



# Pharmaceutical drug transport: The issues and the implications that it is essentially carrier-mediated only

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All cells necessarily contain tens, if not hundreds, of carriers for nutrients and intermediary metabolites, and the human genome codes for more than 1000 carriers of various kinds. Here, we illustrate using a typical literature example the widespread but erroneous nature of the assumption that the 'background' or 'passive' permeability to drugs occurs in the absence of carriers. Comparison of the rate of drug transport in natural versus artificial membranes shows discrepancies in absolute magnitudes of 100-fold or more, with the carrier-containing cells showing the greater permeability. Expression profiling data show exactly which carriers are expressed in which tissues. The recognition that drugs necessarily require carriers for uptake into cells provides many opportunities for improving the effectiveness of the drug discovery process.

## Introduction

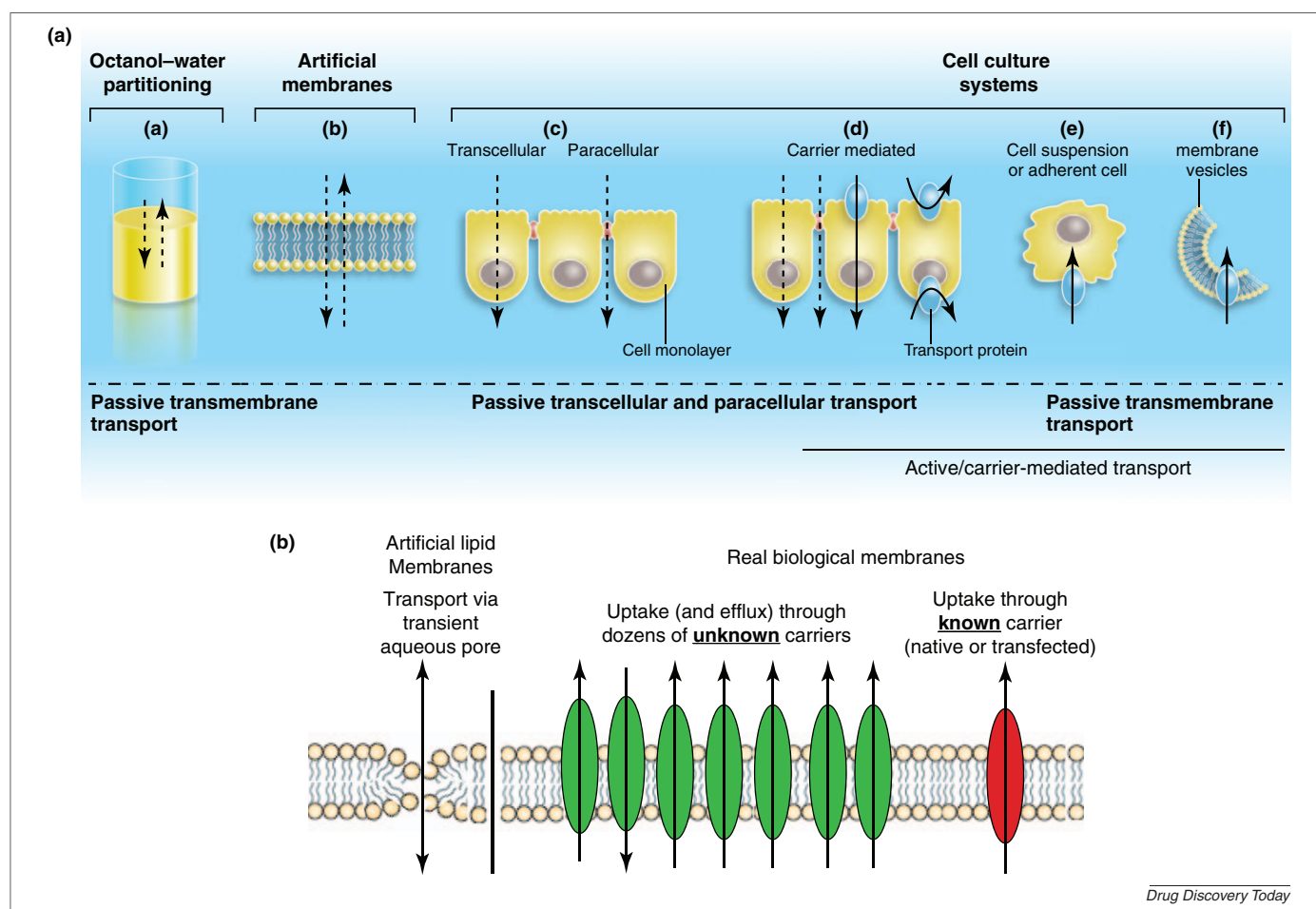
There is considerable and increasing evidence that drugs get into cells more or less solely by hitchhiking on carriers normally used for the transport of nutrients and intermediary metabolites [1–38]. This said, one can find reviews (e.g. [39,40]) that play up the importance of a different cellular route of uptake of pharmaceutical drugs considered to be occurring by simple diffusion through the hydrophobic lipid bilayer portions of biological membranes (and thereby strongly dependent on lipophilicity). Such works also lay stress on studies using artificial membrane systems lacking any proteins, although, as we shall see below, the relevance of such systems (given that the protein:lipid ratio in biological membranes is 1:1–3:1) is at best questionable.

A particular example of a detailed review seeking to provide evidence for the 'mainly bilayer' mode of transport is a recent article by Sugano *et al.* [40], which sought to show that the well-established carrier-mediated transport effecting the cellular uptake of pharmaceutical drugs [1,10,13,14,17] nevertheless coexists with a so-called 'passive' uptake of comparable magnitude, mediated

via the bilayer portions of biological membranes. (In the literature, 'passive' normally means non-concentrative, including facilitated diffusion via a carrier; however, where appropriate in this article, we adopt the usage of Sugano *et al.* [40] to imply, as in their Fig. 1 and Box 3, transport through a bilayer portion of a phospholipid cell membrane.) That review [40] served to highlight some of the reasoning used by those who believe in the importance of trans-bilayer transport and the evidence that is purported to underpin it; therefore, the article is helpful in highlighting where the intellectual issues lie. Consequently, we focus several strands of our argument here on those detailed by Sugano *et al.* [40].

At the outset, one should comment that, despite the considerable literature surveyed [40], the title assertion of [40] [a coexistence of such 'passive' and carrier-mediated processes in (cellular) drug transport] fails, because it is simply that (an assertion). Specifically, the authors (and much of the literature they cite) make (and repeat) the same assumption throughout, which is that anything that is not taken up using a carrier that they know about therefore goes through the presumed bilayer portion of the relevant biological membrane, rather than through carriers they either do not know about (in biological membranes) or through

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**FIGURE 1**

Different views of the transport of pharmaceutical drugs across biological and artificial membranes. **(a)** The mechanisms discussed by Sugano *et al.* [40], in which it is assumed that: (i) drugs can transfer directly across the lipid bilayer portion of artificial membranes; (ii) such bilayers are present in natural membranes with properties little or no different from those of the artificial membranes (e.g. that the high protein content of biological membranes does not influence them); and (iii) biological membranes exist with negligible carrier activity. **(b)** A view in which we recognize that: (i) all biological membranes have tens if not hundreds of carriers (even mitochondria have more than 50 carriers [166]); and (ii) most are of as yet unknown specificity and can therefore contribute to the 'background' uptake of any drug, occurring in the absence of any known native or transfected carrier. This view also recognizes that much of the transport, especially of the more hydrophilic molecules, that occurs 'through' artificial membranes in fact occurs via transient aqueous pores that form on a nanosecond timescale [117,118].

transient aqueous pores that occur widely in artificial lipid membranes but not in real biological membranes. When these features are taken into account, the actual requirement, and any evidence, for such 'passive' permeation disappears. Although it is possible that such trans-bilayer 'passive' permeation occurs at meaningful rates, there appears to be no compelling evidence as yet that any pharmaceutical drug crosses real cell membranes by passing through the hydrophobic portion of a phospholipid bilayer. However, as noted above, [40] does provide an excellent basis to help focus on some key issues. We therefore compare the assumptions made by Sugano *et al.* (Fig. 1a; redrawn from Fig. 1 of [40]) with the mechanisms that they did not include (Fig. 1b) and which, in our view, are dominant.

### Nature of 'passive' transport in different cell types

Sugano *et al.* [40] assert 'basic passive transcellular transport occurs regardless of cell type (for example between *in vivo* organs and *in*

*vitro* cells). The extent of passive transcellular transport could be dependent on the lipid composition of the membrane, but is usually of comparable magnitude between different cell types'. However, the well-established existence of the blood-brain barrier (BBB) shows that it can vary considerably between different cell types. The BBB is generally impermeable to most drugs (e.g. [41–45]) as tight junctions preclude paracellular transport; however, although its lipid composition is not seen as particularly atypical [46], the BBB does contain many transporters that do allow selective uptake (e.g. [47–59]) as well as catalyzing efflux (e.g. [60–68]). In addition, methods that use chemoinformatic substructural analyses (which would detect determinants of transporter substrates [12]) are at least as effective at predicting BBB uptake as are those based on descriptors such as lipophilicity (e.g. [59,69]). Finally, several studies (e.g. [70–74]) demonstrate the utility of delivering drugs to the central nervous system (CNS) as prodrugs designed to be taken up via known carriers. This shows that,

although there are efflux carriers at the BBB, they alone cannot account for the apparent impermeability of most drugs observed. The BBB also contains many tight junctions, but these would affect only paracellular and not transcellular permeability (Fig. 1a).

### The permeability of Caco-2 versus MDCK cells to drugs

One way of assessing the claim that 'background' rates are similar in different cells is to compare them directly. Thus, Sugano *et al.* [40] state 'For instance, Caco-2 cells (derived from the human colon) and Madin–Darby canine kidney (MDCK) cells show similar magnitudes of passive transcellular transport [75]. Therefore, in general, a lipophilic drug that displays a high passive transcellular transport across the intestinal epithelial cell membrane may also display a high passive transcellular transport across, for example, the sinusoidal and canalicular cell membranes of the hepatocyte.' However, the reference cited [75] presented no evidence that the drugs they labeled 'passively permeable' (in any case an ambiguous term; see above) did cross the bilayer 'passively', that is, without a carrier. Moreover, a careful analysis of their data suggests that carrier-free 'passive' permeability is unlikely to be the transport mechanism. First, the relationship between the uptake into the two kinds of cell is obscured by the use of a log–log plot; for 24 of the 55 drugs tested (Table 1 of [75] and the present Fig. 2a), the rates vary more than twofold between the two cell types. Secondly, comparison of the data in Table 1 and Fig. 1 of [75] enables one to identify which (11) of the 55 drugs are claimed to be uptake (seven) or efflux (four) transporter substrates and which (44) are thus supposedly taken up 'passively'. Of these 44, we can find clear literature evidence (see also [10]) for the carrier-mediated transport of 18 of them, namely acebutolol [76], acyclovir [77], atenolol [78], bretylium [79–81], bupropion [82], cefuroxime [83], chlorothiazide [84,85], gabapentin [86,87], hydrocortisone (cortisol) [88,89], ketoprofen [90], mannitol [91,92], methylprednisolone [93,94], phenytoin [95–97], progesterone [98], propranolol [99], testosterone [100] and trimethoprim [101].

The assumption that these molecules cross membranes only 'passively' is, therefore, incorrect. Of course, we cannot say much about the other drugs for which carriers have not (yet) been found. Nevertheless, it would be surprising if the others did not have carriers, because, for instance, we know of many carriers for substances that are closely related structurally to the substrates for which we did not find explicit literature evidence for carrier-mediated uptake. Thus, there are carriers for steroids [102], penicillin G [103] (rather than penicillin V as studied by Irvine *et al.* [75]), as well as for many other  $\beta$ -lactams [104–108], and so on [23]. We thus re-plot the data of Fig. 1 of [75] in Fig. 2b, where it can be seen that the general shape of the plot (as in the original, in fact) is the same whether carriers are known to be used or not. The most obvious interpretation of this plot, then, is that all the drugs tested are using carriers, but that, in some cases, it is not known which ones.

The membranes of any cells, including Caco-2 and MDCK, necessarily contain tens, if not hundreds, of carriers for all kinds of nutrients and intermediary metabolites and it is probably with these 'unknown' carriers that pharmaceutical drugs hitchhike a lift to effect their entry into cells (e.g. [4,9,10,12,13]). More generally, it is becoming increasingly clear from genome-wide association studies that many individual proteins contribute what are individually small amounts to what might be a sub-

stantial phenotype [109–111]. Drug transporters are unlikely to be an exception.

### What are the proper controls for so-called 'passive' uptake?

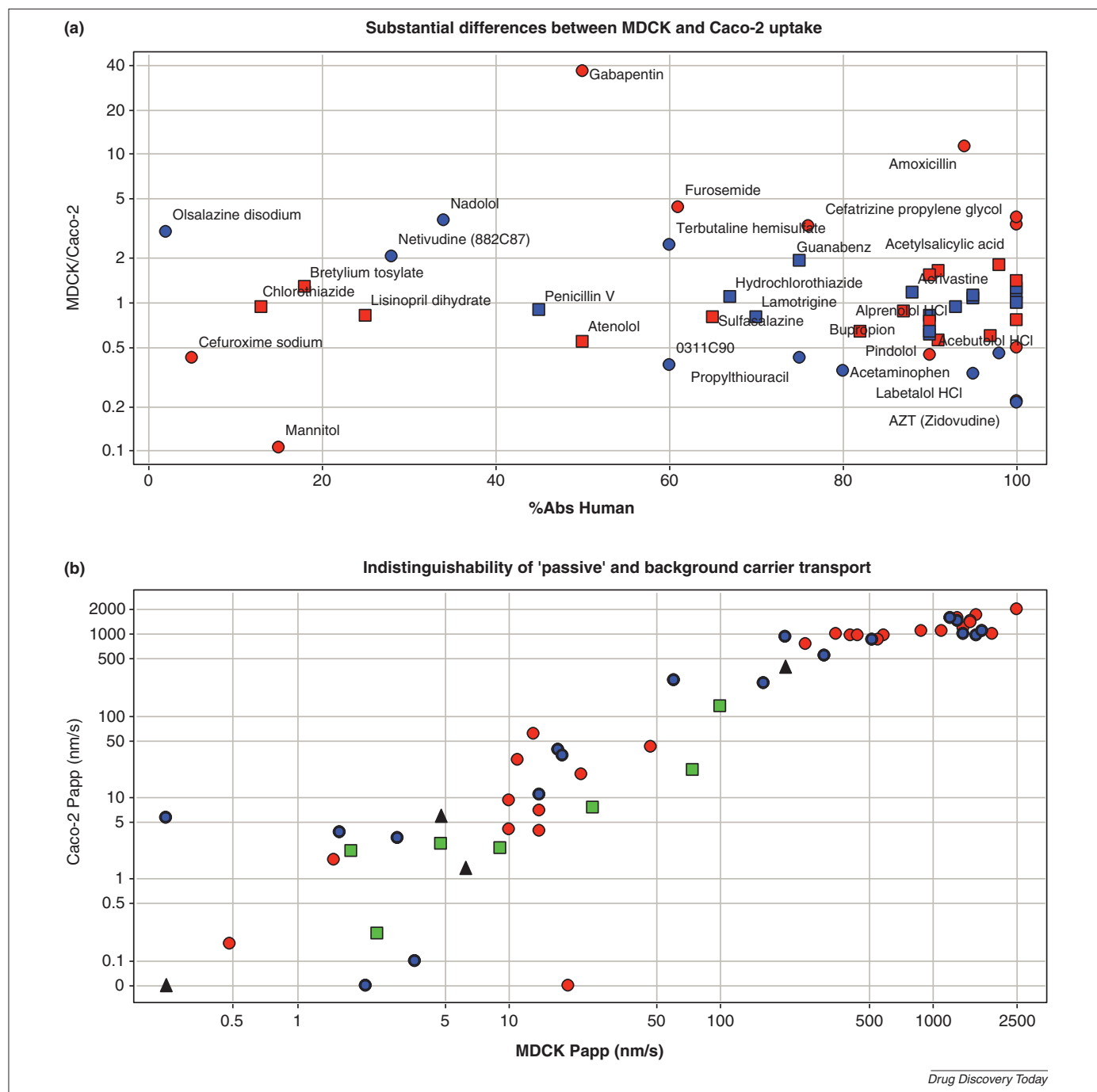
Sugano *et al.* [40] assert 'usually when transfected cells are used, non-transfected cells (mock) are simultaneously used as a control experiment to evaluate the contribution of passive transcellular membrane transport.' However, non-transfected cells are emphatically not such a control, because they naturally contain many carriers (scores if not hundreds), and it is not correct to assume that unknown carriers are either absent or irrelevant when assessing 'background' rates of transport in the absence of any known transfected carrier. Although such non-transfected cells might provide a background that enables the determination of the incremental contribution to drug transport of a transfected carrier, they tell one nothing about the mechanisms of any 'background' uptake. The same applies to the assertion [40] (from a survey of carrier-mediated uptake papers) 'Clear indications of the involvement of passive transport were presented in 81 cases. In 46 cases, passive transcellular transport contributed to more than 30% of total uptake and/or permeance.' However, this 'background' uptake could have occurred by any means, most plausibly via the numerous carriers that were present but not identified, as in the case of [75] discussed above.

### Known transporters encoded by the human and other genomes

Much is now known about the transporters encoded by the human [112] and other genomes. Thus, the analysis given online at <http://www.membranetransport.org/> indicates that the human genome encodes 1022 (uptake and efflux) membrane transporters (0.32 per Mbase genome), with the numbers for some common model eukaryotes being *Arabidopsis thaliana* 1210 (9.68), *Caenorhabditis elegans* 654 (6.74), *Drosophila melanogaster* 603 (5.03), *Mus musculus* 1090 (0.4) and *Saccharomyces cerevisiae* 318 (24.46). This set of eukaryote data is shown in Fig. 3, from which it is clear that all higher organisms contain genes for hundreds or thousands of carrier proteins.

### The properties of artificial phospholipid bilayer membranes lacking proteins

One potential way round the issue of transport by unknown carriers is to study it in artificial 'membranes' that do not contain proteins. Thus, Sugano *et al.* [40] state 'A good, but not perfect, choice for such a reference membrane is the black lipid membrane model and unilamellar vesicles (liposomes)'. Unfortunately, such models are entirely inappropriate, precisely because, by lacking proteins, whose ratio to lipids in real membranes is in the range 1:1–3:1, they do not represent biological membranes. Moreover, they are essentially leaky via the formation of transient hydrophilic pores (Fig. 1b). Although these enable molecules to cross such membranes, this is by a mechanism that does not involve their transfer through the hydrophobic portion of the lipid bilayer. This is most obvious when these molecules are charged, because they do appear to cross bilayer membranes (e.g. [113]); however, the enormous Born charging energy required [114–116] means that this cannot be other than via hydrophilic pores, which form



**FIGURE 2**

Reanalysis of the permeability properties of MDCK and Caco-2 cells as published in [75]. **(a)** Comparison of the rates of drug transfer in the two cell lines, expressed as a ratio, as a function of the percentage absorbed in humans. It is clear that, far from being similar as claimed [40], the rate of uptake of individual drugs across the two cell lines varies considerably [by a factor of at least twofold (circles) in 24 of the 55 drugs tested]. The degree of variation does not appear to depend on whether a carrier is already known (red) or not (blue). The permeability of mannitol in MDCK cells is indistinguishable from zero (unsurprisingly, as it is a well-known osmoticum); in addition, three drugs (acyclovir, ranitidine and sumatriptan succinate) are not shown because their permeability through Caco-2 cells is indistinguishable from zero. The abscissa reflects the percentage of an oral dose that is absorbed by humans *in vivo*. **(b)** Independence, on whether a carrier is known, of the relationship between drug uptake by MDCK and Caco-2 cells. Drugs are classified into whether they were assumed [40,75] to be 'passively' permeable through a membrane bilayer portion (circles), actively taken up (squares), or actively effluxed (triangles). Of the 'passively permeable' drugs, 18 have known carriers (blue), whereas, for others, no carrier is, as yet, known (red).

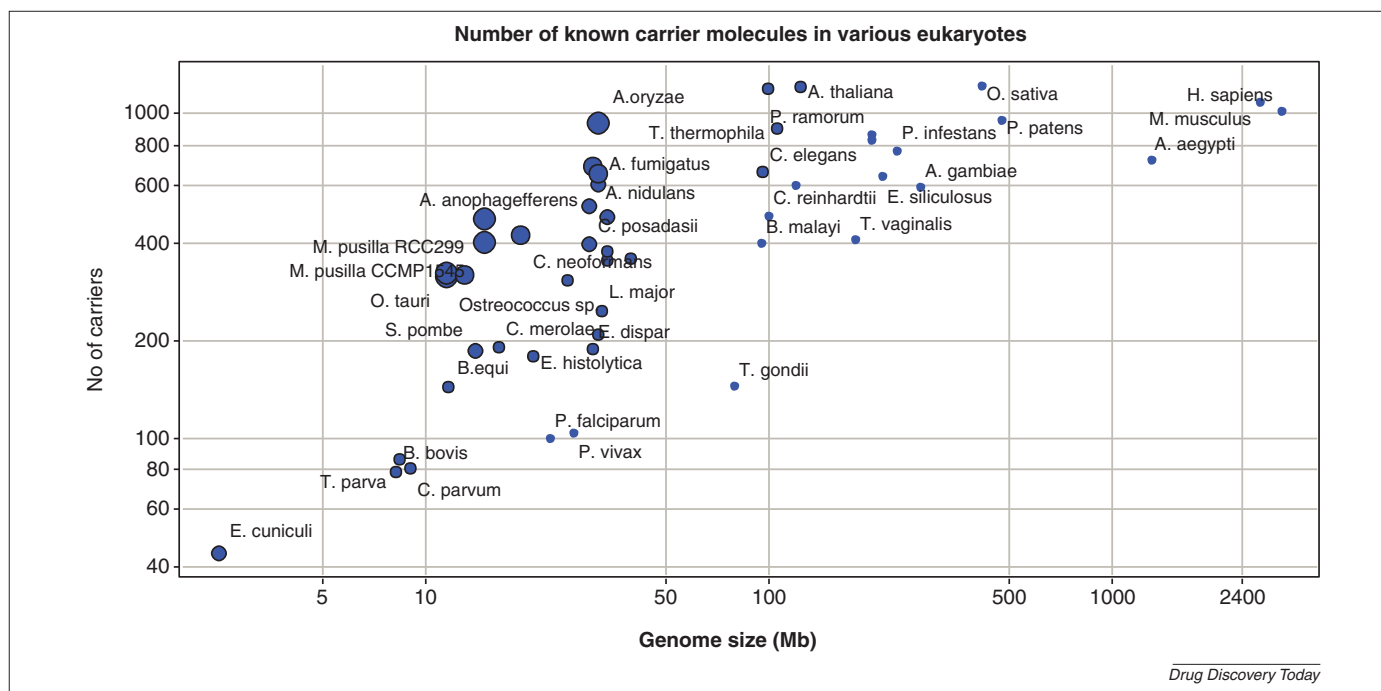


FIGURE 3

The number of carriers known to be encoded within the genomes of various eukaryotes, plotted against their genome size. Data are taken from <http://www.membranetransport.org/>. In addition, the size of the symbols is proportional to the number of carriers per Mbase of genome. It is evident that, in most cases, hundreds of carriers are already known from each type of organism.

frequently and spontaneously (on a nanosecond timescale) in artificial phospholipid bilayer membranes [117,118]. However, this cannot be occurring in real biological membranes because, if it did, they would exhibit no significant osmotic properties and any osmolytes added externally (such as mannitol or sorbitol, as in typical biochemical incubation media) would be able to cross the membrane freely and would thus not serve as osmotic.

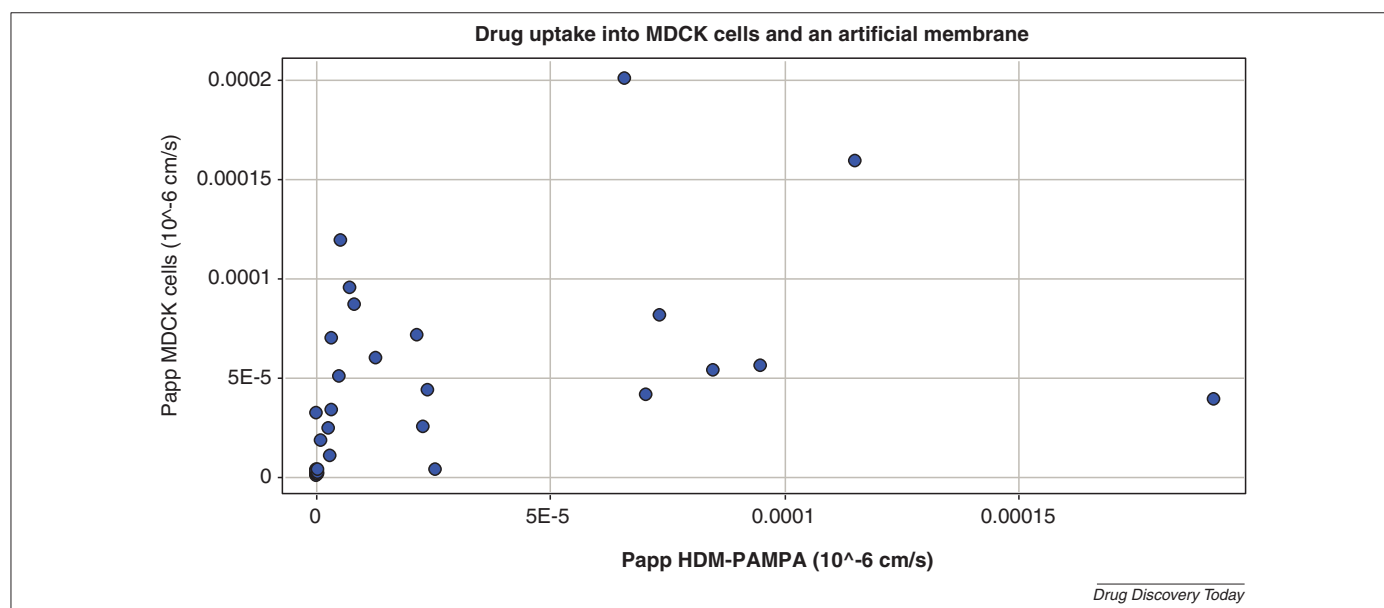
### Black lipid membranes and liposomes versus real biological membranes

Sugano *et al.* [40] also state 'In black lipid membranes and liposomal membranes, numerous reports suggest that compounds with mid to high lipophilicity [e.g. a  $\log D_{\text{oct}}$  {i.e. octanol:water distribution coefficient} > 0] [119] rapidly permeated, whereas compounds with low lipophilicity slowly permeated (for example, glycerol ( $\log D_{\text{oct}} = -1.76$ ) and urea ( $\log D_{\text{oct}} = -1.66$ )...). These studies indicate that many drug-like compounds can pass through the lipid bilayer in proportion to their lipophilicity [120]'. Although these studies do indicate this (although there are plenty of outliers, and in fact an entirely different relationship is shown in Fig. 4 of ref. [40]), they indicate neither how this is done, nor what relevance this has to real biological membranes. It is well known that many hydrophobic molecules can disrupt membrane integrity, the effect being greater, within a given structural class, for more hydrophobic molecules [121]. Thus, more hydrophobic molecules are more likely to disrupt the black lipid membrane or liposome structure and thereby induce aqueous pores. A plot is also given (Fig. 5a in [40]) that, although not identifying the specific drugs representing each data point, does imply a 'correlation' (coefficient) of 0.84 between the permeability of drugs into

MDCK cells and their permeability in an artificial membrane assay containing hexadecane, 'suggesting that passive transcellular transport is dominant in the permeation of these compounds in MDCK cells' [40]. Although this interpretation would not follow from the data even if the correlation coefficient were unity, the relationship is far from being close. This is because the slope of the log-log plot is not close to 1; instead, it is approximately 0.5. Thus, at the lower end, there is nearly a 100-fold discrepancy in the absolute fluxes; that is, the real biological MDCK cells, which contain carriers, take up the less permeable drugs approximately 100-fold more rapidly than do the artificial membranes. If there were a true correlation (whatever its interpretation), it should manifest itself linearly. We plot the digitized data in linear coordinates in Fig. 4, where it can be seen that the linearity of the relationship is poor; the slope of the 'best' straight line fit is again 0.5, but the correlation coefficient is now only 0.45.

Table 1 of [40] also contains a list of papers that seek to relate various measures of cellular uptake to the behavior of drugs in various artificial membrane assays. Leaving aside the fact that there are many flavors of these assays, such that they are often 'tuned' to optimize the correlations that can be found [10], and there is rarely a separate validation set [122], it is of interest that correlations are poor. Let us take one example ([123], Ref. 100 of [40]), chosen because the artificial membrane is referred to as 'biomimetic'. What this paper (and our Fig. 5) shows (before extra corrections are introduced) is similar to what is described in the previous paragraph: a log-log plot in which the permeability of the less permeable molecules is (in this case) typically greater in the Caco-2 cells, in which the slope is 0.59 and the linear correlation coefficient is again 0.45.



**FIGURE 4**

Relationship between the rate of drug uptake by MDCK cells and by an artificial membrane system containing hexadecane (HDM-PAMPA). Data are re-plotted (using the Ungraph software from Elsevier Biosoft) from Fig. 5 [40], but on a linear scale. The slope of the 'best fit' line is 0.5, but this is not shown as the correlation coefficient is only 0.45. The low correlation coefficient is not surprising because there are discrepancies of typically two orders of magnitude in the absolute rates (see also [40]), with the MDCK cells (which contain carriers) effecting the faster uptake.

### The non-significance of structural specificity to enzymes

Much is made in these kinds of discussion (e.g. [40]) of the supposed difference between the assumed structural specificity towards the substrates of carrier molecules and its essential independence (other than on lipophilicity) from 'passive' transport: 'Correlation between indicators of biological membrane permeation and passive permeation (the whole molecule physicochemical property and artificial membrane permeation) for structurally diverse compounds also suggests that passive transcellular membrane transport of drugs exists' [40]. Balaz [39] also focuses on the importance of specificity in carrier-mediated uptake. However, this ignores the fact that biological membranes are replete with transporters and that many enzymes, such as various cytochromes P450 (e.g. [124]), or transporters, such as the organic anion transporting proteins [125], PEPT1/2 [106–108] and the P-glycoprotein carrier [63–67,126–128], have very broad specificities [129]. Furthermore, narcotics (general anesthetics), which are a structurally diverse set of molecules once thought [130] to depend for their activity solely on lipophilicity, interact rather specifically with their target proteins (e.g. [10,13,14,131–139]). Thus, although a degree of specificity (especially stereospecificity) can be used to imply a role for enzymes and carriers, its absence (especially in the absence of knowledge of the actual specificities of the carriers involved) cannot really tell one anything.

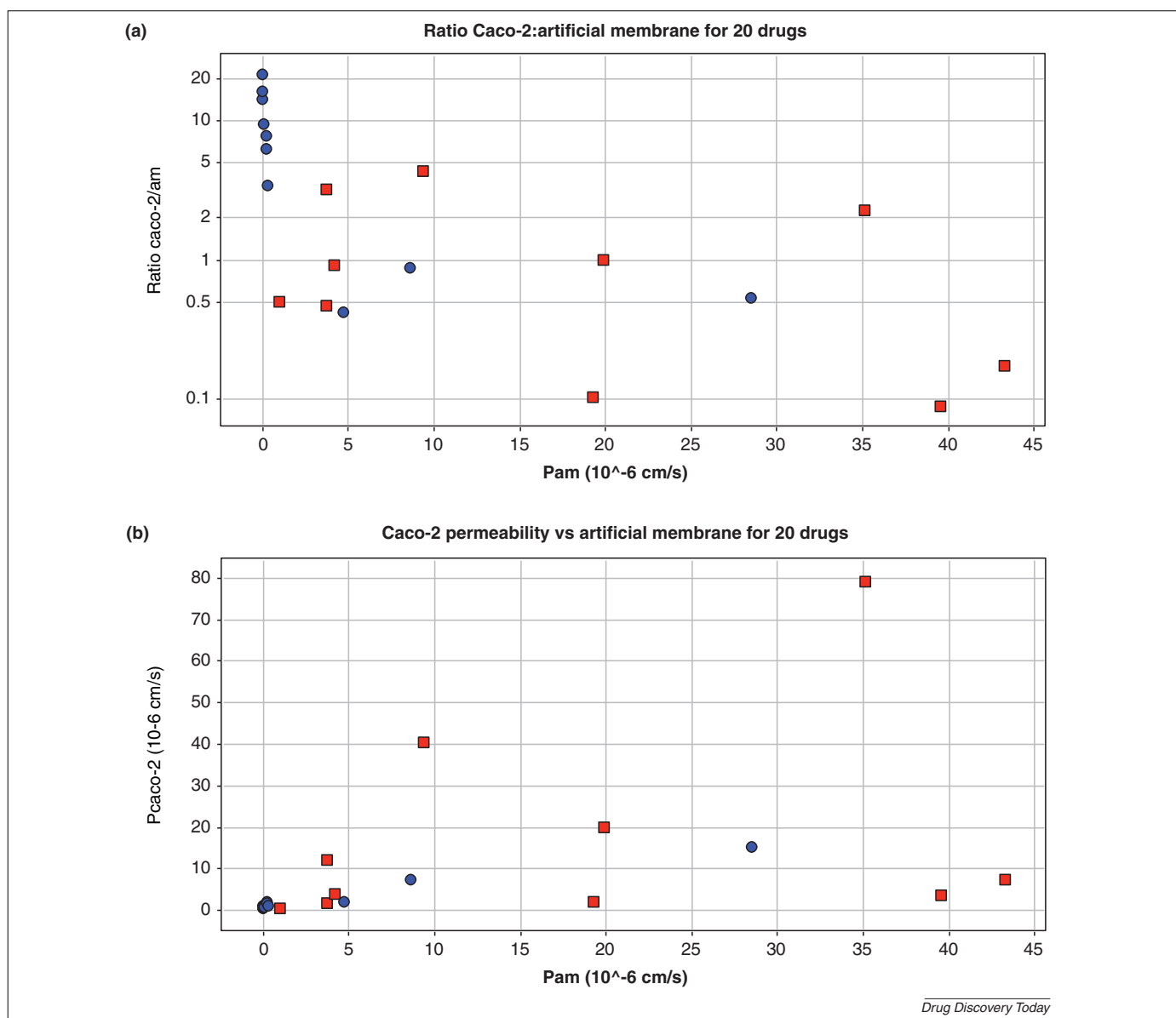
### Expression profiling of solute transporters in biological cells

A useful method to find out which carriers are present in real (biological) cell lines and membranes, and, therefore, to study their properties, is to carry out expression profiling analyses. This is starting to be done, for instance via transcriptomics [140–143], and

it is known that the plasma membrane of Caco-2 cells contains over 1000 proteins, including several hundred transporters [144–148], of broad (but usually unknown) specificity, whereas the membranes of MDCK cells contain over 800 proteins [149]. Searching the Pharmacogenomic Knowledge Base with the query 'transporter' and limiting the results to genes (<http://pharmgkb.org/search/search.action?typeFilter=Gene&exactMatch=false&query=transporter>) gives more than 500 separate genes (500 being the maximum that can be displayed in this way). The antibody-based Human Protein Atlas (HPA; <http://proteintlas.org/>; [150,151]) now lists expression profiles in multiple cells and tissues of more than 350 solute carriers, and of over 240 of the 461 proteins labeled ([http://www.tcdb.org/hgnc\\_explore.php](http://www.tcdb.org/hgnc_explore.php)) as transporters. Here, we highlight two examples from searching the HPA. The first ([http://proteintlas.org/tissue\\_profile.php?antibody\\_id=15468&g\\_no=ENSG00000197208](http://proteintlas.org/tissue_profile.php?antibody_id=15468&g_no=ENSG00000197208)) shows that the organic cation transporter (member 4 of the SLC22 solute carrier family [152]) is expressed in most cells. A second (using the 'advanced search' facility) shows that HeLa cells express 154 different transporters. Although data are not yet available for cells such as MDCK or Caco-2, the expression profiles available in the HPA no longer make it possible to claim that transporters are not expressed in very large numbers in all cells tested. We also highlight here the experimental analyses of transporter proteins in mice [55], the BBB [57] and human intestine [153], all of which illustrate the presence in every cell studied of a considerable number of 'background' transporters. To take just one example, MDCK cells even benefit from a special carrier for urea [154].

### Stereospecificity and enzyme kinetics

Although stereospecificity of uptake is unlikely to be observable for any passive diffusion mechanisms, any stereospecificity of uptake

**FIGURE 5**

Relationship between Caco-2 permeability ( $P$ ) and permeability through an artificial membrane ( $am$ ) in a published experiment [123]. Data were digitized from Fig. 6a of [123]. **(a)** Ratio of uptake into Caco-2 cells to that of the artificial membrane versus that of the artificial membrane alone. Only four of the 20 drugs are within a factor 2 of each other. **(b)** Poor linear 'correlation' between Caco-2 cell and artificial membrane permeability. The slope is 0.59 and the correlation coefficient is 0.45. Drugs are classed (by [123]) as hydrophobic ( $prologD > 0$ ; blue squares) or hydrophilic ( $prologD < 0$ ; red circles).

observed could be taken as good evidence for carrier mediation (albeit that the converse is not true, as many enzymes are highly promiscuous [129,155,156]). However, Sugano *et al.* [40] also ascribe some unusual properties to carriers. Thus, 'as carrier proteins are made of chiral amino acids, carrier-mediated transport is stereospecific' [40]; however, stereoselectivity comes from the three-dimensional arrangement of atoms in the protein that bind the substrates, and the fact that there are multiple interactions between protein and substrate [157–159]; this would be a sufficient condition whether the building blocks of proteins were inherently chiral or otherwise. Another comment reads 'Analyses of concentration dependency data using kinetic models, such as the Michaelis–Menten equation, are also used to differentiate carrier-mediated transport from passive transcellular membrane transport. According to the Michaelis–Menten equation, the fraction of a permeant that crosses the membrane by passive transcellular membrane transport can become more significant when the concentration of the permeant is higher than the  $K_m$ ' [40]. This is a curious interpretation of standard enzyme kinetics, because the rate of an enzyme-catalyzed reaction obeying the Michaelis–Menten equation continues to rise monotonically with its substrate concentration, the amount being transported therefore increasing with its substrate concentration, and with the absolute rate being determined more by  $k_{cat}$  than by  $K_m$ .

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## Concluding remarks

Given that the kinds of experiment being claimed, repeatedly, to support a significant 'passive' (carrier-independent) permeability of pharmaceutical drugs across the bilayer portion of biological membranes are both commonly performed and commonly misinterpreted, we have found it useful here to highlight our view of some of the issues, which we now do in summary form below:

- 1 All biological membranes contain tens and possibly hundreds of different kinds of transporter molecule.
- 2 The presumed absence of evidence for transporter molecules (or the ignoring of it) is not evidence of their absence.
- 3 Modern expression profiling methods, especially proteomics, can determine which transporters are present in different cell types.
- 4 Most of these proteins are of unknown specificity and catalytic power ( $k_{cat}/K_m$ ).
- 5 Some proteins have wide substrate specificity, and will use a suite of substrates with imperceptible structural relatedness.
- 6 Consequently, these many native transporters, each possibly contributing only weakly (but with some predominating in specific cases) are most likely, together, to be responsible for the 'background' permeability of most cells to various drugs. This can be tested by determining their expression profiles and studying the properties of the carriers directly.
- 7 Artificial (lipid or other) membranes lacking these or other proteins cannot be used to assess the role of transporters in pharmaceutical drug uptake in biological cells.
- 8 Any correlations between transport across an artificial membrane and a real biological membrane cannot have a mechanistic basis because the membranes are different; for example, real biological membranes have protein:lipid ratios of 1:1–3:1, not 0:1.
- 9 Unlike biological membranes, artificial bilayers are leaky and form transient aqueous pores that enable the transfer of small molecules across the membrane; such molecules do not *per se* cross the hydrophobic core of the lipid bilayer.
- 10 We now know of carriers for all kinds of molecule [160], from water, urea and glycerol to highly hydrophobic molecules, such as the dibenzylidimethylammonium cation [161], as well as for hundreds, if not thousands, of different drugs [1–4,6–10,17,23].
- 11 Because of the enormous Born charging energy, small charged molecules cannot diffuse through the hydrophobic portion of a phospholipid bilayer.
- 12 It is well known that many hydrophobic molecules can disrupt membrane integrity, the effect being greater within a structural class for more hydrophobic molecules [121]; thus, small molecules might themselves induce such hydrophilic pores, possibly as a function of their lipophilicity.
- 13 Cells lacking a transfected protein are not suitable controls for the same cells containing them.
- 14 Suitable controls would involve using cells that had a negligible background activity.

Given the continuing huge attrition rates in the pharmaceutical industry [162–165], it is important to find out which carriers are used in which cells. Ignoring their existence serves only to prevent their identification and exploitation in improving the safety and efficacy of new drugs.

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