivo* conditions [2]. A discussion of these permeation processes is presented in their literature, see Ref. [2]. Various *in vitro* and *in vivo* methods are available to assess the extent of contributions from passive diffusion and CM transport [4–11]. Kell *et al.* [3] strongly disagreed with the view of Sugano *et al.* [2] and argued that drug transport is essentially CM-only. It is our opinion that some of the arguments made by Kell *et al.* [3] are not generally applicable and certain statements might be scientifically unsound and potentially misleading.

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Kell *et al.* [3] disregard a large body of established evidence that supports passive diffusion mechanisms and instead propose a new theory that membrane permeation occurs exclusively via transporters, most of them as yet unknown with uncharacteristic substrate specificity, even for compounds with transport mechanisms that have been thought to be dominantly linked to passive diffusion. In this article, the case is made that Kell *et al.* [3] did not adopt a balanced view on the roles of passive diffusion and CM processes in drug transport. The following sections systematically refute the major tenets of the theory put by Kell *et al*. regarding exclusive CM transport and present numerous compelling examples of passive permeation, supported by sound physicochemical principles.

Lack of published evidence for CM-only drug transport theory

Kell *et al*. [3] asserted that: ''There is considerable and increasing evidence that drugs get into cells more or less solely by hitchhiking on carriers...'', and they cite 38 references as support. A new theory of drug transport requires considerable supporting evidence that correlates well with observed drug transport. Many references cited by Kell *et al.* are opinion pieces and not research articles (refs. 10, 12–15, 26, 35 and 38 in Kell article – ref. [3]). Other references are not based on a premise of supporting a theory of CM-only uptake. Some of the references cited review known transporters and correlation to specific observations of absorption, distribution or excretion of specific drugs (refs. 3, 5–7, 9, 11, 16–21, 23–26, 30–33, 36 and 37 in Kell article – ref. [3]). Evidence that some drugs pass membranes via transporters is not evidence that all drugs do. Other references review how knowledge of substrate specificity and expression profile are used opportunistically by drug researchers for: (i) enhancing drug absorption; (ii) increasing exposure to specific tissues [e.g. central nervous system (CNS)]; (iii) reducing clearance; (iv) designing drugs by modifying structures to change transporter interactions and effects; or (v) explaining and avoiding potentially toxic drug–drug interactions (refs. 1, 4, 5, 8, 15–17, 25, 30, 34 and 38 in Kell article – ref. [3]). Transporter genomics references focus on explaining inter-individual differences in pharmacokinetics (refs. 2, 4, 22, 28 and 29 in Kell article – ref. [3]). It is our opinion that the references cited in the article by Kell *et al.* [3] are not sufficient to support a CM-only theory that explains known drug transport observations. The idea that new transporters will be discovered that account for documented drug transport observations appears to be speculative.

Cell membranes have higher molar ratio of lipid to protein

Kell *et al.* [3] suggest that lipid bilayer permeation is not relevant in biological membranes because they consist of protein:lipid ratios of 1:1–3:1. This range gives the impression that there are not enough lipid molecules to form bilayers or a significant surface area between the proteins for passive diffusion through the cell membranes. On the basis of this value, CM processes are thus said to be the sole mechanism for cellular transport. Artificial membrane systems lacking proteins, for example black lipid membranes (BLMs), unilamellar vesicles and parallel artificial membrane permeability assays (PAMPAs), are reported to be entirely inappropriate and not representative of biological membranes [3]. However, the stated 1:1–3:1 protein:lipid ratio could be misleading. A typical porcine brush border membrane has a protein:lipid (phospholipids plus cholesterol) mass ratio of 2.3:1 [12]. This results in an estimated lipid:protein molar ratio of 40 when assuming an average molecular weight of 550 Da for the lipids (56 mol% cholesterol, 44 mol% phospholipids) and 50 kDa for the membrane proteins [13]. Thus, lipids contribute significantly to the barrier function of a membrane. The ratios 1:1–3:1 previously stated [3] are mass ratios where the protein mass includes the cytoplasmic and exoplasmic protein fractions of membrane proteins, not just the relevant transmembrane fraction. In addition, the stated range also covers protein-rich membranes, such as the inner mitochondrial membrane, which is not of direct interest when cellular drug uptake and efflux are considered. Because the lipids are the major component of biological membranes, in terms of molar ratio, their contribution to barrier passage by passive diffusion or flip–flop events are of major pharmacological importance [14]. Furthermore, the passive permeability data obtained by artificial membrane systems consisting of lipids and lipid-like structures are relevant to that in biological membranes because they chemically, structurally and physically mimic the lipid bilayer portion of the biological membranes.

Permeation of drug molecules through transient water pores in artificial lipid membranes is insignificant

Artificial lipid membranes (i.e. BLMs, liposomes and PAMPAs) that are models of passive diffusion have been correlated in numerous publications to membrane transport for a large portion of drug compounds. Kell *et al*. [3] attempted to negate artificial membranes by suggesting that they contain transient hydrophilic pores, by which drug molecules transit the membrane, and such pores are absent in cell membranes. For example, Kell *et al*. [3] asserted: ''...transient aqueous pores that occur widely in artificial lipid membranes but not in real biological membranes'' and ''...they [BLM model and unilamellar vesicles] are essentially leaky via the formation of transient hydrophilic pores...''.

Two references [15,16] are cited to support this assertion; however they describe computational simulations of Na^+ and Cl^- ion transport partly under significant physical stress conditions (i.e. high surface tension). These references are relevant neither for relaxed membranes nor for the permeation of drug-like solutes. In fact, the permeability of ions is orders of magnitude lower than that of drugs. None of the references cited in Ref. [3] investigated drug permeation through membrane pores, except in the presence of excess organic solvent. In fact, studies [17–20] indicate the insignificance of transient aqueous pores in normal artificial lipid membranes. Other examples include the following:

- Liposomes were impermeable to the hydrophilic terbium (III) ion and EDTA, but were permeable to aromatic carboxylic acids in a pH-dependent manner [21–23].
- In a comparison of permeability coefficients of various compound types in artificial membranes, phosphate, sodium and potassium ions had permeability coefficients of 0.1–1.0×10^{-12} cm s^{-1}, hydrophilic amino acids had permeability coefficients of 5.1–5.7×10^{-12} cm s^{-1} and lipophilic amino acids permeated at rates of 250–410×10^{-12} cm s^{-1} [24]. Similar values were reported for enkephalin peptides [25]. Higher values would have been seen if there were aqueous pores.
- When $(NH_4)_2SO_4$ was in solution adjacent to a BLM, permeation of neutral NH_3 was proven by the accumulation of H^+ on

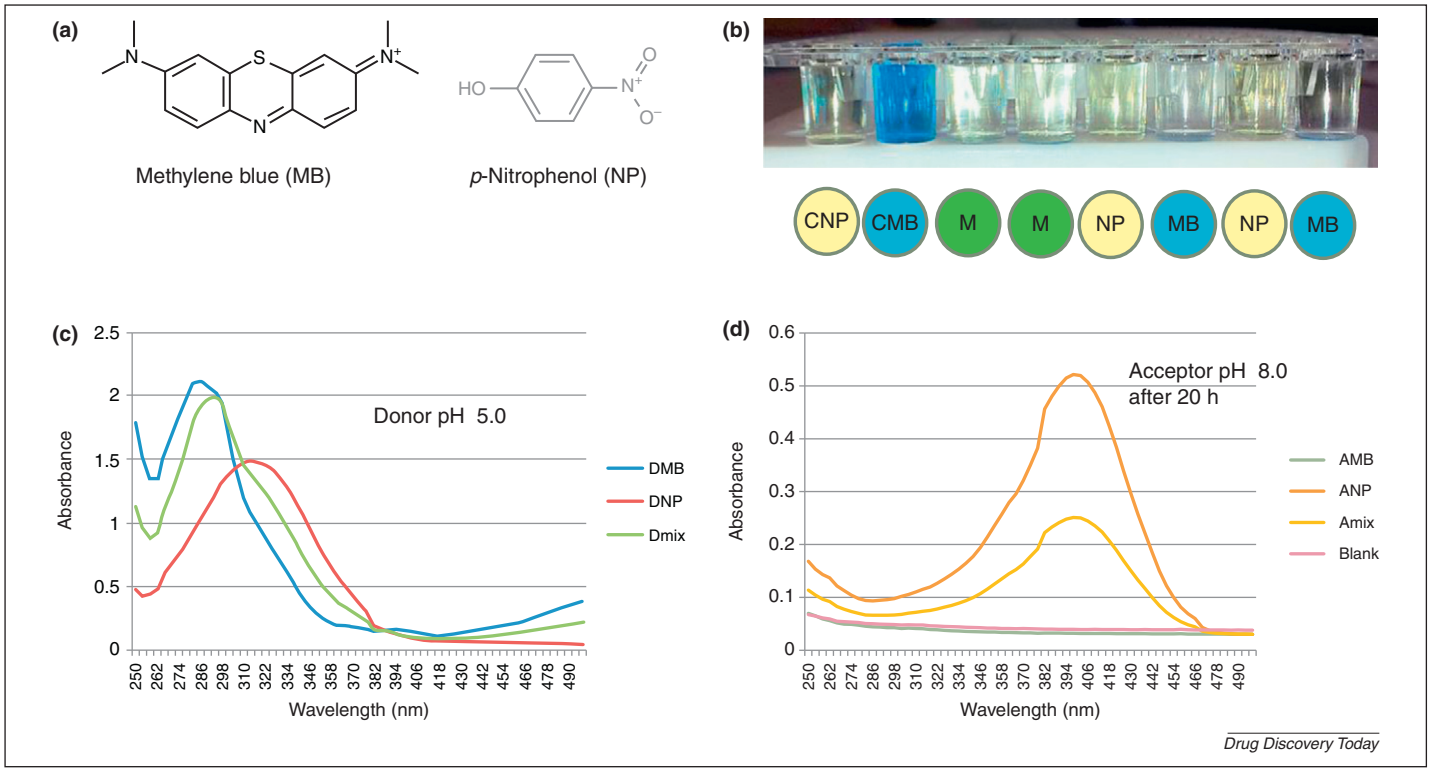

FIGURE 1

Standard PAMPA experiment used for the determination of compound flux across a hexadecane layer. Methylene blue (MB) and *p*-nitrophenol (NP) are used separately and as mixtures (M). Donor pH 5.0. Acceptor pH 8.0. **(a)** Compound structures. **(b)** Acceptor plate (after 20 hours) on top of a PVDF donor plate. Well designations below. Control *p*-nitrophenol (CNP) at equilibrium; control MB (CMB) at equilibrium; mixture MB + NP (M). **(c, d)** Absorbance as a function of wavelength in donor (c) and acceptor compartments (d). Donor MB (DMB); donor NP (DNP); donor M (Dmix); acceptor MB (AMB); acceptor NP (ANP); acceptor M (Amix). Results: NP passes the hexadecane layer by passive diffusion whereas MB is unable to pass. From the mixture solution only NP is able to pass, excluding pore formation as the mechanism because MB would pass through the pores. Owing to different pH in donor [54–56] and acceptor wells, UV maximum of NP is shifted to higher wavelength.

the donor side and depletion of H^+ on the acceptor side [26], which would not have been observed if aqueous pores were the transport mechanism.

- When the transport properties of a series of α-substituted *p*-methylhippuric acids (i.e. –H, –Cl, $–OCH_3$, –CN, –OH, –COOH and $–CONH_2$) were measured in artificial bilayer membranes, with and without bilayer-spanning protein [27,28], permeability in the neutral form (P_0) ranged from 4.6×10^{-4} to 8.4×10^{-8} cm s^{-1} and in the anionic form (P_i) ranged from 4.4×10^{-10} to 2×10^{-13} cm s^{-1}, with a pH dependence exactly consistent with the pH-partition hypothesis. The difference between the permeability of the neutral and anionic forms was an almost constant six orders of magnitude. Hydrophilic D-mannitol had a permeability of 1.0×10^{-10} cm s^{-1} and was independent of pH. The presence of the bilayer-spanning protein had little effect on the most permeable molecules but increased the permeability of the least permeable molecules by an order of magnitude at most. It was concluded that the barrier domain is similar for neutral and anionic forms of the drug-like molecules and substantially more hydrophobic than octanol, clear evidence for the absence of water-pore mechanism of transport [18].
- BLMs have a high direct current resistance and very high capacitance, demonstrating low permeability for ions [29] and this is inconsistent with the suggestion of 'essential leakiness' of BLMs [3].
- PAMPA studies using hexadecane membranes showed a good correlation between P_0 and $\log P_{HXD/W}$ (partition coefficient measured in *n*-hexadecane/water) [30]. No such correlation would be expected if transient aqueous pores were the major mechanism of permeation.
- PAMPA studies with hexadecane membranes (Fig. 1) determined that methylene blue (MB), a permanently charged organic molecule, did not pass the membrane, whereas *p*-nitrophenol (NP), a weakly ionizable organic acid, passed the membrane, whether the two compounds were alone in solution or as a mixture.
- Pores are generally not seen in a PAMPA permeability measurement. When pores exist in poorly prepared PAMPA membranes it is easy to recognize them by (i) the permeability values being abnormally high in the region where the molecule is essentially charged, and (ii) the lack of pH dependence in the permeability–pH plot in that same region. For ionizable molecules, the plot of log permeability versus pH has a characteristic hyperbolic shape in the absence of pores, consistent with the expectations of the pH-partition hypothesis. Pores can increase permeability in the pH region where the molecule is predominantly charged. Furthermore, inefficient stirring can lower the permeability in the region where a lipophilic molecule is predominantly uncharged, when the unstirred water layer (UWL) becomes rate-limiting in

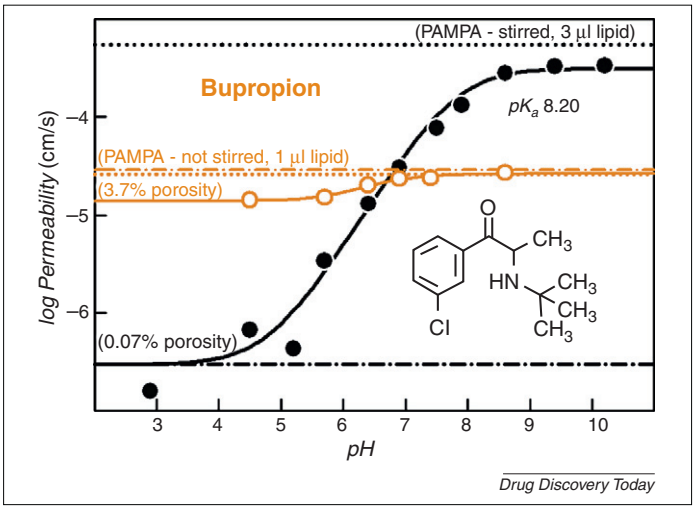

FIGURE 2

This figure explains how to recognize water pores from permeability–pH data analysis of bupropion. The log permeability versus pH plots of bupropion determined by the PAMPA method (figure drawn from data in Ref. [31]). The solid black curve and symbols are based on 'thick' coated filters, whereas the solid brown curve and symbols are based on 'thin' pre-coated filter plates. The pH was varied to assess the contribution of the unstirred water layer (pH > 8) and the shunting effect of the paramembrane aqueous pores (pH < 6). The best-fits of the measured effective permeability–pH are represented by the solid curves; the paramembrane permeability is indicated the by dash-dot lines; and the UWL permeability is indicated by the dotted lines. The leaky thin-membrane case is easily recognized (and rejected) by the lack of prominent permeability–pH dependence.

permeability for lipophilic molecules. These two combined effects can make the log permeability curve nearly pH-independent. Figure 2 illustrates how to recognize water pores based on the permeability–pH data analysis of two sets of bupropion PAMPA measurements [31]. The solid black curve and symbols are based on 'thick' 3 µl porcine brain lipid extract coated filters, whereas the solid brown curve and symbols are based on the 'thin' 1 µl 4% w/v dioleyoylphosphatidylcholine (DOPC) in hexadecane pre-coated filter plates (available from BD Biosciences; http://www.bdbiosciences.com/home.jsp). The pH was varied to assess the contribution of the UWL for pH > 8 and the shunting effect of the paramembrane aqueous pores for pH < 6. The best-fits of the measured effective permeability–pH are represented by the solid curves; the paramembrane permeability is indicated the by dash-dot lines; and the UWL permeability is indicated by the dotted lines. The leaky thin-membrane case is easily recognized (and rejected) by the lack of prominent permeability–pH dependence. Thin membranes are more likely to possess pores than thick membranes. Figure 2 shows the presence of water-pore permeation and absence of pH-dependent permeation effects (unfilled circles) for a thin membrane in which pores were estimated as 3.7% of the membrane surface. For thicker membranes the water hole porosity dropped to 0.07% or lower (filled black circles in Fig. 2) and permeability values showed a pH-dependence [31]. It is abnormal to see pores in PAMPA measurements using established protocols.

The assertions made by Kell *et al.* [3] regarding transient lipophilic pores is based on extrapolating computational simulation for ions, but it is not consistent with other published data. The above described evidences are consistent with the passive diffusion of neutral lipophilic and ionizable drug molecules across membranes at a rate dependent on lipophilicity and ionization state.

Ionizable drug molecules permeate membranes in the non-ionized form

Kell *et al.* [3] state: ''This is most obvious when these molecules are charged, because they do appear to cross bilayer membranes; however, the enormous Born charging energy required means that this cannot be other than via hydrophilic pores, which form frequently and spontaneously (on a nanosecond timescale) in artificial phospholipid bilayer membranes.'' Although this point has been explained previously by Sugano *et al.* [2], it will be discussed further because it is one of the most frequently asked questions about passive permeation. An ionizable drug is in equilibrium between the non-ionized and ionized forms, with the ratio of molecules in the two forms being determined by the solution pH and pK_a of the molecules. The non-ionized molecules have higher lipophilicity and permeate the membrane faster than ionized molecules, as described by the 'pH-partition theory'. For example, an acidic drug with a pK_a of 4 and a basic drug with a pK_a of 9 are 99.7% ionized at pH 6.5 (the intestinal microclimate pH). In this case, only 0.3% is in the uncharged form. However, there is significant permeation because the intrinsic permeability of non-ionized species is very high. For example, the uncharged form of a compound with a log *P* of 3 can have an intrinsic permeability as high as $10{,}000 \times 10^{-6}$ cm s^{-1} [32]. The reason why this high level of permeability is rarely observed, even at a pH where the drug exists completely uncharged, is that the UWL determines the upper limit of directly observable permeability. UWL is a region adjacent to the membrane where the compound concentration is reduced by penetration into the membrane and there is insufficient diffusion to maintain the concentration equal to that of the surrounding solution. It was observed that when the UWL decreased by vigorous stirring the permeability increased to this range [32–34]. Also, when the UWL was subtracted from the apparent permeability, a linear relationship between log $P_{HXD/W}$ (a measure of lipophilicity) and intrinsic permeability, log P_0, was observed [30]. Therefore, ionizable molecules permeate lipid membranes in the non-ionized form by passive diffusion rather than via hydrophilic pores. Kell *et al.* [3] also mentioned that the water pores that they believed to exist in artificial membranes disappear in biological membranes, but this factor is unexplained. Experimental evidence would be required to support their theory.

Coexistence of passive diffusion and carrier-mediated mechanisms are evidenced by permeation saturation

Kell *et al.* [3] claimed that passive diffusion does not exist in the biological membrane and that drugs are transported by CM processes only. This appears to be based on speculation rather than experimental evidence. Passive diffusion, by contrast, is not concentration-dependent or saturable (i.e., rate of transport increases hyperbolically with concentration and transport has limited capacity) at therapeutic and even toxic drug concentrations. Experimentally, many compounds demonstrate concentration independence and lack of saturation in biological membranes. This is consistent with nonsaturable (transport has unlimited capacity) passive diffusion. The high local concentrations in the gut after oral dosing

of drugs will saturate active drug transporters. This is illustrated by studies using the Caco-2 permeability experiment with high (i.e. mM level) drug concentrations, which saturate active transporters yet demonstrate non-saturable transport, suggesting passive diffusion. For example, in Loc-I-Gut experiments lack of interaction after inclusion of inhibitors of active transporters indicated that drugs classified as biopharmaceutics classification systems (BCS) III and IV, such as fexofenadine, cimetidine, atenolol, terbutaline, amoxicillin and methyldopa, were absorbed across the gut wall by passive diffusion as at least one major route [16].

Artificial membranes can exhibit higher permeability compared with cellular systems

Kell *et al*. [3] asserted that artificial membrane systems have lower permeability compared with cellular systems: ''Thus, at the lower end [of permeability values], there is nearly a 100-fold discrepancy in the absolute fluxes; that is, the real biological MDCK (Madin–Darby Canine Kidney) cells, which contain carriers, take up the less permeable drugs approximately 100-fold more rapidly than do the artificial membranes''. This statement is not true, because some compounds are known to have higher permeability in artificial membrane systems than cellular systems. For instance, alfentanil has a higher permeability value in hexadecane artificial membranes (HDMs) than in the cell systems [35]. The HDMs were impermeable to hydrophilic fluorescent probes [35] but, when properly stirred to reduce the UWL, showed very high permeabilities for some highly permeable compounds. Furthermore, this permeability was of the same order as those observed in Caco-2 and 2/4/A1 cells [36]. In a comparison between 24 human jejunal permeabilities and Caco-2 permeabilities the *in vivo* and *in vitro* drug permeability measurements correlated well for passively absorbed drugs ($R^2 = 0.85$), when removing compounds that are mainly transported by CM processes [37]. These reports suggest that passive diffusion is the major mechanism for the uptake of the compounds rather than CM processes.

Permeability differences among cell lines do not support CM-only transport mechanism

Kell *et al*. [3] reanalyzed the comparison between the permeability (P_{app} values) data from Caco-2 and MDCK cells as discussed by Sugano *et al*. [2]. Sugano *et al*. correctly stated that 24 out of 55 drugs (i.e. 44%) had more than a two-fold difference in permeability between the two cell lines. Kell *et al*. [3] ascribed these differences to CM transport and concluded that passive diffusion does not exist for those compounds in the two cell lines. As pointed out previously by Sugano *et al*. [2], because lipid membranes are universal for living cells, passive diffusion is, therefore, ubiquitous for cell permeation. Caco-2 (derived from the human colonic adenocarcinoma) and MDCK cells have qualitatively similar potential for permeability by passive diffusion. However, because of the differences between the two cell lines (i.e. lipid composition, cell surface morphology, and barrier thickness) and variable experimental conditions (i.e. UWL, temperature, and additives), there will be some inherent differences in measured passive permeability data between the two cell lines. All things considered, the correlation between the two cell lines across a large range of permeability is good for this set of structurally diverse compounds ($R^2 = 0.90$ for 79 compounds [38]). Kell *et al*. [3] pointed out that many of the compounds in the set are transport substrates. It has been well documented that Caco-2 and MDCK cells express different transporters and, even for the same transporters, the two cells lines can have different expression levels, and certain substrates can exhibit species differences in transport (e.g. human versus canine) [39]. If permeation of these model drugs is solely governed by CM processes, as Kell *et al*. assert [3], more-significant permeability differences between the two cell lines would be observed, reflecting the transporter differences (i.e. difference in pattern of expressed transporters, species difference plus quantitative and qualitative expression differences). Being a CM substrate does not mean that CM is dominant. The correlation between Caco-2 and MDCK rather suggests that, even though these drugs have been diagnosed as transporter substrates, the predominant permeation mechanism is passive diffusion.

This is also the case when substrates with differences in transport mechanisms are compared. Drug transport rates in Caco-2 monolayers were compared with those obtained in the human jejunum *in vivo*. Permeability coefficients unbiased by the hydrodynamic conditions were calculated to enable direct comparison of the two models. The rapidly (passively) permeating drugs naproxen, antipyrine and metoprolol had comparable permeability coefficients in Caco-2 cells and in human jejunum. The permeability coefficients of the slowly (passively) permeating hydrophilic drugs terbutaline and atenolol were 79- and 27-fold lower, respectively, in Caco-2 cells than in human jejunum owing to narrower paracellular tight junctions in Caco-2. The CM transport rates of L-dopa, L-leucine and D-glucose were also much slower in Caco-2 cells than in human jejunum. The lower permeability of the CM-transported compounds and of atenolol and terbutaline, which were considered to be transported via the paracellular route, is consistent with the colonic origin of the Caco-2 cells. The results indicate that Caco-2 monolayers can be used to predict passive drug transport in humans, whereas prediction of transport by CM systems might require a scaling factor, owing to an altered expression of carriers in this cell line [40].

Artificial membrane permeability and lipophilicity are consistent with biological membrane permeability

Kell *et al*. [3] stated that artificial membrane permeability does not correlate with cellular membrane permeability. However, there are experimental results showing a good correlation between artificial membrane permeability and cellular membrane permeability [2]. Furthermore, this work also demonstrates a good lipophilicity–cellular-membrane permeability relationship [2]. Lipid bilayer permeation can be considered as the sum of three processes: (i) partitioning of the permeant into the *cis*-lipid layer; (ii) flip–flop or translocation to the *trans*-lipid layer (diffusion in the lipid phase); and (iii) partitioning out of the *trans*-lipid layer into the *trans* aqueous phase. Each of the three processes can be rate-determining, depending on the properties of the permeant and the membrane, the system geometry, the pH, the temperature and the ionic strength [41]. However, all processes depend, among other factors, on the lipophilicity of the permeant. Because the contributions of permeant lipophilicity, charge, size, among others, are different for the three processes and all processes can be rate-determining, it is not surprising that there is never a perfect correlation between lipophilicity parameters and permeation or between permeation

coefficients of systems with differing geometry. However, this imperfect correlation should not be interpreted to deny passive diffusion. As discussed above, the correlation is significant and covers a wide range of permeability values of structurally diverse drugs.

Passive diffusion is the major mechanism for blood–brain barrier permeation of lipophilic small molecules

In the discussion regarding the blood–brain barrier (BBB), Kell *et al.* [3] minimized the contribution of passive diffusion for brain uptake with the following statements: ''Methods that use chemoinformatic substructural analyses (which would detect determinants of transporter substrates [42]) are at least as effective at predicting BBB uptake as those based on descriptors such as lipophilicity'', and ''. . .several studies (e.g. [43–47]) demonstrate the utility of delivering drugs to the central nervous system (CNS) as prodrugs designed to be taken up via known carriers''.

The BBB uptake transporters have unique substrate specificities and require specific structural motifs for transportation to be possible. A few drugs, such as gabapentin or L-dopa, are transported via L-type amino acid transporter 1 (LAT1) [48]. However, there is no concrete evidence for uptake transporters being responsible for the passage of most drugs across the BBB, at least not as the sole or dominant mechanism. Significant efforts have been applied to the process of designing drugs that would use uptake transporters to enhance brain uptake; however, there are only a few successful stories. Prodrug approaches that use uptake transporters to increase brain penetration are scarce and have limited success. Although research into BBB transporters is a very active area, the transporter functions in moving drugs across the BBB are not well defined and currently have limited application in drug discovery. By contrast, there is a large body of strong evidence that suggests many lipophilic small molecules cross the BBB by passive diffusion. This has been the corner stone in designing successful CNS drugs with sufficient brain exposure [49–52]. It has been shown that, when efflux transporters are inhibited or saturated at the BBB, permeability across the BBB, as measured using *in situ* brain perfusion studies, shows an excellent correlation with PAMPA-BBB, suggesting passive diffusion is the dominant mechanism for brain uptake of most lipophilic drugs [31] (Fig. 3). It will be interesting to see whether Kell *et al.* can predict the rate of brain penetration *in vivo* with such accuracy using 'chemoinformatic substructural analyses for transporter substrates'. It is our opinion that the hypothesis of CM-only in BBB transport is misleading.

Unknown transporters cannot explain all the drug transport phenomena

Kell *et al.* [3] stated that: ''We now know of carriers for all kinds of molecule, from water, urea and glycerol to highly hydrophobic molecules, such as the dibenzyldimethylammonium cation, as well as for hundreds, if not thousands, of different drugs''. Moreover, they put forward the existence of unidentified transporters to explain all experimental observations of membrane permeation that have been previously ascribed to passive diffusion bilayer permeation.

Although hundreds of carrier proteins exist in many organisms, it is unlikely that the majority of these transporters recognize drugs as substrates. A closer look at the transporter database

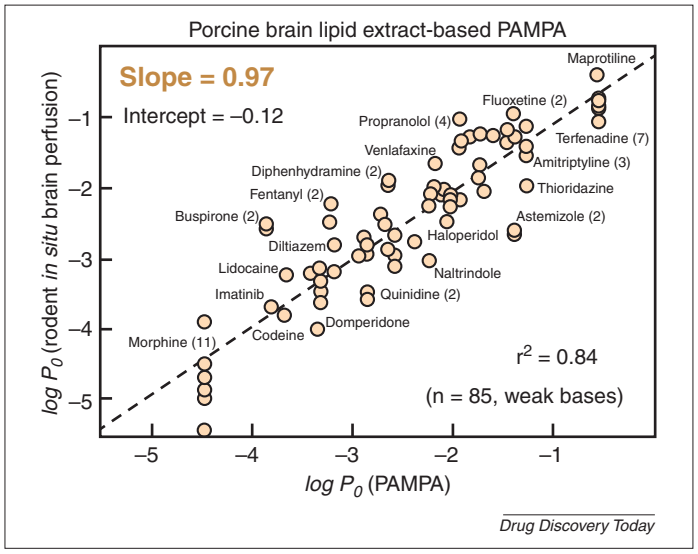

FIGURE 3

Correlation of intrinsic BBB permeability between rodent and PAMPA–BBB (figure drawn from data in Ref. [31]).

(http://www.membranetransport.org) reveals that the substrate information is largely predicted rather than derived from experimental evidence. The extent to which the transporters can recognize drug molecules in addition to their endogenous substrates is, at best, questionable. A recent investigation of the large solute carrier superfamily indicated that the majority of solute transporters have substrates ranging in size from small ions, such as metal ions, to amino acids (i.e. compounds that are much smaller than the average drug). Further, solute transporters with unknown function were more often predicted to transport molecules that are smaller than drugs. This analysis predicts that a large fraction of solute-transporting proteins with unknown function will probably not transport substrates the size of drugs [53]. The transcriptomic and proteomic data, although interesting, have a long way to go before they can be translated into meaningful functional data. The examples indicated by Kell *et al.* [3]: ''. . .urea and glycerol to highly hydrophobic molecules, such as the dibenzyldimethylammonium cation. . .'' are not typical drugs and are significantly more hydrophilic compared with the majority of membrane-permeable drugs.

On the basis of the considerable body of data available regarding permeability in the scientific literature (Ref. [2] and therein), here is a summary of the characteristics that such unidentified transporters proposed by Kell *et al.* [3] that would be required (to mimic the process that is commonly ascribed to passive diffusion):

- Ability to identify (bind and transport) substrates based on the same correlation with molecular properties (lipophilicity, MW, polar surface area, etc.) as ascribed for passive diffusion
- Coverage of the same chemical space as all permeable compounds.
- No energy source beside a concentration gradient.
- To be expressed and functional in all cell types, including dead cells (ghost red blood cells and snake skin).
- To have comparable characteristics in all species (mammals, insects, bacteria, etc.)
- To have the same density in apical and basolateral domains of all plasma membranes (resulting in equal permeation rates from apical to basal and vice versa).

- To have exactly the same rate constants in both directions (exoplasmic to cytoplasmic and vice versa).
- Not to be saturated or inhibited.

Although researchers are actively engaged in identifying and characterizing new transporters, which improves our understanding of drug transport and disposition, it seems that the proposed broad theory of CM transport only by unknown transporters [3] possessing all the characteristics listed above is pure speculation without scientific basis or evidence. The references cited by Kell *et al.* [3] to support unknown transporters actually support nonexistence of such unknown transporters that have the observed membrane permeation properties that have been correlated to passive transmembrane diffusion, as described above.

Conclusion

On the basis of a thorough analysis of published *in vivo* and *in vitro* transport studies (Ref. [2] and therein), including those publications that were recently stated to support the CM-only hypothesis, we come to the conclusion that experimental evidence for CM-only permeation of drugs across biological barriers is nonexistent. Published data (Ref. [2] and therein) and our own observations are in complete agreement with the co-existence of passive diffusion and CM transport of drugs across cell membranes. Artificial membranes such as BLMs, liposomes and PAMPAs are valuable tools for predicting passive permeation of drugs across biological membranes. The CM-only hypothesis would require transporter characteristics that have not been described for any known transporter to date. The choice whether to favor co-existence of passive diffusion and CM transport or CM-only permeation has a major impact on the strategic decisions in drug discovery and development, at all levels from drug design to the post-marketing phase.

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