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Does transbilayer diffusion have a role in membrane transport of drugs?

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Abstract

The existing consensus on coexistence of transbilayer diffusion and carrier-mediated transport as two main mechanisms for drugs crossing biological membranes was recently challenged by a systems biology group. Their transporters-only hypothesis is examined in this article using published experimental evidence. The main focus is on the key claim of their hypothesis, stating that ‘the drug molecules cross pure phospholipid bilayers through transient pores that cannot form in the bilayers of cell membranes, and thus transbilayer drug transport does not exist in cells’. The analysis shows that the prior consensus remains a valid scientific view of the membrane transport of drugs.

Introduction

The coexistence of transbilayer diffusion and carrier-mediated transport as two main mechanisms by which small-molecule drugs cross intact cell and organelle membranes is a generally accepted concept[1]. Recently, this consensus was challenged by a systems biology group, which hypothesized that all transmembrane transport of drugs requires the use of transporters, and no diffusion through the bilayer occurs [2–4]. The authors’ views gradually polarized over time, and culminated in their latest paper, where, in the abstract, they claim ‘the widespread but erroneous nature of the assumption that the ‘background’ or ‘passive’ permeability to drugs occurs in the absence of carriers. Because of the impact, which ‘the transporters-only hypothesis’, if proven, would have on the drug development process, I examine their arguments in the light of previous scientific observations, and present the results in the order of importance for supporting their hypothesis.

To avoid confusion, the two transport processes are coarsely defined as follows. Transbilayer diffusion is permeation of small-molecule drugs across the intact bilayer, in any molecular form or species, without interacting with any membrane components besides the lipids. Carrier-mediated transport requires binding of drug molecules to native, membrane-bound carrier macromolecules, which then translocate the drug molecules across the membrane using their conformational movements.

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Key issues of transporters-only hypothesis

The main pillar of the transporters-only construction is the proposition that drug molecules cross phospholipid bilayers through transient aqueous pores, which only exist in pure phospholipid bilayers but cannot form in biological membranes. As a consequence, transbilayer drug diffusion should only take place in pure phospholipid bilayers and not in membranes. Let us analyze the pertinent claims and their support by published experimental studies step by step.

Transbilayer drug passage through aqueous pores

Transbilayer drug transport can readily be observed experimentally in planar bilayers and liposomes. Planar bilayers formed on a small opening in a teflon barrier separating two aqueous compartments are often called black lipid membranes (BLMs) because of their appearance caused by interference of light reflecting from their front and back surfaces [5]. In contrast to the mainstream view of this process as diffusion across the intact bilayer, the authors claim that drugs pass through the bilayer using transient pores. The pore-permeation mechanism was proposed for water, inorganic ions (Na^+ , K^+), and water-soluble, low-molecular-weight compounds [6,7]. However, the agreement on the mechanism even for these small ions and molecules is not equivocal. The pore-permeation mechanism can be examined experimentally: the pore formation rate decreases with the bilayer thickness faster than the rate of diffusion. Transport measurements in bilayers made of phospholipids with varying fatty acid lengths support the pore-permeation mechanism for K^+ and H^+ in thinner bilayers only, whereas for the bilayers with longer fatty acids, diffusion prevails [8]. For halide anions Cl^- , Br^- , and I^- [9], as well as for water, urea, and glycerol [8], the experiments indicate that diffusion is the main transport mechanism.

Drug transport rates depend on the organization of phospholipids in the bilayer. They increase with increased bilayer fluidity, most notably by transition from gel to liquid state, and also when the bilayer structure is disturbed (e.g. by swelling or shrinking) and at the interfaces between lateral patches of separated lipids also called lipid rafts. However, the transport rates through the disturbed bilayer still maintain the strong dependence on drug structure that is incompatible with the aqueous pore formation [1].

What evidence do the authors present to justify the extension of the pore-permeation mechanism to drugs? They cite, at different places in their publications: (i) a book [10] without giving the page numbers and an edited book [11] without even mentioning the chapter, so the actual data are difficult to track down; (ii) a bilayer patch-clamp study of the transport of small inorganic ions (e.g. Na^+ , Cl^-), not drugs [12]; and two computational molecular dynamics studies, which simulate pore formation under conditions of mechanical stress [13] and as a transport mechanism for small inorganic ions [14].

In contrast to the arguable support for the pore-permeation mechanism, there is overwhelming evidence that the drug molecules actually become embedded in the intact bilayer upon contact. Drug accumulation in the bilayer can be significant: the partition coefficients in liposomes span 5+ orders of magnitude [15]. Accumulation is in contradiction to the pore forming hypothesis: accumulated molecules are in direct interactions with phospholipids and practically no water molecules are seen in the core region.

Spectroscopic techniques provide a plethora of evidence of the ability of drug molecules to diffuse through the heterogeneous bilayer and can be used to monitor the kinetics of individual steps in this process. Spectroscopic signals change in response to (i) drug interactions with specific groups [nuclear magnetic resonance (NMR) [16], and electron

paramagnetic resonance (EPR) [17]], (ii) drug presence in aqueous and nonpolar environments (ultraviolet and visible difference spectroscopy [18], fluorescence [19] and circular dichroism [20]), and (iii) drug effects on excimer formation [21] in the bilayer and fluorescence lifetime data [22]. Molecular character of the environment changes significantly as drug molecules pass through the headgroup and core regions. However, for the transport through aqueous pores, the changes in the character of the environment would be much less dramatic and should not lead to the observed significant changes in spectroscopic signals.

Direct evidence against the pore-formation hypothesis is represented by observations of drug molecules embedded in the intact bilayer upon contact, in atomistic detail, which were made in experiments utilizing neutron diffraction [23], small-angle X-ray diffraction [24], various NMR and EPR techniques, in addition to fluorescence quenching [25] (the past two techniques use phospholipids containing labels in different positions). Some examples of the bilayer locations are given in Figure 1, where the cephalophilic, lipophilic, and amphiphilic molecules are shown located in the preferred positions in the headgroup region, in the hydrophobic core, or spanning the headgroup and/or core interface, respectively.

Why do transbilayer drug diffusion rates vary widely?

The diffusion equilibria in two aqueous compartments of subcellular-sized volume, separated by a bilayer of corresponding area are achieved by different drugs in the time periods varying from a split second to days. The difference in the transport rates can be explained by varying solvation of drug molecules in individual bilayer regions (Box 1). Slow transbilayer transport is observed for very hydrophilic or very lipophilic molecules because of weak or strong interactions with the hydrocarbon bilayer core, respectively [26]. The transport of amphiphiles is hindered by their strong interactions with the headgroup and/or core interface [27]. Strong interactions with the headgroups should have similar consequence for cephalophilic compounds [1].

The well-known pH-partition hypothesis [28] describes numerous observations (for pure phospholipid bilayers, biological membranes, cell monolayers, and biological sheets) that charged molecules are transported at a slower rate than their nonionized species. Charges of ionized drug molecules significantly affect the drug–bilayer interactions: they increase the strength of interactions with the headgroups and suppress the interactions with the bilayer core. This interaction pattern leads to either (i) a surfactant-like adsorption at the interface if the rest of the molecule is lipophilic and solvates well in the core or (ii) to strong solvation in the headgroups in other cases. The flip-flop of surfactant-like amphiphilic molecules from one interface through the core to the opposite interface can be very slow for compounds interacting strongly with the interface [27]. However, the authors' generalization that the presence of charges would completely halt the transbilayer diffusion has no published experimental support. Transport rates of several charged molecules through BLM are straightforward to measure and vary significantly with drug structure in a way that is incompatible with the pore permeation [29].

How does the variability of transbilayer diffusion rates fit in the transporters-only hypothesis? To explain the different transport rates, the authors postulate changes in the pore formation rate as a consequence of bilayer destabilization, which should be proportional to lipophilicity. There are several problems with this assumption.

The main issue is the lack of any experimental evidence, despite the fact that the conductance measurements, which would have recorded the increase in pore formation, were performed frequently. Namely, these measurements are used routinely in the BLM experiments to monitor the integrity of the bilayer between the aqueous phases containing

inorganic salts. Any change in pore formation rate would affect transport of small inorganic ions and increase conductance. No significant conductance changes were observed experimentally during drug transport, unless the drug molecules themselves were ionized [30].

The next problem is with the explanation of the pH-partition hypothesis by the asserted permeation through the pores. The charges of ionized molecules should not significantly affect the permeation through the pores, and the decrease in the transport rate for charged molecules should not be as significant as seen in the experiments.

The authors' proposal about compounds affecting the pore formation exhibits significant dichotomy in reasoning. The suggested dependence of bilayer destabilization on lipophilicity implies that the drug molecules need to accumulate in the bilayer to affect the pore formation rate. If the drug molecules are able to enter the bilayer from the donor aqueous phase, why could not they exit on the opposite side of the physically symmetrical bilayer, into the acceptor aqueous phase?

The authors' assumptions that both the rate of transbilayer diffusion and the extent of accumulation within the bilayer are proportional to lipophilicity are both incorrect. Lipophilicity (the logarithm of the reference partition coefficient P) alone cannot account for all drug–phospholipid interactions. Transport and accumulation depend on the affinity of drug molecules for headgroups (cephalophilicity), core (lipophilicity) or both phases (amphiphilicity), as illustrated in Box 1. For a complete description of drug accumulation in and transport through the bilayer for diverse compounds, all three types of interactions need to be quantified. Currently, these data or estimates are not available and the work using surrogate phases for the hydrated headgroups [31] is in progress.

If a studied data set is small and homogeneous so that all studied drug molecules exhibit intermediate-strength interactions with the headgroups and/or the headgroup and core interface, lipophilicity is the only rate-determining property. However, the logarithmic dependence of drug concentrations at a given time (a measure of transport rate) on lipophilicity is nonlinear and specific for the number of bilayers crossed and exposure time (Box 2). So the transbilayer transport rates of drugs and their accumulation in the bilayer do not change in parallel with drug lipophilicity, as the authors expect.

The inability of lipophilicity to completely describe transbilayer diffusion should not be construed as a proof that transbilayer diffusion in membranes does not exist. The authors' expectation that permeability through cell monolayers will be in exact correlation ('the slope and the correlation coefficient equal to unity') with the permeability through model membranes in the PAMPA assays or using lipophilicity ($\log P$) is unrealistic. Such correlation cannot be expected even for diffusion through or accumulation in two bilayers formed of different pure phospholipids, unless the studied series is small and homogeneous so that all drugs maintain invariant amphiphilicity and cephalophilicity, and the range of lipophilicity is narrow.

Transient pores present in phospholipid bilayers and absent in membranes

To make a case against transbilayer diffusion in membrane transport of drugs, the authors needed to explain, why diffusion that is readily observable in pure phospholipid bilayer systems cannot happen in physically similar bilayers of biological membranes. They argue that the pores are only formed in pure phospholipid bilayers and not in biological membranes, mainly because the proteins in membranes affect the properties of phospholipid bilayers. What is known about protein–phospholipid interactions?

Biological membranes are composed of a bilayer with embedded or adsorbed proteins in the amounts varying between 20–80 % w/w, with myelin sheath and mitochondrial membrane being the examples of the low-protein and high-protein extremes, respectively. This protein-lipid ratio leaves a significant portion of the bilayer surface exposed and available for diffusion. How do proteins affect the bilayer characteristics? Typically, integral membrane proteins interact with the bilayer core through hydrophobic residues and with the bilayer headgroup region through aromatic and charged residues [32]. From a lipid mixture in the membrane bilayer, proteins selectively attract the components with the molecular length of the lipid part similar to the height of their hydrophobic perimeter, to avoid hydrophobic mismatch [33]. This phenomenon may give rise to lipid rafts, which exhibit a tendency to structural defects at the interfaces with other lipid phases. Therefore, if the pore permeation of drugs would be a viable transport mechanism, it could actually be more pronounced in the cells than in the bilayers, which were made of pure phospholipids or defined mixtures of lipids not forming lipid rafts. This possibility is documented by the increased efflux of several compounds from phospholipid vesicles following incorporation of gramicidin A, an ion channel unsuitable for drug transport, while the data trends excluded the pore formation [29].

Other claims meant to support the transporters-only hypothesis

In addition to the main line of arguments described above, the authors present two other claims in support of their hypothesis: tissue accumulation of drugs and the asserted inability of quantitative structure–activity relationships (QSAR) to distinguish between the transbilayer diffusion and carrier-mediated mechanisms in membrane transport of drugs.

Drug accumulation in tissues caused only by transporters?

As a support for the transporters-only hypothesis, the authors claim that the drug accumulation in tissues is ‘often greater than any possible number of intracellular binding sites’, although no quantitative data or estimates were provided either for accumulation or the binding sites. Interactions with membrane phospholipids, which can occur both inside and outside the cells, are also an important accumulation mechanism [34]. At typical drug concentrations in the body, these interactions are not saturable and, hence, not characterized by specific binding sites. Most importantly, drug accumulation is often caused by solvation in specific media, in addition to the binding to macromolecules. A prominent example is drug accumulation in the fat tissue, mainly in triglyceride droplets representing up to 85% of the adipocyte volume. This environment accumulates lipophilic drugs to a significant extent because of much stronger drug solvation than in other tissues containing mostly water. The fat content of organs correlates well with accumulation of neutral, lipophilic compounds [35].

Ionizable molecules prefer to accumulate in the media where they are more ionized, again, because of more favorable aqueous solvation after ionization. Some cell organelles contain aqueous phases with the pH values differing from that of cytoplasm: lysosomes are at the low end with pH ~ 5 and the interior of mitochondria is at the opposite end of the range with pH ~ 8. Therefore, dissolved weak bases prefer lysosomes [36,37] and dissolved weak acids accumulate in mitochondria [48]. For instance, the cells of liver and kidney have a higher concentration of lysosomes [34,39] and mitochondria [40] than those of heart and muscle, and uneven accumulation of ionizable compounds in these organs can be explained by the solvation of ionized species. Finally, several drugs accumulate in cells simply because they precipitate in certain environments [41].

Summarizing, drug accumulation in tissues can be caused by various phenomena. Therefore, accumulation itself cannot serve as evidence that all accumulating drugs are actively transported.

Are QSAR equations really the same for transporters and transbilayer diffusion?

The authors state that the QSAR equations cannot be associated with the specific transport mechanism, because biophysical forces, hydrophobic and H-bonding interactions, are involved in both transbilayer diffusion and binding to transporters. The authors apparently do not distinguish between structure-specific binding of drugs to transporters and structure-nonspecific interactions with the bilayers, which is a concept that has been well recognized in the QSAR community for several decades [42]. For transporters, precise spatial positions of drug molecule parts are required to establish the binding with the binding site, whereas interactions with the headgroup and core regions of the bilayers do not have such limitations. Carrier-mediated transport is one of the reasons for the occurrence of cliffs in structure–activity relationships [43], whereas transbilayer diffusion results in smooth dependencies.

Structure-nonspecific interactions represent the basis for fragment-based approaches to quantitative prediction of the partition coefficients in the two-phase systems, with the 1-octanol/water system being the best known example. Such approaches do not work for structure-specific protein binding because of the importance of spatial locations of interactions. These locations can be specified by positions of the interacting groups on the parent skeleton, if the binding modes do not change [44]. Otherwise, or for more diverse skeletons, 3D-QSAR techniques [45] need to be used.

The Bio-Loom database [46] contains more than 500 examples of smooth, nonlinear dependences of bioactivities on the partition coefficients, mostly in homogenous series of compounds. The specific shapes of these smooth dependences can hardly result from structure-specific, cliffs-producing interactions with transporter binding sites. Rigorous simulations have been published, demonstrating that these shapes are in many cases in close agreement with transbilayer diffusion models (Box 2).

We have shown that in several cases of drug action in cell suspension, the kinetics of membrane transport does not need to be considered and a pseudo-equilibrium accumulation is sufficient to create good-quality, model-based QSARs for a limited series of compounds [47,48]. The agreement of the model with experimental data indicates that drugs were distributed according to the lipophilic-hydrophilic equilibrium, which eliminates the involvement of transporters. The advanced, model-based QSAR techniques, such as our cell-QSAR approach [48], are able to resolve whether the disposition underlying the analyzed biological effect is caused by passive diffusion or transporter-mediated processes. This issue may only represent a problem for low-resolution QSAR approaches, which select significant descriptors from large descriptor pools using usually linear combination of descriptors and neglecting possible mechanisms.

Claims with insufficient evidence value

The authors also present two types of arguments, which do not contribute to resolving the transport mechanisms question. The first type analyzes cellular data, which lack sufficient detail to address the title question. The second type of arguments simply emphasizes the growing number of known transporters but does not exclude transbilayer diffusion *per se*.

Drug transport through two cell monolayers

The authors expect that the permeabilities of drugs in Caco-2 and MDCK cell monolayers [49] would exhibit an exact match in case of transbilayer diffusion. They consider the two- to five-fold differences in drug permeabilities through the two cell monolayers on a hundred-fold scale as evidence against passive transbilayer diffusion. This opinion neglects the fact that transbilayer diffusion rates depend on the lipid composition of the bilayer, which varies widely depending on the function and differs especially between the barrier membranes and other membranes [50]. For cell monolayers, additional factors come to play, such as the bilayer area that is unobstructed by proteins; the number of crossed bilayers, including those of organelles and endoplasmic reticulum; and composition of intracellular aqueous phases. The difference in bilayer areas, which are available for drug diffusion, could explain the observed leveling-off for high Caco-2 permeabilities, which do not keep up with increasing MDCK permeabilities. The authors are of different opinion: 'The most obvious interpretation ... is that all the drugs tested are using carriers, but that, in some cases, it is not known which ones.' To support their statement, the authors attempted to identify transporter-substrate pairs beyond those mentioned in the original study [49]. They appear unconcerned with the fact that they cite transporter data for completely different cell types (e.g. corneal epithelium, aortic strips, adrenergic neurons, placenta, hepatocytes, kidney cells, adrenal cells, fibroblasts) from different organisms (e.g. human, rabbit, rat, mouse, *Escherichia coli*, even celery phloem). In addition, for quantitative agreement, not only expression but also concentrations and activities of the transporters must be similar in both cell lines. This seems to be a tough-to-meet requirement for the cell lines, which are derived from human colon (Caco-2) and dog kidney (MDCK) [49].

The authors apparently adopt different attitudes when making the conclusions about the transport mechanisms: they are overly rigorous when evaluating the diffusion and very tolerant when analyzing transporters. Although the presented evidence favors transbilayer diffusion mechanism, a fair conclusion is that there are insufficient data to decide this case. The measurement of transport through polarized cell monolayers in both directions would provide a decisive answer: if both the rates for a drug are equal, transbilayer diffusion is observed; otherwise the transporters are involved.

New data on transporters do not invalidate transbilayer diffusion as transport mechanism

On several occasions, the authors select specific facts they claim as the evidence in favor of the transport-only hypothesis. Usually, these cases only document the involvement of transporters but do not confirm exclusivity of the carrier-mediated transport.

The authors state that the significant number of studies reporting participation of specific transporters in membrane transport is the 'the first and most direct line of evidence' supporting the transporters-only hypothesis. They remind the reader of situations when transporter activities were discovered for compounds originally deemed to be transported passively (e.g. unspecified lipophilic cations, water, urea, glycerol). However, these studies do not exclude parallel involvement of transbilayer diffusion and cannot be generalized to other drugs.

The arguments about the number and promiscuity of transporters do not compromise the existence of transbilayer transport in cells. They would not need to be mentioned here, if not for the way the transporter numbers in individual species are reported. For instance, predictions based on sequence similarity to known or putative transporters and predicted transmembrane topology (TransportDB: www.membranetransport.org) are treated as confirmed values, although the TransportDB creators state that their automatic annotation pipeline still needs extensive curation for eucaryotes. The authors count in small-ion

transport proteins, improbable carriers for drug-like molecules, although their fraction among predicted transport proteins is significant and reaches 30–40%. They neglect the fact that all genes are not expressed in all cell types within an organism, and so the number of functional transporters in any given cell will usually be lower than those predictions. Another neglected aspect is represented by the concentrations of expressed transporters, which are much higher in barrier tissues than in other cells.

A few other specific cases that also have no evidence value are summarized below. In these issues, the conclusions are affected by the fact that the cited references are not complete. The authors state that glycerol is an osmolyte in yeasts but not in liposomes because of fast transport. Actually, a fast transbilayer transport of glycerol was observed only in experiments associated with the osmotic volume change of liposomes [50]. The shrinking and swelling bilayer in these experiments can hardly be similar to its natural state, and the occurrence of structural defects is highly probable. When measured in BLM, glycerol permeation is very slow – 26,000 and 12,000 times slower than that of codeine and nonionized butyrate, respectively [51].

The authors claim that only cells exhibit volume changes caused by osmolytes. However, liposomes exhibit similar changes as well [52,53]. In fact, light scattering, which monitors the liposome size changes caused by osmotic shrinking or swelling, was one of the first techniques for measurement of the transport rates of osmolytes.

The authors even brought in the case of general anesthetics and ethanol, reminding the reader that their effects are mediated by binding to specific receptors rather than by simple accumulation in membranes. They do not mention that this issue has nothing to do with the transport mechanism of general anesthetics.

How nonspecific must transporters be for transporters-only hypothesis to hold?

Many transporters, especially efflux pumps but also numerous influx transporters are promiscuous, while others (e.g. GLUT1) [54], are quite specific. The transporters-only hypothesis requires that the substrate specificity is as broad as possible, whereas the transporter mission of selective transport calls for limited promiscuity.

Published data on actives causing intracellular effects in high-throughput screening campaigns provide an estimate of substrate specificity, should the transporter-only hypothesis hold. For instance, the PubChem Assay ID 504834 (<http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid5504834>, assay provided by D. Fidock and E. Eklund, Columbia University) data set shows that 22,335 diverse compounds out of 323,212 tested compounds were active after 96 hours against the malarial parasite *Plasmodium falciparum* growing in erythrocytes. It can be assumed that the monitored inhibition of the development of apicoplast requires an action of these compounds inside *P. falciparum* cells, because the membrane-active compounds would attack the erythrocyte membrane first and be eliminated from the assay as toxic. Then all actives need to cross both the erythrocyte and protozoal cell membranes. Interestingly, only 100 transporter proteins are predicted for *P. falciparum* by genomic analyses summarized in TransportDB (www.membranetransport.org), among them 37 small-ion transporters, which are generally unsuitable careers for drug-like molecules. This means that each *P. falciparum* transporter would transport, on average, approximately 350 diverse compounds. In reality, this number must be higher because (i) some substrates of *P. falciparum* transporters would not pass through erythrocyte transporters, (ii) not all predicted transporters may be expressed in the *P. falciparum* cell membrane, and (iii) some *P. falciparum* transporter substrates may be inactive. Even though much remains to be learned about specificity of transporters, the minimum average requirement of several hundred diverse substrates per transporter still

seems to be an unrealistically high number. The promiscuity of most transporters would need to be enormous, to make the transporters-only hypothesis valid.

Concluding remarks

A comparison of the main claims of the authors of the transporters-only hypothesis with published results is summarized in Table 1. Available experimental evidence contradicts the authors' assertion about 'the widespread but erroneous nature of the assumption that the 'background' or 'passive' permeability to drugs occurs in the absence of carriers'. What is the relationship between transbilayer diffusion and transporter-mediated transport in the light of the presented evidence?

- i. Transbilayer diffusion proceeds even in the presence of transporters because the bilayers are present in all membranes and there is no evidence against drugs using transbilayer diffusion as a transport mechanism.
- ii. Transbilayer transport rates vary, depending on drug structure, in the range of a second to days. The contributions of transbilayer diffusion and carrier-mediated transport to overall membrane transport vary from drug to drug. The effect of transporters is most significant for compounds with slow transbilayer transport [55].

There is still a long way to go until we will completely understand diffusion and accumulation in bilayers in terms of drug properties. There are many gaps to be filled but these gaps should not be used to undermine the existing, experimentally confirmed elements of the phenomenon. A steady development of the knowledge about the transbilayer diffusion and transporters is a condition for our understanding of the complexities of membrane transport of drugs. Unjustified generalizations, such as the transporters-only hypothesis do not help this cause.

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Box 1. Interactions of drug-like molecules with the bilayer: transport versus accumulation

Drugs can accumulate in the bilayer to a significant extent: the molarity-based liposome/water partition coefficients span 5+ orders of magnitude. There is a connection between transport rate and the extent of equilibrium drug accumulation at the interface (amphiphilicity) and in the core (lipophilicity), as illustrated in Figure 2:

- Fast transbilayer diffusion is observed for drugs with intermediate strengths of interactions with all three bilayer regions, assuming that the conclusion can be extended to the headgroups.
- Too weak or too strong interactions with any bilayer region cause a slowdown.

Box 2. Transport-based activity-lipophilicity relationships for homogeneous drug series

Rigorous simulations using the transport rate constants k_i and k_o (Figure 2, row 2) measured in the two phase systems produced the concentration-lipophilicity profiles shown in Figure 3 (A, C) and other profiles. The dependencies are valid for the compounds which do not have extreme (too low or too high) amphiphilicities and cephalophilicities. The profiles have specific shapes: the slopes of the left and right (leftmost and rightmost in Figure 3C) linear parts are associated with the number of crossed bilayers as follows: in aqueous phases, the slopes are equal to $\pm n$ (n is the number of crossed bilayers); in bilayers, the slopes are n and $(1-n)$ [56]. The equation, describing the profiles is an extended form of the Kubinyi's bilinear equation [57]:

$$\log c \text{ or } \log(1/c_x) = A \log P + \sum_{i=1}^3 B_i \log(C_i P^\beta + 1) + D$$

Here, c are the drug concentrations and c_x are the isoeffective drug concentrations, eliciting the effect equal to the fraction X of the maximum effect. The slopes are given by the values of adjustable coefficients A and B_i . Coefficients C_i determine the positions of the curvatures. Coefficient β relates the bilayer lipophilicity to the reference partition coefficient P .

The close fits of the equation to the experimental data is shown in Figure 3 (B, D).

Highlights

The transporters-only hypothesis of membrane transport of drugs shows serious flaws

No published data support the proposed bilayer transport via transient aqueous pores

Drug molecules do not need pores to enter bilayer - accumulation is easy to observe

Tissue accumulation is caused by other mechanisms in addition to active transport

Trans-bilayer diffusion remains indispensable contribution to transport of many drugs

Figure 1. Experimentally determined locations of several compounds in the headgroups, interface, and core of phosphatidylcholine bilayer
The references and additional 30 examples, including the used techniques and references, can be found in Charts 1–3 and Tables 2–4 of a recent review [1]. These experiments require certain time for data collection therefore the drugs are captured in preferred, equilibrium positions.

Lipophilicity	Amphiphilicity	Overall Transfer Time	Compartment Number	Rate Constants in One Direction	Bilayer Compartment Model						
					Bulk Water	Headgroups	Interface	Core	Interface	Headgroups	Bulk Water
low	l/m	minutes to hours	2	PC							
medium	l/m	seconds	3	I_i, I_o							
high	l/m	minutes to hours									
low	high	minutes to days	4	I_a, I_f, I_d							
medium	high	minutes to hours	5	I_a, I_{dc}, I_{ac}, I_d							
high	high	hours to days									

Figure 2. Overall transfer times through the bilayer and compartmentalization of the bilayer for the description of passive transport of drugs as dependent on their lipophilicity and amphiphilicity

Lipophilicity, expressed as the 1-octanol/water partition coefficient, characterizes the accumulation in the core. Amphiphilicity as the tendency to accumulate at the core/headgroups interface has no established measure. High amphiphilicity is deduced from clear spatial separation of heteroatoms and carbons in the molecule; for other cases, l/m indicates low to medium amphiphilicity. The arrows indicate the transfer between two sites of accumulation. The placement of schematic phospholipid structures in the bottom right corner indicates their correspondence to bilayer regions. The overall transfer time is the time needed for a complete equilibration of a drug across a liquid-ordered bilayer in cell-sized compartments. The transfer rate parameters I are listed in the order of individual steps, from the donor aqueous phase (left) to the acceptor aqueous phase (right). The subscripts indicate: i – input, o – output, a – adsorption, f – flip-flop, d – desorption, dc – desorption to the core, and ac – adsorption from the core. PC stands for the permeability coefficient, which is a complete characteristics of transport only if the bilayer behaves as an inert barrier and no significant accumulation is seen in any region. For the backward processes, the order of individual steps is in the right-to-left direction.

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Figure 3. Comparison of simulated (A,C) and experimental (B, D) concentration-lipophilicity profiles

Simulations were performed in a system of alternating water (5) and bilayer (5) phases after different periods of distribution: (A) 10 time units and (C) 600 time units. The colors indicate the concentrations in the 1st (red) and 5th (green) bilayers. The blue lines are valid for the 3rd bilayer in (A) and for the preceding aqueous phase in (C). The equilibrium curve for all bilayers (A, C, D) is shown in black. (B) Mutagenicity of alkyl amides of 3-(5-nitro-2-furyl)-acrylic acid against *Salmonella typhimurium* TA100 rfa1 (green, contains two bilayers) and rfa- (blue, contains one bilayer), as dependent on the partition coefficient P [37]. The concentration c_X elicits 600 revertants per plate. (D) Growth inhibition of *Ctenomyces mentagrophytes* by alkyl amines (c_X is the minimum inhibitory concentration) as dependent on P [56]. The blue curve represents the mixed period of distribution (i.e. 2), when some compounds already reached the equilibrium (where blue and black curves overlap). The slopes of the full curves (B, D) are in perfect agreement with the simulation results, although in used scaling, they are not identical because the parameter $\beta=1$ for simulations and $\beta\sim 0.6$ for experimental data. Additional 13 cases of very close fits of model-based equations to the dependencies of antimicrobial activities on lipophilicity are summarized in Tables 8 and 9 of ref. [1].

Table 1

The transporters-only hypothesis and experimental evidence.

Claim	Analysis summary
Drug transport through pure phospholipid bilayers proceeds only through transient pores	<ul style="list-style-type: none"> No valid experimental evidence presented This mechanism is experimentally only documented for H⁺ and K⁺ in thinner bilayers, and considered for other small ions and small molecules Drug accumulation in bilayers shows that drug molecules do not need transient pores to enter bilayers Transport through transient pores does not explain <ul style="list-style-type: none"> the widely varying rates of transbilayer drug transport pH-partition hypothesis Spectroscopic techniques capture changes in the polarity of environment of transported molecules, which are more pronounced for the diffusion than for transient aqueous pores
Transient aqueous pores are present only in pure phospholipid bilayers and are absent in membranes	<ul style="list-style-type: none"> Bilayers in membranes are more prone to structural defects than the phospholipid bilayers because of selective accumulation of certain lipids around membrane proteins to avoid hydrophobic mismatch This view is supported by the increase in transport rates of several drugs by incorporation of gramicidin A, an ion channel that is unlikely to transport drugs, into a bilayer, while the pore formation was excluded.
Drug accumulation in some tissues is caused solely by active transport	<p>Carrier mediated transport is not the only mechanism responsible for drug accumulation in tissues. Other mechanisms include:</p> <ul style="list-style-type: none"> solvation of lipophilic drug molecules in fat tissue accumulation of ionizable drugs in cell organelles where they are more ionized (weak bases in lysosomes, weak acids in mitochondria) – tissues rich in these organelles may exhibit accumulation precipitation of some drugs in some environments
QSAR equations cannot distinguish between transport mechanisms because the involved drug interactions are the same	<ul style="list-style-type: none"> True only for low-resolution QSAR approaches applied to large data sets QSAR modeling of transbilayer transport is simpler than that of carrier-mediated transport yet there are unsolved problems Rigorous models derived for simpler cases or transbilayer transport are in close agreement with experimental data
Transporters are numerous and promiscuous	<p>Although this statement is true in general, it does not rule out transbilayer transport. Some related observations:</p> <ul style="list-style-type: none"> The cited predictions include the transporters of small ions (approximately 30–40% of transport proteins), which are unlikely to carry drugs Substrate specificity of transporters varies and is difficult to assess Available experimental data on growth inhibition of <i>Plasmodium falciparum</i> in erythrocytes indicate that several hundred diverse active molecules would need to be transported by each <i>P. falciparum</i> transporter on average, if the transporters-only hypothesis holds.