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REVIEW

On a biophysical and mathematical model of Pgp-mediated multidrug resistance: understanding the "space–time" dimension of MDR

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Abstract Multidrug resistance (MDR) is explained by drug transporters with a drug-handling activity. Despite much work, MDR remains multifaceted, and several conditions are required to generate drug resistance. The drug pumping was conceptually described using a kinetic, i.e., temporal, approach. The re-emergence of physical biology has allowed us to take into account new parameters focusing on the notion of space. This, in turn, has given us important clues regarding the process whereby drug and transporter interact. We will demonstrate that the likelihood of drug-transporter meeting (i.e., the affinity) and thus interaction are also driven by the mechanical interaction between drug molecular weight (MW) and the membrane mechanical properties. This should allow us to mechanically control drug delivery.

Keywords Physical biology · Pharmacokinetic · Membrane · Drug delivery · Multi-drug resistance · Lipinski's second rule

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Position of the problem

Multidrug resistance (MDR) mediated by drug transporters is a two-sided problem. The first difficulty is to understand the relationship between the structure and the function of transporters. The second difficulty is to understand how and why drugs diffuse toward transporters given the complex requirement needed for their handling and extrusion. This review will focus on the concept of drug diffusion toward transporters.

MDR was initially explained by the overexpression of drug transporters with a drug-handling activity. It seems today that MDR is multifaceted and that several conditions are required to generate drug resistance. Beyond the strict field of MDR, it is interesting to note that a similar problem exists in the field of drug bioavailability where similar transporters impair drug chemicals from reaching their target. Recently the re-emergence of physical biology (i.e., trans-disciplinary research fields), also named "complex fluids or soft-matter (bio)physics," has allowed new parameters involved in complex biological processes such as MDR to be taken into account. Interestingly, these parameters are linked to physics and therefore widen the conceptual notion of space at the cellular level.

MDR transporter activity, namely drug pumping, was conceptually described using classical pharmacokinetics, which uses specific mathematical tools from chemistry that are focused on kinetics, i.e., time. As chemistry is chiefly a science of contact, it informs whether, or not, a reaction has occurred once chemicals meet together, classically given by Gibbs' energy. Therefore, unless considering averaged and approximated bulk diffusion, chemistry does not deal with the probability of two compounds meeting, or the reason why a drug and a transporter meet, especially in spatially heterogeneous systems of biology.

The science that deals with the notion of "space" is physics, the role of which is to provide an understanding of the optimal spatial organization of homogenous and heterogeneous systems composed of molecules. Given this simple thought, it follows that the current challenge in modeling is to combine the notion of space, recently developed by complex- or soft-matter (bio)physics, with the notion of time (kinetics). We will show that this type of modeling can provide fundamental insights about the efficiencies of time- or space-related biological processes in MDR: specifically, whether or not the pumping kinetics or probability of meeting is the most limiting factor in MDR.

Affinity versus specificity in MDR

In very simple terms, biochemistry is the field that studies the chemistry of biological elements. In the cellular context, this means that biochemistry aims to describe how and why biological molecules can interact together, based on structural and molecular aspects. Accordingly, biochemistry is a science of contacts. For example Eyring's theory on the transition-state/complex activation demonstrates that the kinetic constant, k, of a reaction is always given by: $k = k_B T/h \cdot \exp(-\Delta G/k_B T)$ where k_B, T, h and ΔG are, Boltzmann's constant, the absolute temperature, Planck's constant and Gibbs' energy of the transition state, respectively. This theory forms the basis of any kinetic studies, and it follows that the limitation of the kinetics of a reaction is given by the occurrence of the transition state, providing chemicals are already in contact.

Therefore, to interact, chemicals have to have some ability to react, which is described by Gibbs' energy and sometimes catalyzed by enzymes. However, prior to reacting, chemicals have to come into contact with one another, which can only be achieved by a specific spatial organization of systems. Experimentally, it is the term of "affinity" that best describes the notion of interaction. However, this term deduced experimentally does not differentiate between the ability of two molecules to interact and to react and accordingly, in the notion of "affinity," the likelihood (probability) of a reaction is as important as Gibbs' energy.

Therefore, although two chemicals may look highly specific and complementary to one another chemically, the resulting experimental affinity can be very low if the spatial organization[1] of the system impedes their ability to meet and thus react together. The converse is also true and has direct application in drug resistance. Indeed, it may well be that two chemicals are forced to come together because of the spatial organization of the system, even if they are not that specific to one another. In turn, this would likely elevate the experimental affinity measured.

Multi-drug resistance corresponds to a state whereby an organism is resistant to many drugs that are not necessarily chemically related. This means that the specificity between a drug and a transporter, although important, is unlikely to wholly explain the levels of resistance that result from the interaction between drugs and transporters. Therefore, it follows that changes in the spatial organization of MDR cells have to happen for high affinities between drugs and transporters to develop and to generate, in due course, high levels of *multi*-drug resistance. Again, a reaction resulting from the collision of two chemicals may not be that efficient[2]; nonetheless, if one increases the meeting probability between these two chemicals, or makes sure that they repeatedly collide to trigger the reaction, then a reaction can look highly efficient. Based on this, it follows that a cell will be likely to sustain high levels of resistance if it has found a way of forcing drug chemicals and transporters to meet together.

The fundamental role of spatial dimensions in the "multi" of multi-drug resistance

The "multi" of multi-drug resistance can be explained by finding a way of forcing drugs and transporters to meet. Whatever the biological complexity involved in a given process, the probability of a simple drug and a complex protein meeting is essentially driven by Brownian (or equivalently random) diffusion. It follows that the diffusion itself has to hold a fundamental key to the concept of *multi*-drug resistance. Random processes have been studied for more than a century, and it is now well established that the mathematical properties of Brownian diffusions are fully dependent on the dimensions of space. In particular, there is one theorem, known as Polya's Theorem, that states that portions of space are always left unvisited (whatever the visitation time considered) if the Brownian particle diffuses in dimensions higher than 2, and that conversely, in dimensions smaller than or equal to 2, all the space will be visited possibly more than one time over a long enough period of time. A "hand-waving" explanation is given in Fig. 1.

If one associates this later mathematical theorem with drug resistance, it follows that *multi*-drug resistance could be the result of mathematical properties of random

[1] Note that "spatial organization of a system" does refer also to dimensions that we will see are crucial to drug transporter meeting probability. Indeed, diffusion properties are different in 1, 2 or 3 dimensions.

[2] The efficiency defined at the molecular levels is referred as the number of products created per unit of time.

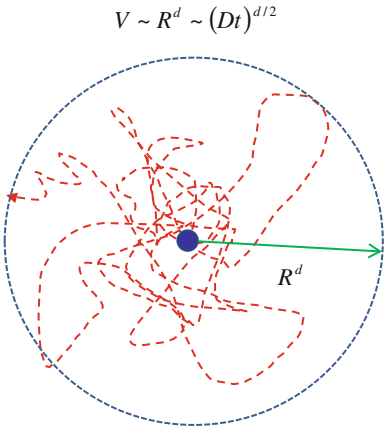

Fig. 1 This is certainly not a proof of Polya's theorem. Nonetheless, it is a very good exercise to visualize how the spatial dimensions generate interesting properties regarding Brownian diffusion. Thus, this is a hand-waving explanation of Polya's theorem. Let's consider a Brownian diffusion in a space for which the dimension is "*d*." After a sufficiently long time, the Brownian particle would have diffused within a volume $V \sim R^d$. The volume of diffusion is necessarily related to time, and as far as Brownian diffusion is concerned, the radius of the volume of diffusion is expressed as $R \sim t^{1/2}$. Thus, the volume is related to time by the following relation: $V \sim t^{d/2}$. As seen in the figure, as time goes on the particle will visit more and more dots composing the volume. By dots one means the number of particles that can be inserted in the defined volume. In actual fact, the number of dots (N) visited in the volume of diffusion is proportional to time: $N \sim t$. Consequently, the density of dots visited in the volume of diffusion is: $N/V \sim t^{1-d/2}$. As a result, when $d > 2$, the "density of visitation" decreases with time, which means that many sites will be left unvisited. Conversely, when $d \leq 2$, it can be proven that the density of visitation is always one, which means that all the sites will be visited at least one time; even so, if for this to happen, we have to wait long enough. All this formalism is further developed in Rudnick and Gaspari (2004)

diffusions unrelated to the complex biology of drug resistance (i.e., transporters in our case). Indeed, Polya's Theorem suggests that a large (and thus relatively static) membrane transporter would meet a small diffusing drug embedded in the membrane more than one time. The only condition for this to happen is that the residence time of the drug in the membrane has to be long enough. Of course, the cell membrane has a thickness and is not totally a 2D object. Nonetheless, if the residence time in the membrane is long enough, namely that the 2D diffusion occurs over long distances, the thickness can be neglected; see (Rauch and Pluen 2007). Note that as the ability of drugs to diffuse over long distances is fundamentally linked to the physical interaction between the drug and the membrane, it is the physics of drug-membrane interaction that would drive, via Polya's Theorem, the "*multi*" of *multi*-drug resistance.

Of course, Polya's Theorem and the associated explanation of *multi*-drug resistance would be better applied to specific cases where the interaction between a drug and a transporter is supported by the membrane. As we shall see, this necessitates specific drug transporters similar to the well-known Pgp (P-glycoprotein) that expels drugs from the membrane. Finally, saying that Polya's Theorem is a strong argument in favor of drug extrusion from the membrane does not rule out the possibility that drugs may also be extruded from the cytosol; nonetheless, this theorem simply suggests that the efficiency would be lower (see Fig. 1).

Finally, if Polya's Theorem (including the underlying physics of drug membrane interaction) has any meaning in drug resistance to describe drug-transporter affinity, the notion conveyed by this theorem must already exist in this field, albeit under a different name. In the field of drug resistance, an argument similar to Polya's Theorem has always been used—say invoked—to represent drug resistance. This argument is the well-known "vacuum cleaner" hypothesis. To fully appreciate this point it is important to develop the initial steps that have led to the discovery of drug transporters.

Drug-transporter affinity driven by the physical biology of the "vacuum cleaning" process

It was in 1973 that Dano Keld suggested that the mechanism of resistance was due to an outward efflux (Dano 1973). This hypothesis clearly took off when a few years later P-glycoprotein (Pgp) was identified by Juliano and Ling (1976) as the membrane protein overexpressed in MDR cancer cells that actively extrude membrane amphipathic drugs. Since this first discovery, many biological, biochemical and structural studies have been carried out on this transporter. Pgp consists of a duplicated structure (tandem structure) composed of around 1,280 amino acids with a MW ~170 kDa (Fig. 2). Each half of the molecule contains a nucleotide-binding domain (NBD) including six highly hydrophobic transmembrane domains (TMDs) (Fig. 2) (Borst and Elferink 2002; Shilling et al. 2006). The TMDs are considered to form a cavity through which hydrophobic drug molecules cross the membrane. The NH2- and COOH-termini and NBDs are located intracellularly, and the first extracellular loop is N-glycosylated. Each NBD consists of two Walker A and B motifs, common to most proteins that bind nucleotides typical of ATPases (Walker et al. 1982). The two half molecules are separated by a highly charged 'linker region' or C motif, a signature of ABC transporters (Higgins 2007). Several studies have indicated that a conformational change in the structure of Pgp upon ATP binding allows access from the lipid bilayer inner leaflet to the internal cavity of volume ~6,000 A^3 that can accommodate up to two hydrophobic drugs (Aller et al. 2009; Buxbaum 1999; Romsicki and Sharom 1998; Rosenberg et al. 2001). It has

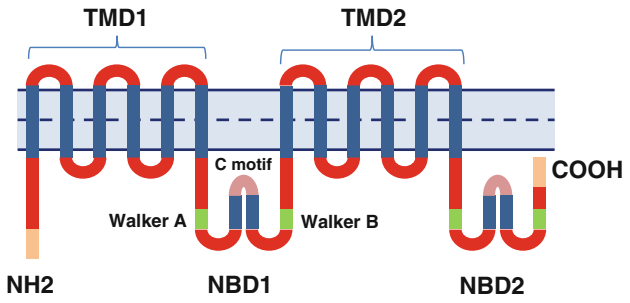

Fig. 2 Sketch of the biochemical structure of a P-glycoprotein

also been demonstrated that the drug-binding affinity is more sensitive to ATP binding rather than hydrolysis and that two ATP molecules need to be bound on each part of the Pgp to allow its full activation (Kimura et al. 2007; Martin et al. 2000a, b, 2001; Rosenberg et al. 2001; Sauna and Ambudkar 2000). Finally, the ability of many drugs to bind the internal cavity of Pgp is supposed to be linked to the number of potential binding sites available on the wall of the internal cavity composed of hydrophobic, aromatic, polar and charged amino acid residues (Aller et al. 2009). In addition to the use of crystallography methods and basic biology, by using reconstituted proteoliposomes as model systems, it was found that the turnover rate of Pgp ATPase at maximal drug stimulation is in the range of ~1–15ATP/s (Borgnia et al. 1996; Shapiro and Ling 1995), with a near stoichiometric substrate transport to ATP hydrolysis ~2ATP/drug [reviewed in (Ambudkar et al. 2006)]. Although Pgp with an extrusion activity from the inner leaflet is considered as the archetype of drug transporters, today we also know that other transporters may be involved in drug resistance as well, be it directly or indirectly, and that a drug extrusion from the cytosol is not ruled out (Ayrton and Morgan 2008).

In conclusion, these works on Pgp seemed to have determined fully the biochemical reason whereby drug resistance can exist, including the order of magnitude of the pumping kinetics of Pgp transporters, which in turn seemed to have solved the problem of MDR. However, there was a problem not related to the pumping kinetics but to the fact that Pgps do not cover all the cell membrane of drug resistant cells. Therefore, the recurring question was why would Pgps and drugs meet?

It was demonstrated that overexpression of drug transporters, leading to a very high level of resistance, corresponds to ~18% of membrane proteins (Borgnia et al. 1996), but that among these ~18%, only ~70% are located on the outer plasma membrane (Kim et al. 1997). Given that on average proteins represent between 20 and 50% of membrane surface area (Koval and Pagano 1991; Lange et al. 1989; van Meer 1989), it follows that between ~2.5 and ~6% of the plasma membrane area contributes in drug efflux in highly resistant cells. If one excludes drug diffusion in the membrane, this means that the probability that a drug incorporates into the membrane in the vicinity of a Pgp (prior to being extruded) is at most ~6%, i.e., much less than 1. In addition, it was also found that to be extruded by a Pgp, the drug has to be in the inner leaflet of cells (Shapiro et al. 1997; Shapiro and Ling 1997; Sharom et al. 2005). Thus, for a drug to interact with a transporter, many non-trivial conditions are required.

These problems lead to the hypothesis initially postulated by Dano Keld, that is, that drug transporters "vacuum clean" drugs. This hypothesis, besides characterizing the biochemical extrusion of drugs from the inner leaflet by transporters, informs indirectly on the ability of two molecules to meet together (i.e., likelihood of a reaction), which occurs before any biochemical/contact reactions. As seen above, this notion of "vacuum cleaning" is very similar to Polya's Theorem as long as diffusion and drug-membrane interaction are considered.

It is interesting to note that if we were in a position of controlling how drugs and transporters meet together, we would be in a position of harnessing, albeit indirectly, the drug pumping as well. Thus, by providing a clear explanation of the elusive "vacuum cleaner" hypothesis, we should be able to control MDR, at least in theory.

It is important to recall that along with the discovery of Pgp functionality, results from various laboratories demonstrated that many other physicochemical cellular parameters are altered in drug resistant cells. For example, membrane potential and intracellular pH alteration are important features in MDR (Roepe 1998; Roepe and Martiney 1999; Roepe et al. 1993, 1996; Santai et al. 1999), involved in lowering the influx of charged drugs into cells and trapping drugs in acidified compartments (Simon 2001; Simon et al. 1994). This trapping was associated with the fact that MDR cells re-organize the architecture of their intracellular organelles, including changes in the kinetics of membrane recycling, i.e., the "nonspecific adsorptive endocytosis," which is known today as fluid-phase endocytosis (Sehested et al. 1987a, b). In addition, and in an attempt to better understand drug transporters' function, it was also found that drugs are extruded from the inner leaflet of the cell membrane (Shapiro et al. 1997; Shapiro and Ling 1997; Sharom et al. 2005) and that the residence time of drugs in the cell membrane was an important factor (Eytan et al. 1997; Regev and Eytan 1997), likely to be tuned with the pumping kinetics. Given these changes it is interesting to ask which of these events could potentially mediate the high affinity expected between drugs and transporters. In other words, which one of these events could be central to the "vacuum cleaning" process or, said differently, makes Polya's Theorem work by reinforcing the drug-membrane interaction?

To finish, we would like to emphasize a particular seminal study on MDR published 3 years before Dano's work, the relevance of which will soon become obvious when the physical interaction between drug and membrane has been worked out. It is important to remember that at that time, i.e., 1970, no membrane drug transporters were suggested or even thought about. This study published by Biedler and Riehm (1970) investigated the cross resistance to actinomycin D using cancer cells (which were later found to express Pgp). Their conclusion was that the level of drug resistance could be correlated to the size (MW) of drugs used, namely that the larger the drug the higher the resistance, and conversely, the smaller the drug the better their sensitivity. It is important to emphasize that given the limited data on MDR, their study did not focus on complex cellular processes, but on drugs' physicochemical properties. We focus on this particular study for two main reasons. The first reason is linked to the fact that a similar conclusion relating the size (i.e., MW) of a drug and its ability to traverse bilayer membranes was drawn by the pharmaceutical industry $\sim$30 years later, when it became interested in drug bioavailability (see below). Indeed, drug transporters like Pgp are naturally expressed by certain cells in our body and are involved in the bioavailability of oral drug chemicals (Chan et al. 2004; Fromm 2003; Schinkel 1999; Zhang and Benet 2001); it is known that the MW of drugs is a fundamental factor affecting their bioavailability that cross-talks with Pgp functionality (Gleeson 2008; Gombar et al. 2004; Hou and Xu 2003). The second reason is related to the fact that the increase in the apparent affinity between drugs and transporters in MDR can be explained physically by the mechanical interaction among the drug size (i.e., MW), membrane endocytosis (altered in drug resistant cells) and, of course, drug transporter surface density and functionality (i.e., drugs' extrusion from the inner leaflet) (see the part after next).

Lipinski's rules and the bioavailability of drug chemicals

The pharmaceutical industry has adopted a "megabrand" marketing concept that advocates a strong focus on single products yielding significant returns at peak sales ($\sim$\$1 billion/annum) (Drews 2003; Oprea 2002). Accordingly, this vision has emulated the notion of "blockbusters." It is therefore understandable that the strategies adopted by these companies are those that provide information, in advance of costly clinical trials, about which chemicals are most likely to become blockbusters. Thus, the properties that make a chemical a "likely" drug predefine and are central to the "blockbuster" product. Lipinski's strategy answered a real demand in an innovative way. Lipinski and his collaborators from Pfizer analyzed the physicochemical properties of marketed oral drugs. Lipinski found that marketed drugs follow four rules. The first rule is based on the lipophilic index of drugs (octanol–water partitioning: log$P < 5$), the second rule is based on the drugs' MW, which needs to be <500, and the third and fourth rules are based on the drugs' state of charge (number of hydrogen-bond donors, i.e., number of OH + NH bonds >5; and number of hydrogen bonds acceptor, i.e., number of O + N atoms >10). Together, these rules define the physicochemical properties drugs should have to achieve the 90th percentile of bioavailability (Lipinski et al. 2001). These rules, which concerned synthetic chemicals, are also found for natural compounds (Quinn et al. 2008). Given the potentially huge return from Lipinski's rules, which predicts inefficient compounds prior to them reaching the development stage, this specific route regarding the determination of drug likeliness properties has been further developed and refined by others (Proudfoot 2002; Veber et al. 2002), extending these initial rules to other physicochemical properties that compounds should have (Palm et al. 1997; Veber et al. 2002). Today, the application of these guidelines to determine the drug likeliness properties of potential lead compounds have been largely embraced by the pharmaceutical industry and, accordingly, Lipinski-type rules are now an integral part of the decision-making process in this industry and are now considered drug discovery paradigms. To conclude, we can say that the up-side of Lipinski's rules is related to the fact that his study has focused on the physicochemical properties of drug chemicals, bypassing (but not ignoring) complex biology such as drug transporters. The down-side of Lipinski's rules, however, is that (1) only physicochemical margins are defined (e.g., $0 < \text{MW} < 500$) and (2) rules are not laws, i.e., there is no explanation provided, albeit the understanding of some rules can be inferred to the membrane (e.g., rule one) or pH changes in the GI tract (e.g., rules three and four).

If these rules pre-define what chemicals should look like to cross cell membranes, a similar conclusion is likely to be valid for chemicals crossing Pgp-expressing MDR cells. This was indeed shown for the drug MW (see Biedler and Riehm's study). The question now is: can we use basic physics to merge all this different data into one coherent body of evidence?

Merging Lipinski's second rule and Biedler and Riehm's study

In both the Lipinski (basic drug delivery) and Biedler and Riehm (drug resistance) studies, the MW of chemicals is

central. Drugs that are small enough have a MW that is proportional to their volume, suggesting that drug volume is a limiting parameter when they cross biomembranes. In thermodynamic physics, the conjugated parameters of spatial dimensions, as for example the *volume* of a drug, is either the pressure (c.f., the Law of ideal gas: $PV = nRT$) or the surface pressure (i.e., mechanical surface tension) if the membrane is concerned. Given that, to reach their targets, chemicals have to cross many membrane barriers, it is very likely that the membrane, and thus the mechanical surface tension, will be centrally involved in the alteration of drug transverse movement. Therefore, in the sum of energies making up the total activation energy required for a drug to cross cellular membranes, there must exist an energy term that is a specific function of the drug's dimension so that the drug/membrane interaction yields a significant energy $\geq k_B T$ (k_B is Boltzmann's constant and T the temperature in Kelvin). Before entering the field of physics, we will recall in the next part the most important results (for us) regarding the (bio)physics of the cell membrane.

A very short history of membrane physics and its role in endocytosis: from Lipinski's second rule to Lipinski's second law?

Since the mosaic model of the cell membrane, we have learned a lot from the physical properties of a cell membrane. An essential aspect of the biological matter like the membrane when considered at the mesoscopic level, namely between the micro- and macroscopic levels, is that it is neither totally hard nor totally liquid water. In fact, it is between both and is thus called soft matter. A property that is central to these soft objects is that a rather mild change can trigger an important effect. This is down to the way entities composing these soft objects self-assemble and organize themselves to minimize the system energy. The bilayer membrane, i.e., the cell membrane, is a very good example (Israelachvili et al. 1980). Based on their amphipathic properties, lipids self-assemble to form bilayer membranes. In addition, bilayer membranes are soft and we know that a small change in the lipid asymmetry between leaflets triggers drastic shape changes (Farge et al. 1990; Farge and Devaux 1992; Seifert et al. 1991). In fact we now know that the lipid asymmetry between lipid leaflets of the cellular membrane, mediated by lipid flippases activity (Seigneuret and Devaux 1984; Seigneuret et al. 1984), allows the creation of fluid-phase vesicle ($\sim$50 nm radius) (Farge et al. 1999; Rauch and Farge 2000) (Fig. 3a). As said previously, if the drug dimension and the plasma membrane are considered, the physical parameter that best fits such an interaction is the leaflets' surface pressure, σ,[3] that is related to how packed lipids are. However, in cells, two types of membrane tension can be distinguished, the mean surface tension, noted σ_0, which corresponds to the sum of leaflet surface tensions, and the difference in surface tensions, $\Delta\sigma$, between the inner and outer leaflet. Cells have a large reservoir of membrane and an average membrane tension that is remarkably low, $\sigma_0 \sim 10^{-2}$–10^{-3} mN/m (Hochmuth et al. 1996; Raucher and Sheetz 1999), compared to the magnitude of the difference in surface tensions between leaflets, $|\Delta\sigma| \sim$ 0.9 mN/m (Rauch and Farge 2000). Accordingly and given the magnitude of this parameter, $\Delta\sigma$ is more likely to be involved in impairing the transverse movement of chemicals. Dimensionally speaking, the magnitude of the drug critical cross-section area, a_c, can be defined by:

$$a_c = -k_B T/\Delta\sigma \quad (1)$$

In Eq. 1, the minus sign indicates that the membrane is compressed when drugs traverse it. The difference in surface tensions, $\Delta\sigma$, is associated with the role of lipid flippases that maintain membrane lipid asymmetry (Seigneuret and Devaux 1984). In particular, it has been demonstrated that a particular membrane flippase actively relocates phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the outer into the inner leaflet of the cell membrane. A consequence of this inward pumping is a constantly more highly packed inner leaflet as it contains more phospholipids than the outer leaflet. It has been demonstrated that this lipid packing asymmetry between the membrane leaflets leads to fluid-phase endocytosis (Devaux 2000; Farge 1995; Farge et al. 1999; Rauch and Farge 2000) (Fig. 3b) and that the vesicle radius, R, can be expressed as (Rauch and Farge 2000):

$$R = -8k_c/h\Delta\sigma \quad (2)$$

where k_c and h are, respectively, the membrane bending modulus and membrane thickness. As for drugs small enough, their MW is proportional to their Van der Waals volume (expressed in $\AA^3$), i.e., MW $\sim V \sim a^{3/2}$, using Eqs. 1 and 2, a critical MW (MW_c) can be determined (Rauch and Pluen 2007):

$$MW_c = (4/3\sqrt{\pi})(hRk_B T/8k_c)^{3/2} \quad (3)$$

Equation 3 provides a law with regard to the drugs' size (or MW) selectivity on their permeation across cellular membranes (Fig. 3c). Using the numerical values of physical constants or biological parameters from drug-sensitive cells, it follows $MW_c \cong 240$ at 37°C (Rauch and Pluen 2007). Note that this formula would explain why the

[3] Note that in the following text, surface pressure or tension will be used without conceptual difference. In both cases they refer to the mechanical packing of lipids in membrane leaflets.

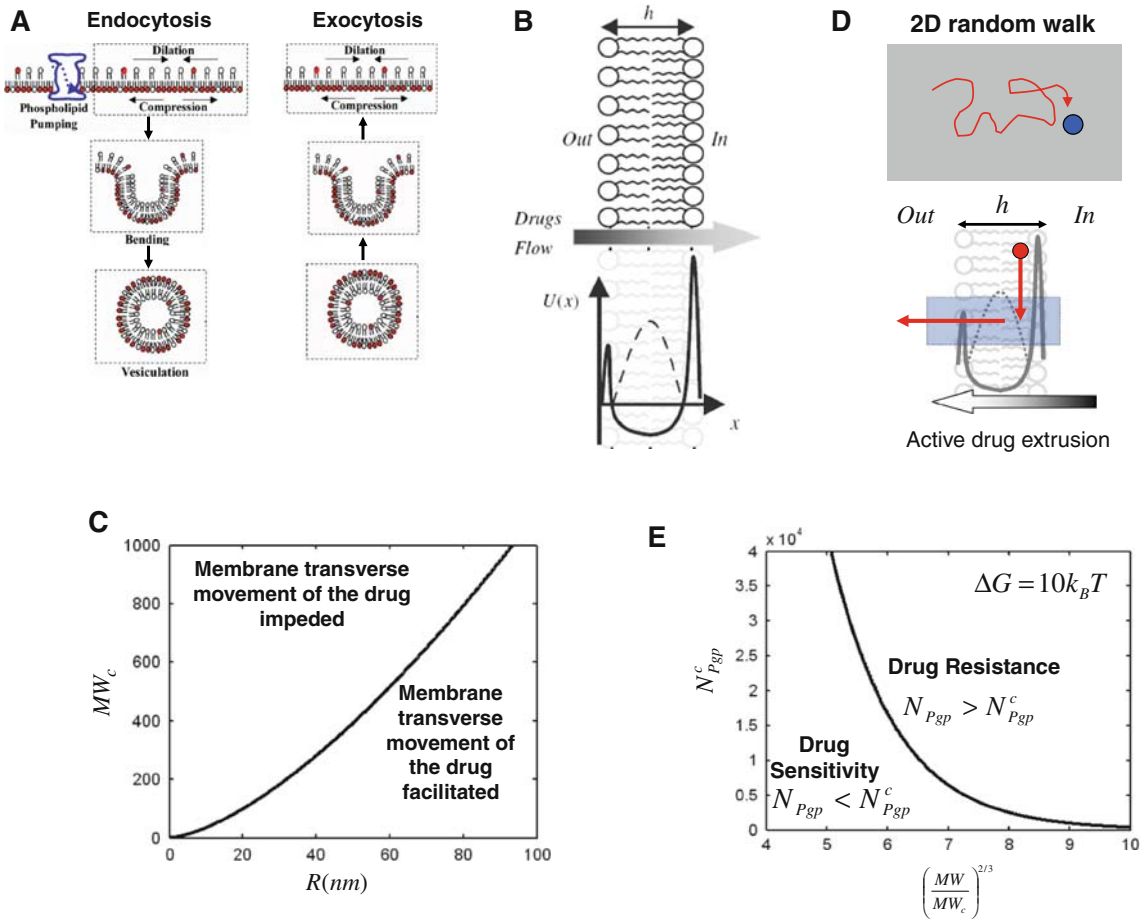

Fig. 3 **a** Sketch representing the current model linking fluid phase endocytosis to the membrane phospholipid number asymmetry. In the *left panel*, the translocation of dark-headed lipids into the inner leaflet induces a differential packing of lipids between leaflets leading to membrane bending and vesiculation. Note that the membrane recycling that occurs in cells (*right panel*), i.e., the exocytosis of vesicles with a size similar to endocytic vesicles, allows the maintenance of the lipid asymmetry and thus the maintenance of the differential packing of leaflets at the level of the plasmalemma. The relationship existing between the lipid number asymmetry and the vesicle radius is given by Eq. 2. Accordingly, the lipid number asymmetry has been experimentally deduced from studies on drug-sensitive cells with a value providing a vesicle radius of about ~35 nm. **b** Representation of the different energy barriers involved when a drug traverses the bilayer cellular membrane. Two leaflets have been represented with an inner leaflet containing more phospholipids related to the increase in the difference in surface tensions (*upper graph*). Energy profiles of lipid packing in both leaflet (*plain curve-middle graph*) and hydrophobic core of membrane (*dashed curve-middle graph*) are both involved in providing penalty energies with regard to the transbilayer movement of drugs. As the inner leaflet is packed, drugs crossing the membrane will be trapped in this leaflet, which will delay and impair their flow into the cytosol. The latter effect will be dependent on the size of drugs as bigger drugs will "feel more strongly" this mechanical barrier. **c** Plot of Eq. 3, namely the membrane permeability to drugs related to drug MW and vesicle radius (i.e., inner leaflet packing). **d** In the presence of transporters (*blue* in the figure), it is expected that the mechanical activation energy needed for a drug to cross the membrane when cells are resistant will: increase the residency time of drugs in the membrane and allow the drug to diffuse laterally in the membrane (*upper panel*). In turn this will increase the meeting probability between a drugs and transporters followed by extrusion. **e** Plot of Eq. 4 that predicts the relationship among drug resistance, Pgp expression level and drug MW. Note that the critical MW is defined by the vesicle radius (i.e., endocytosis) (see **c**)

drug size is important for their transverse movement. It is important to note that if the vesicular radius generated by the membrane decreases, then the mechanical packing of the inner leaflet increases which, in turn, decreases MW_c. This last result suggests that cells are able to set up mechanical barriers to chemicals, simply by changing the lipid asymmetry they have in the membrane. It is noteworthy that the kinetics of endocytosis is increased in MDR cells and that, as this parameter is inversely related to the vesicle radius (Farge et al. 1999; Rauch and Farge 2000), this suggests strongly that this packing is increased in the MDR state. It is now worth asking whether one can address the low specificity/high affinity linked to the "vacuum cleaner" hypothesis, on which Pgp activity is totally reliant.

Explaining the "vacuum cleaner" hypothesis using Lipinski's second law?

As stated before, the vacuum cleaner hypothesis characterizes the ability of drugs to meet Pgp. Given that membrane-embedded drug chemicals have a non-negligible residence time in the membrane (Eytan et al. 1997; Regev and Eytan 1997), they will diffuse randomly and by chance may meet a Pgp (c.f., Polya's Theorem). The condition that will lead to effective extrusion is that any meeting has to happen within the inner lealfet (Shapiro et al. 1997; Shapiro and Ling 1997). The higher mechanical packing of the inner leaflet is the only parameter that can ensure that Pgp and drugs effectively meet in the inner leaflet. In this setting it is possible to deduce the surface density of transporters needed to trigger drug resistance (Fig. 3d). Without going into too many details, it is possible to demonstrate that the critical surface density of transporters, ρ^{c}_{Pgp}, covering cells must satisfy (Rauch and Pluen 2007):

$$\rho^{c}_{Pgp} \sim \left[\frac{\Delta G}{k_B T} + \left(\frac{MW}{MW_c}\right)^{2/3}\right] e^{-\left(\frac{MW}{MW_c}\right)^{2/3}} \quad (4)$$

where ΔG and MW are the drug dehydration energy and the drug molecular weight. Note that if the MW of a drug increases (or equivalently that the vesicle radius decreases—see Eq. 3), then the surface density of Pgps can decrease exponentially (Fig. 3e). At constant surface density of Pgp, this result means that if larger MW drugs were used, at least larger than the ones used to generate MDR, then they would, with no doubt, reach transporters and be extruded. This formula provides a simple explanation of Biedler and Riehm's study, based on Lipinski's second rule (Eq. 3), namely, the reason why drug size matters in MDR (Rauch 2009a). Note, however, that Eq. 4 provides the minimal condition required between the surface density of drug transporters and the MW of drugs to trigger drugs extrusion; nonetheless, in practice what is measured is the IC_{50} of drug chemicals. Thus, to link this theory to experiments and test how robust the model is, one needs to define how IC_{50s} are expected to vary as a function of parameters discussed above.

IC_{50}s in living systems resistant to drugs

The cellular sensitivity or resistance to drugs is usually determined using the IC_{50}s method. The IC_{50} is an indicator of drug efficiency as it provides the drug concentration needed to kill 50% of cells in a given population. When IC_{50}s are compared for an identical drug between sensitive and resistant cells, the ratio $(IC_{50})_{MDR}/(IC_{50})_{non\text{-}MDR}$ is thus related to the amount of drugs able to cross the membrane per unit of time. In addition, this ratio also represents the level of resistance. Accordingly, the higher the $IC_{50,}$ the lower the sensitivity, and thus an inverse relationship exists between the ability of drugs to cross the membrane of cells and the IC_{50}s. Given Pgp with a "drug handling" activity in MDR cells, the kinetic rate of drug membrane permeation has already been determined (Rauch and Pluen 2007):

$$\frac{r_{MDR}}{r_{non-MDR}} \cong e^{-\left(\frac{MW}{MW_c}\right)^{2/3}\left(\frac{\Delta\sigma_{MDR}}{\Delta\sigma_{non-MDR}}-1\right)}\left(1-\frac{\rho_{Pgp}}{\rho^{c}_{Pcp}}\right) \quad (5)$$

r_{MDR} and $r_{non\text{-}MDR}$ are, respectively, the kinetic rates of drug membrane transverse movement in resistant (subscript "MDR") or sensitive (subscript "non-MDR") cells; $\Delta\sigma_{MDR}$ and $\Delta\sigma_{non\text{-}MDR}$ are, respectively, the difference in surface tensions in resistant (subscript "MDR") or sensitive (subscript "non-MDR") cells. Note that the differences in surface tensions have been left in the formula as they correspond to the physical nature of the packing. Finally, ρ_{Pgp} and ρ^{c}_{Pcp} are, respectively, the surface density of drug transporters and the critical surface density of transporters. The critical surface density of transporters is the surface density of transporters needed to trigger full drug resistance. For example, when $\rho_{Pgp} \to \rho^{c}_{Pcp}$, then $r_{MDR}/r_{non\text{-}MDR} \to 0$, i.e., the kinetics of membrane, is null. Note, however, that in a living system resistant to drugs, there is always the possibility of killing cells by increasing the extracellular amount of drugs and therefore the ultra-resistant state, i.e., when $\rho_{Pgp} = \rho^{c}_{Pcp}$ as defined by Eq. 4, is never totally reached. Thus, in practice, $\rho_{Pgp} < \rho^{c}_{Pcp}$. It has to be noted as well that the drug dehydration energy does not intervene in Eq. 5 as it is cancelled upon division.

Considering that the IC_{50}s are inversely proportional to the kinetic rates of the drug's transverse movement across the membrane, it follows that:

$$\begin{aligned}\ln\left(\frac{(IC50)_{MDR}}{(IC50)_{non-MDR}}\right) &= -\ln\left(\frac{r_{MDR}}{r_{non-MDR}}\right)\\ &\cong \left(\frac{MW}{MW_c}\right)^{2/3}\left(\frac{\Delta\sigma_{MDR}}{\Delta\sigma_{non-MDR}}-1\right)\\ &\quad -\ln\left(1-\frac{\rho_{Pgp}}{\rho^{c}_{Pgp}}\right)\end{aligned} \quad (6)$$

Equation 6 states that drug resistance levels (IC_{50}s) would result from the interaction between the lipid packing, the drug size (or MW) and the surface density of drug transporters. Equation 6 should allow us to test experimentally the robustness of the model.

Importantly, Eq. 6 contains two major terms, the first is related to the mechanical interaction between a drug and membrane, the second to drug transporters. If drug resistance

occurs without the expression of transporters, then the second term must be omitted by posing: $\rho_{Pgp} = 0$. Note, however, that if drug transporters are expressed, the first cannot be omitted as it represents the "vacuum cleaner" effect upon which transporters activity rely.

Future perspective: challenges ahead for the next 10 years

The points that will be developed now are what remain to be done. Of course, proving totally Eq. 6 remains central, but there is now enough evidence that shows that the theory is sound (see (Rauch 2009a, b; Rauch and Pluen 2007) and unpublished observation). Therefore, the next paragraphs will focus on the future challenges.

First challenge ahead: introducing pumping kinetics

What has been exposed so far does not contain any references to the pumping kinetics of drug transporters. In fact the model assumes that the pumping kinetics is instantaneous, namely, that each time a drug meets a Pgp in the inner leaflet, it is also extruded. Equivalently, this assumption supposes that the concentration of membrane embedded drugs is low enough that only one drug molecule at a time will meet a transporter. Taking into consideration the drug pumping kinetics will allow for the fact that a drug can meet a transporter, but that this transporter is in an occupied state while pumping another drug. This point is important as not only will it allow us to consider higher concentrations of drugs, but it will also allow the design of potentially new therapeutic strategies based on drug size and ability to move in the membrane.

Second challenge ahead: introducing pH

Up to now the model has been restricted to the mechanical properties of the cell membrane. Nonetheless, we know that recurrent features exist in MDR cells, and cytosolic pH alkalization is one of them. The next step will be to determine how a higher pH as observed in MDR can affect the packing of lipids, thereby influencing the transverse movement of drugs as a function of their size. As there are negatively charged lipids in the inner leaflet of the membrane (e.g., phosphatidylserine), it may well be that a change in pH will affect the way lipids repulse each other, thereby defining new differences in surface tensions that are critical for Pgp activity. This will also likely interact with the drug charge, affecting drugs' flip-flop rate across the membrane and their on–off association with the membrane.

Third challenge ahead: introducing the membrane potential

What has been exposed so far does not contain any references to the charge of drugs. However, in some cases a change in the membrane potential has been noted that may facilitate or impede the influx of drugs across the membrane of cells. Note, however, that it is unclear as to whether the change in membrane potential is direct, e.g., mediated by an extra activation/transcription of ion channels, or indirect and related to the change in pH. This point will have to be addressed, which will introduce electrostatic interactions between the drug and the membrane potential within the model, providing a better picture of MDR.

Fourth challenge ahead: mechanical control of drug delivery

The new prediction given by the model presented here is that a physical mechanical interaction takes place when a drug crosses the cell membrane. In turn this means that if we could harness this mechanical interaction we should be in a position of controlling the delivery of drugs, especially the larger ones. In terms of drug targets, it seems that the lipid metabolism is one to focus upon. Indeed, it is the cell that controls how many lipids are synthesized and where they will be located, i.e., the inner or outer leaflet. By doing so the cell also controls the mechanical properties of its membrane, which directly affects the mechanics of the transverse movement of drugs. There is a real need to find new biological targets to control the physics of drug delivery.

Fifth challenge ahead: modeling the complexity of MDR

Multi-drug resistance is not only linked to Pgp expression. Other transporters exist that are also involved in drug resistance; MRP1 transports drugs from the cytoplasm and the present model based on drug-membrane interaction do not explain the affinity for such transporters (Chang 2007). In addition, activity of solute carrier proteins (Okabe et al. 2008) is known to affect drug entry into cells, and again the present model does not describe the role of these carriers. Therefore, the ultimate and very long-term challenge is to integrate in a more comprehensive model the true complexity of MDR, which has, on a biophysical level, to

include lipid charge and specific drug structure/chemical moieties as well.

Conclusions

We have tried to highlight the fundamental role of cross-disciplinary research in complex biology. To understand multi-drug resistance, we have started by a fundamental mathematical property of diffusive process. We then have blended this mathematical property to the physical biology of the cell membrane and the biology of multi-drug resistance. It is remarkable that a single cell can create a very complex behavior through relatively simple properties. Finally, we would to emphasize two points:

- Physical biology must now be an integral part of the drug discovery process and decision making in the pharmaceutical industry.
- The physical properties of the plasma membrane including lipid metabolism are obvious targets if one has to ameliorate drug bioavailability or circumvent drug resistance.

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