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On the relationship between drug's size, cell membrane mechanical properties and high levels of multi drug resistance: a comparison to published data

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Abstract Multi drug resistance (MDR) or cross resistance to drugs was initially explained on the basis that MDR cells express drug transporters that expel membrane-embedded drugs. However, it is now clear that transporters are a single piece from a complex puzzle. An issue that has been solved recently is, given that these transporters have to handle drugs, why should a membrane-embedded drug and a transporter meet? To solve this problem, a theory has been suggested considering the interaction between the cell membrane mechanical properties and the size of drugs. In simple terms, this theory proposes that an excess in the packing of lipid in the inner leaflet of the membrane of MDR cells is responsible for blocking drugs mechanically as a function of their sizes at the membrane level, thus impairing their flux into the cytosol. In turn it is expected that this would allow any membrane embedded drug to diffuse toward transporters. The study concluded that the size of drugs is necessarily important regarding the mechanical interaction between the drug and the membrane, and likely to be central to MDR. Remarkably, an experimental study based on MDR and published years ago concluded that the molecular weight (MW) of drugs was central to MDR (Biedler and Riehm in Cancer Res 30:1174–1184, 1970). Given that size and MW are linked together, I have compared the former theory to the latter experimental data and demonstrate that, indeed, basic membrane mechanics is involved in high levels of cross resistance to drugs in Pgp expressing cells.

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Introduction

An essential aspect in the field of drug resistance is that once a cell is resistant to one particular drug, the cell is usually resistant to many drugs that are not chemically and structurally related. This phenomenon, known as multi drug resistance (MDR) or cross resistance to drugs, remains poorly understood. Although drug transporters were initially suggested as the single cause of drug resistance, it seems today that MDR relies on more than drug transporters and related pumping activity.

A problem that is central to drug resistance, if it has to be controlled by transporters via a "drug handling" mechanism, is determining why and how drugs should meet transporters. For that reason, a model has been put forward suggesting that the membrane mechanical properties are likely to be involved in MDR (Rauch and Pluen 2007). More specifically, as drugs are supposedly expelled from the inner leaflet of cells' plasma membrane by transporters (Ferte 2000) and that there are no reasons why a drug should incorporate the membrane in the vicinity of a transporter, it was suggested that the mechanical packing of lipids in the inner leaflet of the cell membrane would help the trapping of drugs, increasing their residency time in the membrane and thus lateral diffusion, prior to meeting and being actively expelled by transporters. As the mechanical packing of lipids in the inner leaflet of the cell membrane drives endocytosis, the higher mechanical packing in drug resistant cells is reflected by a 2- to 10-fold increase in their kinetic rates of endocytosis compared to drug sensitive

cells (see Fig. 1b, c) (Ferte 2000; Altan et al. 1999; Colin et al. 1996).

This model, which aimed to solve a conceptual imperfection in the field of drug resistance by providing a scientific understanding of the "vacuum cleaner hypothesis", has given the theoretical relationship needed between endocytosis, transporters' surface density, size of drugs and dehydration energy, to allow total drug resistance.

To conclude, this theory explains why cells are resistant to drugs when the level of resistance is selected by incubation of a given drug at a given concentration, namely when both drug and transporters' surface density are fixed.

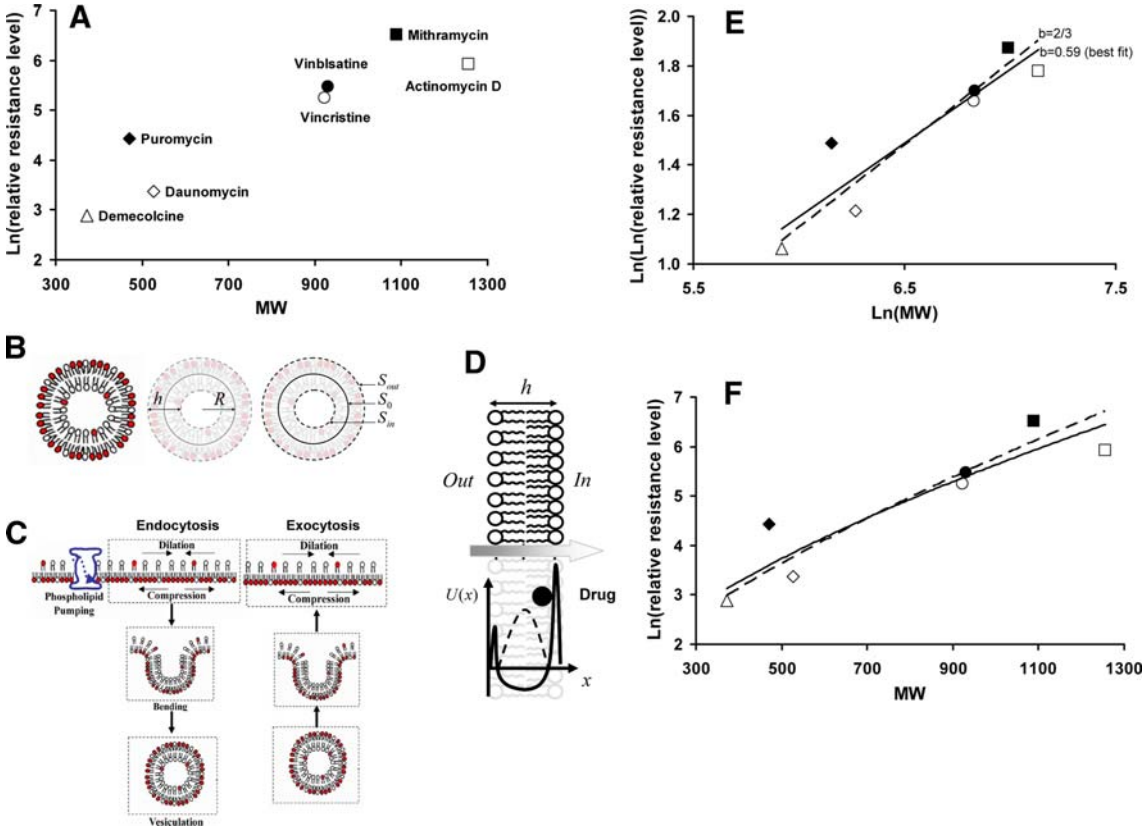

Fig. 1 **a** Data from Biedler and Riehm (1970), i.e. Table 1. The Neperian logarithm of relative resistance levels is plotted against the MW of drugs. Drug's name are also given. **b** Lipid asymmetry at the vesicular scale: given the small size of vesicles, the radius and membrane thickness are relatively close together ($R/h \sim 10$). Thus, the outer leaflet of a vesicle (S_{out}) has significantly more lipid than the inner leaflet (S_{in}). As the vesicle is spherical, noting $S_0 = 4\pi R^2$ the neutral surface area namely the surface area between the outer and inner leaflets, it follows at the first order that $S_{out} = 4\pi(R + h/2)^2 \sim S_0(1 + h/R)$ and $S_{in} = 4\pi(R - h/2)^2 \sim S_0(1 - h/R)$. Thus, $S_{out} - S_{in} \sim S_0 \times h/R$. **c** Sketch representing the current model linking fluid phase endocytosis to the membrane phospholipid number asymmetry (Seigneuret and Devaux 1984). 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 (Farge et al. 1999; Rauch and Farge 2000). 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 $R = 8k_c/hK(\Delta N/N_0)$ (see Appendix). Accordingly, the lipid number asymmetry has been experimentally deduced from studies on drug sensitive cells (K562) with a value $\Delta N/N_0 = 4\%$ providing a $\sim$35 nm vesicle radius (Rauch and Farge 2000). **d** Representation of the different energy barriers [noted together $U(x)$] and 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 (Rauch and Pluen 2007). The latter effect will be dependent on the size of drugs as bigger drugs will "feel more strongly" this mechanical barrier. In the present paper, this effect is supposed to be central for the high levels of cross resistance to drugs that Biedler and Riehm measured. **e** Biedler and Riehm's data were transformed using the double Neperian logarithm in order to determine whether a power law exists. The plain curve corresponds to the best fit following the application of a linear regression ($b = 0.59$, F test $= 27.18$ and p value $= 0.003$). The *dashed curve* corresponds to the theoretical fit given by the optimization of Eq. 6 ($b = 2/3$). **f** Same graph as in (**a**) where the power law predicted by Eq. 6 ($b = 2/3$, *dashed curve*) including the power law deduced from the best fit ($b = 0.59$, *plain curve*) are plotted together and compared against the experimental data. A good conformity is found between the theory and the experiments

A problem arises immediately when cross resistance to drugs is taken into consideration. In this case the theoretical relationship discussed above may not be verified. In particular, the surface density of transporters may not be fitted to expel another drug with different physical properties. The latter scenario would be the case if a smaller drug (i.e. a drug with a lower MW) than the one used to select the resistant phenotype is used, as it would cross membrane more easily, which would decrease the meeting probability between a drug and a transporter. If this scenario occurs, the theory suggests that the influx of drugs into the cytosol, namely levels of cross resistance to drugs, would be solely dependent on the mechanical interaction between the differential lipid packing in the cell membrane and the MW/volume of drugs. The reason being that at given surface density of transporter, the theoretical influx of drugs into the cytosol is exponentially dependent on the MW/volume of drugs (Rauch and Pluen 2007). If this conclusion is true, this means that the theory suggested above should be able to predict the levels of cross resistance to drugs observed; at least by giving the "power law" of the relationship between the MDR levels and the MW/volume of drugs; at best by matching published data on the subject.

For this comparison an experimental study published by Biedler and Riehm (1970) will be used. In agreement with the authors' conclusion, one will demonstrate that the MW or volume of drugs is indeed fundamental in MDR.

The plan will be the following; firstly a very quick introduction to this very important study will be done (Biedler and Riehm 1970). This will be followed by a recapitulation of the theory concerning endocytosis and drug resistance published previously (Rauch and Farge 2000; Rauch and Pluen 2007). Finally, a comparison will be performed.

Biedler and Riehm's study

The role of drug sizes in MDR has been initially demonstrated in 1970 (Biedler and Riehm 1970) and further confirmed in 1990 (Selassie et al. 1990).

Given in vitro observations of MDR in bacteria and mammal cell population, the authors decided to conduct their own study. For this, they generated two drug resistant cell lines but focused on the concept of cross resistance in one cell line only (DC3F lung-derived cell line). As a first step they generated a resistant lung-derived cell line by selective incubation of actinomycin D (MW = 1,255) for several months, which led to expression of Pgp transporters (Safa et al. 1986). As a second step they measured cross resistance levels using several other smaller drugs (MW < 1,255). Given the resistant and sensitive cell lines they looked at how these two cell lines would respond to various drugs.

In the present text, the drug sensitive cell line will be notated DC3F and the resistant one DC3FR. Resistance or cross resistance was determined using the concentration of drugs to kill 50% of the cell population (i.e. ED_{50}, effective dose). As the intention is to understand the physical reason behind the emergence of high levels of resistance, namely >10-fold, only these levels of resistance will be focused upon. The numerical data from the study of Biedler and Riehm (1970) are presented in Table 1 and plotted in Fig.1a. Note that "relative resistance levels" means the ratio between the effective dose of a given drug needed to kill the drug resistant lineage [noted ED_{50}(DC3FR)] divided by the effective dose needed to kill the sensitive lineage [noted ED_{50}(DC3F)].

In order to analyze whether a power law can be deduced from Biedler and Riehm's data, the dependency of the relative resistance levels as function of the drugs' MW was assumed to obey the function $\sim \exp[a(\mathrm{MW})^b]$ (where a and b are unknown variables). The exponential function, i.e. Arrhenius' Law, warrants that basic physico-chemical principles related to the drugs' MW apply when they cross the cell membrane. By taking the double Napierian logarithm of Arrhenius' function, the equation transformed to $\ln(a) + b \ln(\mathrm{MW})$. Therefore, on the double Napierian logarithm of Biedler and Riehm's data a linear regression was carried out. It was found that the transformed data follow a linear trend (Fig. 1e, plain line), with $R^2 = 0.844$ for $b = 0.59 \pm 0.11$ ($p = 0.003$) and $\ln(a) = -2.36 \pm 0.75$ ($p = 0.025$). Finally, the F test provides $F = 27.18$ and $p = 0.003$.

This result suggests, and demonstrates for actinomycin D, that when a drug with a high MW is used to select a resistant phenotype and that thereafter, drugs with a smaller MW are employed to kill cells, the high levels of cross resistance to

Table 1 Data from Biedler and Riehm's study compared against the theory suggested

Drug	MW	ln [ED_{50}(DC3FR)/ED_{50}(DC3F)]		|Δ|(%)[a]
		Experimental values	Theoretical values	
Mithramycin	1,089	6.51	6.16	5
Actinomycin D	1,255	5.92	6.79	14
Vinblastine	929	5.47	5.07	7
Vincristine	923	5.24	5.13	2
Puromycin	471	4.43	3.53	20
Daunomycin	527	3.36	3.81	13
Demecolicine	371	2.89	3.01	4

[a] Absolute relative difference between the theory and experimental data

drugs (>10) can be explained ($p < 5\%$) by $\sim$84.4% by considering the MW of drugs. Accordingly, this phenomenon follows a power law that is given by $\sim MW^{0.59\pm0.11}$.

It is, therefore, worth asking why the cross resistance levels would be so dependent on the MW of drugs and whether it is possible to calculate and predict a similar power law, thus explaining the exponent from a theoretical point of view.

Given that for drugs that are small enough their MW is proportional to their volume, namely that the volume of drugs expressed in Angstrom cube gives also the drugs' MW (Karshikoff 2006), the latter power law suggests that the volume of drugs is a limiting parameter when they cross the membrane. In turn, this suggests that basic mechanical effects are expected. Therefore, it is worth quickly reviewing what is known about the membrane mechanical properties.

On the role of the differential packing of membrane lipids in endocytosis and drug permeability

This part, including Fig. 1b, c, corresponds to the recapitulation of the theoretical basis of the differential packing of membrane lipids and its role and effect on endocytosis and drug permeability.

To introduce how endocytosis is related to the differential packing of lipids, lets consider first a vesicle as found in living cells (Fig. 1b). Once formed, membrane vesicles have a radius of about $R \sim 50$ nm and as the vesicle radius is only 10-fold higher than the membrane thickness ($h \sim 5$ nm), $R/h \sim 10$, the surface area of the outer leaflet is larger than the surface area of the inner leaflet (Fig. 1b). Thus, the outer leaflet of a vesicle must have significantly more lipids than the inner leaflet. Assuming that lipids have a cross section area $\sim a_0$, the difference in lipid number between the outer and inner leaflets (ΔN) is given by $a_0 \times \Delta N \sim S_{out} - S_{in}$, where $S_{out} - S_{in}$ is the difference between the outer and inner surface area. The difference between the surface area is also given by $S_{out} - S_{in} \sim S_0 \times h/R$, where S_0 is defined as the neutral surface area of the vesicle (see Fig. 1b). By equating the above formulas it follows that the vesicle radius is, geometrically speaking, inversely proportional to the lipid number asymmetry between leaflets $R \sim 1/\Delta N$ (Devaux 2000). As the outer leaflet of the vesicle is formed from the inner leaflet of the membrane, this result suggests that for a vesicle to be created, more lipids are stored in the inner leaflet of the cell membrane, relatively to the outer leaflet. This feature has been correlated to the way in which a membrane organizes its own composition in lipids.

It is well known that lipids are not randomly distributed in the cell membrane but follow a strict composition between leaflets due to the action of specific lipid flippase (Pomorski and Menon 2006). For example, phosphatidylserine and phosphatidylethanolamine are maintained in the inner leaflet, and actively relocated from the outer to the inner leaflet if needed, by a lipid flippase (Devaux et al. 1990; Seigneuret and Devaux 1984). The connection between the asymmetry in phospholipid composition of the cell membrane and endocytosis, including the cell membrane mechanical properties, were described in three phases.

Firstly, in well defined model systems it was found that the shape of giant unilamellar liposomes (GUVs) described by physical parameters like the global curvature, could be altered by changing the lipid asymmetry between leaflets (Farge and Devaux 1992) and, by providing an adequate theory based on the mechanical properties of such biomembrane systems [known as the "area-difference elasticity" (ADE) model] it is possible to predict the changes in the GUV's shape as a function of the lipid asymmetry imposed (Miao et al. 1994). This first step allowed to connect membrane shapes and membrane mechanical properties.

The second phase: The relationship between the lipid asymmetry and endocytosis was explained when it was discovered that imposing a sufficient lipid asymmetry in GUVs induced membrane budding (Farge and Devaux 1992), this experiment was repeated in cells and it was found that an excess of phosphatidylserine added in the inner leaflet, namely translocated from the outer into the inner leaflet by a flippase activity (Cribier et al. 1993; Seigneuret and Devaux 1984), increases the kinetic of endocytosis (Farge et al. 1999).

Thirdly, from the connection between living and model systems, it was proposed a theory of endocytosis inspired from the ADE model (Devaux 2000; Rauch and Farge 2000). This theory suggests that to generate endocytosis, namely an inward membrane budding, a constant lipid number asymmetry is needed at the level of the cell membrane, maintained by the flippase activity and the membrane recycling (Fig. 1c). The underlying physical formulas are given in Appendix.

This model was compared to experimental analysis and it was deduced that a lipid number asymmetry exists in the membrane of eukaryotic cells: $\Delta N/N_0 \sim 4\%$, where N_0 corresponds to the average number of lipid in either leaflet (see Appendix) (Rauch and Farge 2000). This theory provides a vesicle radius of $\sim$35 nm similar to what is observed and measured in living cells. Therefore, this model suggests that endocytosis results from a differential packing of lipids between the inner and outer leaflets of the cell membrane (Fig. 1c). It is now believed that a similar mechanism takes place in the formation of coated vesicles (Itoh and De Camilli 2006; Nossal and Zimmerberg 2002).

The role of the differential packing on membrane permeability can now be addressed. The membrane barrier energy, namely the energy required for a drug to cross the membrane, is a complex function of the set of interactions between a drug and the membrane components, which varies along the membrane thickness. Initially, drugs are expected to insert the outer leaflet by optimizing their energy (i.e. chemical potential). Thus the hydrophobic (non polar) part of the drug is presumed to be present in the hydrophobic core of the membrane. Resulting from this assumption is the fact that to incorporate totally the inner leaflet and to traverse the bilayer, the drug must transfer its hydrophilic (polar) part in the hydrophobic core (i.e. oily phase) of the membrane. This is a dehydration process. This will inevitably lead to a penalty energy that usually prevails in the middle of the membrane (dashed curve in Fig. 1d), and that shall be noted as ΔG. Note, however, that this penalty energy will remain constant when a cell switch its state from drug sensitivity to resistant, as it is related to the physico-chemical properties of the drug.

Finally, to pass through the membrane the drug will also have to bypass the barrier energy linked to the differential packing of lipids. This penalty energy is maximal at the level of polar heads, where the repulsion between lipids dominates (plain curve in Fig. 1d). As a result, large drugs will feel more strongly the packing because they will be more strongly squeezed. It can be demonstrated that this energy is: $a \times K \times \Delta N/N_0$, where a is the cross section area of the drug intercalated in the membrane, K the elastic modulus of the membrane and "$\Delta N/N_0$" the lipid asymmetry (see Appendix). Note that contrary to the former penalty energy, the lipid packing is expected to be increased in the drug resistant state, given the higher kinetic rates of endocytosis measured in drug resistant cells. In addition, given that the spatial dimension of the drug is involved (i.e. a), this means that the size of the drug (including its MW) may well be central.

The role of the differential packing of lipids in cross resistance to drugs

Considering the cross resistance to drugs, the power law determined above ($\sim MW^{0.59}$) suggests therefore that drug volume or size is a limiting parameter when they cross biomembranes. In turn, this suggests that a basic mechanical interaction between a drug and the cell membrane is very likely to be involved. 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 an energy $\geq k_B T$ (k_B is Boltzmann's constant and T the temperature in Kelvin). In this case, the physical parameter that best fits this interaction is the differential packing of lipids across the membrane ($\sim$0.9 mN/m) (Rauch and Farge 2000) which prevails over the mean mechanical membrane tension[1] ($\sim$0.003 mN/m) (Hochmuth et al. 1996). Accordingly differential packing of lipids is more likely to be involved in mechanically impairing the transverse movement of chemicals across the membrane (Fig. 1d). It follows that a critical cross section (a_c) can be defined that is function of the differential packing of membrane lipids:

$$a_c = k_B T / K(\Delta N/N_0) \quad (1)$$

Using the latter result and the following power law $MW_c \sim a_c^{3/2}$, it follows that Eq. 1 can be rewritten as:

$$MW_c = (4/3\sqrt{\pi})(k_B T/K(\Delta N/N_0))^{3/2} \quad (2)$$

Equation 2 certifies that a critical MW exists that is related to the differential packing of lipids and that will interfere with the membrane transverse movement of drugs according to their size or molecular weight. This critical MW has been determined in drug sensitive cells: $MW_c \cong 240$ (Rauch and Pluen 2007).

Using Eq. 2, it follows that the influx, J, of a given drug (i.e. given MW) across this membrane mechanical barrier can be expressed using Arrhenius' law:

$$J \cong \exp\left[-(MW/MW_c)^{2/3} - \Delta G/k_B T\right] \quad (3)$$

The ED_{50} is an indicator of drug efficiency as it provides the drug concentration needed to kill 50% of cells in a given population. Accordingly, the higher is the ED_{50} the lower is the sensitivity to drugs and thus, the lower is the influx of drugs. Therefore, there exists an inverse relationship between the ability of drugs to cross the membrane of cells and the ED_{50}s. As a result, it follows that:

$$\ln(ED_{50}) \sim \ln(1/J) \sim (MW/MW_c)^{2/3} + \Delta G/k_B T \quad (4)$$

From Eq. 4, the ratio between ED_{50}(DC3FR) and ED_{50}(DC3F) can be determined:

$$\ln(ED_{50}(DC3FR)/ED_{50}(DC3F)) = \left(MW/MW_c^{DC3FR}\right)^{2/3} - \left(MW/MW_c^{DC3F}\right)^{2/3} \quad (5)$$

In Eq. 5, as ΔG is constant this parameter is canceled upon subtraction. Equation 5 can be re-written to display the variables that are usually measured as far as drug resistance is concerned. In particular, higher kinetics of fluid phase endocytosis are measured in drug resistant cells (Ferte 2000; Altan et al. 1999; Colin et al. 1996). Given that the kinetic rates of endocytosis are proportional to the membrane lipid number asymmetry (Farge et al. 1999), using Eq. 2 it follows:

[1] The mean mechanical membrane tension corresponds to the mean lipid packing of the membrane leaflets.

$$\ln(\mathrm{ED}_{50}(\mathrm{DC3FR})/\mathrm{ED}_{50}(\mathrm{DC3F})) = \left(\mathrm{MW}/\mathrm{MW}_{\mathrm{c}}^{\mathrm{DC3F}}\right)^{2/3}[k_{\mathrm{DC3FR}}/k_{\mathrm{DC3F}} - 1] \quad (6)$$

In Eq. 6, $k_{\mathrm{DC3FR}}/k_{\mathrm{DC3F}}$ is the ratio of kinetic rates of phase endocytosis between drug resistant and sensitive cell.

Consequently, and as long as the membrane mechanical properties and drug sizes are involved, the natural logarithm of ED_{50}s should follow the power law given by Eq. 6: $\sim \mathrm{MW}^{0.66}(2/3 \sim 0.66)$. The latter value is close to the one determined experimentally: $\sim \mathrm{MW}^{0.59\pm0.11}$.

Given Biedler and Riehm's data I found that Eq. 6 fits the best when the multiplication constant of the power law is $[k_{\mathrm{DC3FR}}/k^{\mathrm{DC3F}} - 1]/(\mathrm{MW}_{\mathrm{c}}^{\mathrm{DC3F}})^{2/3} = 0.057 \pm 0.002$ ($p < 0.0001$), which using $\mathrm{MW}_{\mathrm{c}}^{\mathrm{DC3F}} \cong 240$ (see Eq. 2) predicts a ratio concerning the endocytosis kinetic rates: $k_{\mathrm{DC3FR}}/k_{\mathrm{DC3F}} \cong 3.19$. The latter value is coherent with the increases in the kinetic rates of endocytosis measured between drug resistant and sensitive cells (Ferte 2000; Altan et al. 1999; Colin et al. 1996).

Given the power law defined by Eq. 6 and using $k_{\mathrm{DC3FR}}/k_{\mathrm{DC3F}}$ and $\mathrm{MW}_{\mathrm{c}}^{\mathrm{DC3F}}$ numerical values, the theoretical prediction can be compared against the experimental data [Fig. 1f; $R^2 = 0.832$ and $F = 29.63$—note that no p value for the F-test has been determined as there is only one variable in the model as the power law (2/3) is given by the model].

Overall, a good agreement is found.

Discussion

Although the involvement of other drug-related properties different from the MW (e.g. the state of charge and lipophilic index, and possibly the effect of drug handling by transporters) can not be ruled out to fully describe drug resistance levels, nonetheless; selecting drug resistance using a large MW drug (such as actinomycin D) and looking at the high levels of cross resistance using smaller drugs, seems to be reasonably well explained by the MW of drugs.[2] Finally, I agree that to be fully conclusive, the number of drugs studied must as large as possible to improve the statistical relevance of this theory. In addition, this study would certainly gain from experiments on drug permeability of well controlled model membranes that have yet to be done.

Drug's MW and MDR: when the size matter

As already stated, the reason for looking at parameters different from the level of transporter expression and transporter themselves is because small drugs should cross the membrane more easily than large drugs. This notion is intuitive and well described and modeled physically by the mechanical interaction between a drug and the membrane.

It is important to recall that as there is no reason why a drug should incorporate the membrane in the vicinity of a transporter. Thus, membrane-embedded drugs have to diffuse laterally in the membrane prior to meeting and being extruded by transporters.

A parameter that controls the length of diffusion of membrane-embedded drugs, and thus the chance of drug meeting a transporter, is the size of drugs. Thus, by legitimately assuming that the level of transporter expression is set by the drug employed to select the resistant phenotype, if the latter has a large MW, the level of expression of transporter is expected to be set, at least partially, by the MW of the selecting drug. It follows that the surface density of transporters may not be adequate to expel another smaller drug. In this scenario, the drug-membrane mechanical interaction would dominate the cross resistance effect.

To model the role of drug-membrane mechanical interaction I have logically supposed that the ratio between the ED_{50} values (Eq. 6) should follow Arrhenius' law: $\sim \exp[a(\mathrm{MW})^b]$. Note again that this Law warrants that basic physico-chemical principles are valid when drugs cross the cell membrane, and affirms that when $\mathrm{MW} \to 0$ (for very small drugs), then $\exp[a(\mathrm{MW})^b] \to 1$. Therefore, for small drugs the level of resistance should drop to become similar to those measured in sensitive cells. Although reaching $\mathrm{MW} \sim 0$ for drug is, practically speaking, unrealistic nonetheless, the model remains logic and coherent with the initial hypothesis, which is based on the study of Biedler and Riehm (1970). It is, therefore, naturally expected that any theory that models how a drug crosses membrane should ensure that when $\mathrm{MW} \to 0$, the level of resistance vanishes. Conversely, this means that any model that demonstrates a residual level of resistance when $\mathrm{MW} \to 0$, should be disregarded.

If I am insisting on this point it is because by looking at Fig.1a, it would have been trouble-free to plot a straight

[2] A point that certainly needs clarification is related to the affinity between drugs and transporters. It is well known that the binding affinity between drugs and transporters varies between drugs, and it could be postulated that drug with a low MW may have a lower binding affinity with transporters. In turn this could explain why drugs with a low MW cross the membrane more easily as they are not (or less) extruded by transporters (see Fig. 1a). Given that the term "affinity" defined in physics suggests a favorable interaction, if the MW of drugs was to be involved this would mean that the mass of the drug should be responsible for this affinity. The only force that exists and involves the mass as a source of interaction is the gravity. However, application of the gravitational law is only valid over very large scales, well beyond the molecular scale. Thus, the gravity can not be applied in our case. In turn, this means that the assumption suggesting that the binding affinity between drugs and transporters is related to the MW of drugs can be ruled out.

line that, statistically, would have provided a linear equation under the form: 1.968 + 0.003MW, with $R^2 = 0.838$, $F = 25.84$ and $p = 0.004$. Although the statistical parameters deduced are good enough (albeit less significant that those deduced from Arrhenius' law and the power law together), the main paradox arising from this linear fit is that when MW $\rightarrow$ 0 there is a residual constant (i.e. 1.968) level of resistance that is independent of the molecular weight of drugs. As stated above, this is a paradox as when MW $\sim$ 0 no resistance should stand. Thus, this constant contradicts and undermines the logic and coherence of the working hypothesis. Thus, this straight line had to be rejected for the sake of Biedler and Riehm's study and conclusion (1970), and of the importance of MW in drug delivery.

It is noteworthy that, on top of Biedler and Riehm's (1970) seminal study, the size of drugs has been several times highlighted regarding their ability to cross bilayer membranes:

In silico molecular dynamic simulations have shown that the transverse movement of compounds across lipid membrane is affected as a function of their sizes (Bemporad et al. 2004; Mitragotri et al. 1999; Walter and Gutknecht 1986; Xiang and Anderson 1994).

Modulators, i.e. drug resistance reversing agents tend to have a smaller size compared to classical drugs, which contributes to their efficacy (Zamora et al. 1988).

Last but not least, the pharmaceutical industry knows very well that the bioavailability of oral drugs in vivo, which is function of the expression of similar Pgp-like membrane proteins in the intestinal epithelium (Brinkmann and Eichelbaum 2001; Marathe and Rodrigues 2006), is also dependent on the drug's molecular weight (known as Lipinski's second rule, Lipinski et al. 2001).

Therefore, the fact that a growing body of works performed in different research fields has demonstrated that the size of drugs is important for their membrane permeability, suggests that a common mechanism may explain these similar observations. I suggest that the interaction between the size of drugs and the membrane mechanical properties can provide a common explanation for these observations.

Drug size and Lipinski's second rule: application to pharmaceutical science

An aspect of this present work that needs to be underlined is its coherence with studies from the field of drugs' bioavailability and its impact regarding the "drug discovery paradigms". A significant bottleneck in the drug discovery pipeline is determining the properties of a drug that facilitate its delivery to, and uptake by, target tissues/cells. Not surprisingly, drug transporters such as the well known Pgp lining the intestinal epithelium of the gut are directly involved in the impairment of the bioavailability of drugs (Zhang and Benet 2001).

To bypass this barrier and improve the bioavailability of drugs, Lipinski and collaborators (Lipinski et al. 2001), from Pfizer, have produced a set of rules that attempt to identify the physico-chemical properties required for an oral compound to achieve maximum bioavailability, i.e. to cross all biological barriers before reaching its target. They based their study on marketed oral drugs and from the analysis of the physico-chemical properties of these they determined the "drug-likeness" properties.

The first of Lipinski's rules is based on the lipophilic index of the drug, the second on the drug's MW and the third and fourth rules concern the drug's charge properties.

The second rule states that to achieve a 90%-bioavailability drug must have a MW lower than 500. The theory proposed here and based on the membrane mechanical properties and forming a continuity with another work (Rauch and Pluen 2007), suggests that the MW cut off would correspond to MW of $\sim$240, which is similar to the value predicted by Lipinski's second rule.

As stated above, this similarity suggests that Lipinski's second rule and drug transporter mediated MDR may represent the two faces of a single coin in which the mechanical interaction between drug and lipid bilayer is predominant.

Lipid packing alteration in MDR: which origins?

The model proposed in this present paper also suggests that, if the MW is involved and follows a power law under the form: $a \times \mathrm{MW}^b$, then the constant "a" can be explained and numerically valued by the differential packing of membrane lipids between leaflets, which drives endocytosis (Fig. 1b, c). It is therefore worth asking how the membrane is expected to be changed after induction of drug resistance. As the kinetic rate of membrane endocytosis is increased in drug resistant cells, reviewed in Ferte (2000), and that the kinetic rate of endocytosis is proportional to the differential lipid packing in the membrane (Farge et al. 1999), it is expected that the differential packing of lipid in the membrane of drug resistant cells is increased.

There are two ways to increase the differential packing of lipids:

a by adding more lipids in the inner leaflet of drug resistant cells, which equivalently would suggest that the metabolism of lipids is affected in the drug resistant phenotype or;

b by increasing the physical repulsion between lipids in the inner leaflet, the physical repulsion between lipids being responsible for the packing of lipids.

Regarding the first point (a), changes in the metabolism of membrane lipids, including membrane properties, has been noted between resistant and sensitive cells (Hendrich and Michalak 2003; Liang and Huang 2002; Venkatesan et al. 1998); nonetheless, given the very complex subcellular network controlling the exact composition in lipids of the membrane (Bakovic et al. 2007), the amount of a specific lipid including its membrane location (i.e. outer or inner leaflet) has yet to be carried out in cells. However, there are data obtained in cells that tends to suggest indirectly that the alteration of the packing of lipids, via changes in the phospholipid metabolism, is indeed important in drug resistance. For example, it was demonstrated that tamoxifen, which reverses drug resistance, inhibits the metabolism of phospholipids in drug resistant but not sensitive cells, with a predominant effect on phosphatidylethanolamine (Kiss and Crilly 1995). Phosphatidylethanolamine, like phosphatidylserine, is chiefly located in the inner leaflet of the cell membrane. Now, to say that an extra pool of phosphatidylethanolamine exists in the drug resistant state and is the cause of drug resistance because of the higher packing it generates would be rather risky. On the other hand, this raises the possibility that lipid packing and metabolism are linked together in the drug resistant state and likely to be, at least indirectly, involved.

The last point (b) is more focused on the physical packing of lipid and on the parameters that could control its alteration. It is noteworthy that the cytosolic pH has been demonstrated to be essential in drug resistance as pH reversal, from alkaline to acid, increases and controls the sensitivity to drugs (Altan et al. 1998, 1999; Raghunand et al. 1999; Schindler et al. 1996). To consider any effect of the cytosolic pH on lipid packing it is central to clarify the notion of physical packing. At constant membrane surface area, the lipid packing is given by the optimal area per lipid in the cell membrane. The latter is deduced from the balance between lipids repulsion (including hard core or electrostatic effects) and attraction (aliphatic chain(s)/hydrophobic effects), any changes in this balance is expected to affect the optimal area per lipids (i.e. their packing). Therefore, as a non negligible fraction of lipids in the inner leaflet are negatively charged, such as phosphatidylserine for example (Alberts et al. 1994); a slight increase in positive ion concentration (e.g. decrease in pH) is expected to interact with lipids, "masking" their negative charges and decreasing the electrostatic repulsion between them. As a final result, a low cytosolic pH is more likely to be central to collapse the physical repulsion between lipids, and thus to decrease the packing of lipids of the cytosolic leaflet in contact with the low cytosolic pH. Such a relationship between free electrolytes and the cross section area per lipid in model biomembranes is well known experimentally (Petelska and Figaszewski 2002; Petrache et al. 2006; Victorov et al. 1997). Conversely, when the pH increases (i.e. when cells become resistant to drugs in our case), fewer positive charges would be available to mask the lipids charge, which in turn is expected to increase the repulsion between lipids and thus increase their packing. To conclude, this higher differential packing would thus come from the high cytosolic pH of drug resistant cells. This point *b* is being addressed and forms the basis of a future publication.

The two faces of the differential packing of lipids in MDR

The differential packing of lipids in the cell membrane is likely to have two roles in MDR, firstly it is expected to impede the transverse movement of drugs across the membrane but also, and secondly, it is expected to increase the kinetics of membrane endocytosis, which drive the vesicular transport of drugs from the membrane to acidified compartments, where weak base drugs get trapped (Altan et al. 1998, 1999; Raghunand et al. 1999; Rauch and Pluen 2007; Schindler et al. 1996). It is therefore worth asking whether the vesicular transport of drugs, from the membrane to acidified compartments, could not have explained the levels of resistances obtained by Biedler and Riehm. A thorough analysis has compared and weighted the role of endocytosis versus the differential packing of lipids in MDR. The result of this analysis is that the differential of membrane lipids would be a more influential parameter in MDR than endocytosis. The reason for this is that the amount of drugs blocked at the membrane level would follow an exponential function of the drug's MW, whereas the amount of drugs trapped in acidified compartments should be a linear function of the kinetic rates of endocytosis, independently of drugs' MW (see Eq. 19 in Rauch and Pluen 2007). As the present study confirms that the MW of drugs is important in MDR, the kinetic rate of endocytosis is likely to be secondary to the ability of the membrane to block the transverse movement of drugs.

Conclusion

It is true that highly resistant cells often display membrane fragility and many discussions have centered on the pathophysiological relevance of such cell lines. However, if drug transporters extruding membrane-embedded drugs are to be involved in drug resistance whatever the level of resistance, given how they functions, namely pumping out drugs from

the inner leaflet, several implications follow. One of these is related to the mechanism that governs the chance of a drug and a transporter meeting. This mechanism has been demonstrated to rely on the membrane mechanical properties. Here, I further develop and support the role of the membrane mechanical properties in a specific case of cross resistance to drugs, namely when a large drug has been used to select the resistant phenotype and that smaller drugs have been used subsequently.

Accordingly, I suggest that:

1 *High levels* of cross resistance to drugs (MDR) result *also* from the interaction between the cell membrane mechanical properties and the volume of drugs.
2 Cells protect themselves from xenobiotics by altering fluid phase endocytosis, thus putting in place mechanical barriers interfering with drugs' MW/size.
3 The phospholipids metabolism is an obvious target to fight MDR as it can alter the mechanical properties of cells membranes.

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Appendix

Assuming a flat membrane composed of two leaflets and containing, N_{in} lipids in the inner leaflet and, N_{out} lipids in the outer leaflet ($N_{in} > N_{out}$). The relative lipid number asymmetry: $\Delta N/N_0$, with $\Delta N = (N_{in} - N_{out})$ and $N_0 = (N_{in} + N_{out})/2$, generates a differential packing across the membrane of thickness, h. In turn this generates a momentum (i.e. a bending force): $\sim hK\,\Delta N/N_0$, that is proportional to the elastic modulus of leaflets, K, and that tends to bend the membrane inwardly (Fig. 1c).

However, the bending stiffness of the membrane, i.e. the membrane bending modulus k_c, balances the inward bending by an outward bending force that is proportional to k_c and inversely proportional to the radius, R, of the vesicle to be created: $\sim k_c/R$. As such the bending stiffness of the membrane imposes a limit to the creation of too small vesicles.[3] Finally, given this mechanical model of membrane budding, the optimal size of vesicles is dictated by the equilibrium between the inward and outward bending forces, namely: $hK\Delta N/N_0 \sim k_c/R \Rightarrow R \sim k_c/(hK\Delta N/N_0)$. A detailed analysis gives $R = 8k_c/hK(\Delta N/N_0)$(Rauch and Farge 2000).

[3] Mechanically, less energy is required to bend a flat object to give this object a small curvature (i.e. large radius: curvature = 1/radius) than to give it a high curvature.

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