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ORIGINAL PAPER

Toward a mechanical control of drug delivery. On the relationship between Lipinski's 2nd rule and cytosolic pH changes in doxorubicin resistance levels in cancer cells: a comparison to published data

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Abstract Based on molecular and physiological resemblance, the mechanism that controls drug bioavailability and toxicity also shares strong similarities to the one that controls drug resistance. In both cases, this mechanism relies on the expression of drug transporters and the physico-chemical properties of drugs, which together alter the intracellular accumulation of chemicals in cells or tissues. However, a parameter that is central and has received great attention in the field of bioavailability, but almost none in the field of drug resistance, is the molecular weight of drugs. In the former area, it is well known that to achieve a reasonable bioavailability, drugs must have—among other properties—a molecular weight less than 500, known as Lipinski's 2nd rule. Accordingly, it is worth questioning whether a similar rule exists in the field of drug resistance and what subsequent mechanism would control the membrane permeability to drugs as a function of their molecular weight. I demonstrate here that cytosolic pH fixes the molecular weight of drugs entering cells, by altering the cell membrane mechanical properties and that, both cytosolic pH and membrane mechanical properties are needed and sufficient to explain doxorubicin resistance levels in different cancerous cell lines. Finally, I discuss the efficiency of a drug handling activity by transporters in MDR and suggest ways to control drug delivery mechanically. In addition, and for the first time, the literal expression of a Law similar to Lipinski's 2nd rule will be described as a function of cytosolic pH and lipid number asymmetry.

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Introduction

Although drug design now often provides effective pharmacology, the variable absorption and biodistribution of drugs within tissues remain a considerable problem, and the socio-economic impact of drug resistance and variable drug responses worldwide is colossal.

Among the transmembrane proteins initially discovered to impair the transbilayer movement of drugs across cells' membrane, are the multi-drug resistance (MDR) membrane proteins such as the well-known P-glycoprotein (Pgp) expressed in microorganisms or cells (Lage 2003). Historically, and given their homologies to bacterial membrane transporters, these newly discovered membrane transporters were initially postulated to vacuum drugs from cells (known as the "vacuum cleaner" hypothesis) (Gerlach et al. 1986; Gros et al. 1986).

Although primarily embraced, it was later found that the vacuum cleaner hypothesis and the drug handling mechanism/activity may not always be satisfactory to explain drug resistance (Wadkins and Roepe 1997). Therefore, it is now acknowledged that drug resistance is a complex process that requires the existence of synergisms between different biological processes (Ferte 2000; Harguindey et al. 2005; Hoffman et al. 1996; Roepe 2000; Wadkins and Roepe 1997). Among synergisms, it was demonstrated that in cells expressing "drug transporters", alteration of the membrane electrical potential and the alkalization of the cytosolic pH interact with the physico-chemical properties of drugs (including lipophilic and charge properties) and that this set of interactions alter their intracellular accumulation (Altan

et al. 1998, 1999; Chen et al. 1999; Raghunand et al. 1999; Schindler et al. 1996; Wadkins and Roepe 1997).

In addition to this, it was suggested more recently that the size (or volume) of drugs could be an important parameter in drug resistance (Rauch 2008; Rauch and Pluen 2007). These later studies that mostly focused on the drug–membrane mechanical interaction are supported by, and in qualitative agreement with, other works that demonstrated that chemical size (or molecular weight) is central to drug transverse movement across the cell membrane. For example, in silico molecular dynamic simulations have shown that the transverse movement of compounds across lipid membrane is affected by their sizes (Bemporad et al. 2004; Mitragotri et al. 1999; Walter and Gutknecht 1986; Xiang and Anderson 1994). Moreover, 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). In addition, in vitro studies have reported that the drug MW (which is proportional to drug volume) can account for up to 84% of multi-drug resistance levels measured, and that the drug MW predominates over the lipophilic index (Biedler and Riehm 1970; Rauch 2008; Selassie et al. 1990). Finally, the pharmaceutical industry is extremely aware that in vivo oral drug bioavailability, 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 drug MW (known as Lipinski's 2nd rule) (Lipinski et al. 2001).

Therefore, it seems that drug size is central to basic drug delivery. Moreover, as in physics the conjugated parameter of volume or size is the surface pressure (i.e. membrane tension) for lipid biomembranes, it is expected that basic mechanics can be applied regarding drug bioavailability and resistance (Rauch 2008; Rauch and Pluen 2007). If so, it is imperative to determine what controls the drug–membrane mechanical interaction. It is the aim of this present paper to address which recurring features(s) of drug resistance is responsible to make the drug size (or molecular weight) an important parameter.

Among important recurring features, when cells switch their states from drug sensitivity to resistance, are the increases in the kinetic of membrane endocytosis and cytosolic alkalization, reviewed in Ferte (2000).

Regarding membrane endocytosis, it has been demonstrated that the mechanism that drives endocytosis and controls its kinetic is related to the differential packing of lipids between the two leaflets of the membrane (see Fig. 1a) (Farge 1995; Farge et al. 1999; Rauch and Farge 2000). Thus, based on the constant observation that endocytosis is increased when cells are resistant to drugs (reviewed by Ferte (2000)), it has been suggested that the differential packing of membrane lipids between leaflets maybe involved in impairing the transverse movement of drugs across the plasma membrane (see Fig. 1b, e) (Rauch and Pluen 2007). Simply, this packing would mechanically squeeze drugs in the membrane impairing their transverse movement. The ability of the cell membrane to increase the residency time of membrane embedded drugs is central to Pgp-like transporter mediated drug handling and extrusion, as not only it would allow drugs to diffuse randomly toward membrane transporters prior to extrusion (see Fig. 1c), but it would also allow the extrusion from the inner leaflet (see Fig. 1d) (Rauch and Pluen 2007). Accordingly, it is accepted that the ill-defined "vacuum cleaner" notion can be interpreted by a 2D random walk (see Fig. 1c) (Rauch and Pluen 2007). To date however, the mechanism responsible for increasing the packing of lipids in MDR cells, in turn controlling the residency time of membrane embedded drugs, remains unknown, but could well be linked to the cytosolic pH. It is the aim of this present paper to fully address this point.

It is noteworthy that the 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). Thus, given the importance of cytosolic pH in drug resistance, by assuming that the drug transverses movement if affected by the differential packing of lipids, it may well be that this differential packing is governed by pH changes (we shall see that the membrane potential is unlikely to be involved in this packing).

To consider any effect of the cytosolic pH on lipid packing it is central to understand the notion of packing as defined in physics. At a 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 are expected to affect the optimal area per lipid (i.e. their packing). Therefore, as a non negligible percentage of inner leaflet lipids are negatively charged (Alberts et al. 1994), a slight increase in positive ion concentration (i.e. decrease in pH) is expected to interact with lipids, "masking" their negative charges and decreasing the electrostatic repulsion between them. However, it is important to note that given the size of hydrogen ions (i.e. exchangeable protons[1]) they will have a

[1] I agree that the term "hydrogen ion" (i.e. proton) is not adequate when the physico-chemical properties of acid and base solutions are considered (as it is the water molecule that bears hydrogen: H_3O^+). Nonetheless, using the term "hydrogen ion" is easier to represent the electrostatic interaction between proton and negatively charged lipids. For that reason, the term "hydrogen ion" will be used in the text to represent either H_3O^+ (i.e. acid solution) or H^+ (i.e. the electric charge).

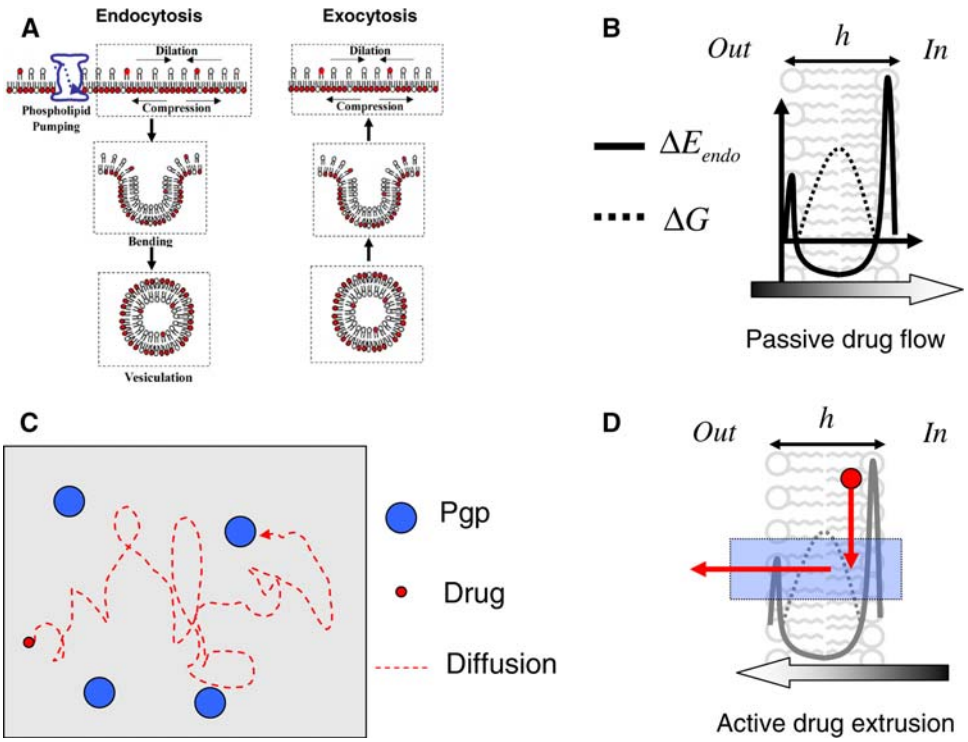

Fig. 1 **a** The lipid number asymmetry induced FPE model: sketch representing the current model that has been applied in living cells linking FPE and the membrane phospholipid number asymmetry thought to be maintained by the aminophospholipid translocase. In the left figure, the translocation of dark-headed lipids into the inner leaflet induces a differential lipid packing between leaflets (namely a difference in surface tensions) 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, also allows the maintenance of the lipid asymmetry at the level of the plasmalemma. The relationship that exists between the lipid number asymmetry and the vesicle radius is given by $R = -8k_c/h\Delta\sigma$ or equivalently $R = 4k_c/hK \cdot 1/(\delta N/N_0)$, where k_c, K, h, $\Delta\sigma$ and $\delta N/N_0$ are the membrane bending modulus, membrane elastic modulus, membrane thickness, difference in surface tensions and the lipid number asymmetry between leaflets. Accordingly, the lipid number asymmetry has been experimentally deduced from studies on drug sensitive cells (K562) with a value $\delta N/N_0 = 2 \pm 0.5\%$ providing a ~35 nm vesicle radius (Rauch and Farge 2000). **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 both surface tension in leaflets (*plain curve-middle graph*) and hydrophobic core of membrane (*dashed curve-middle graph*) are involved in providing a penalty energy with regard to the transbilayer movement of drugs. However, in the present paper, one will mostly be concerned by the surface tension which impedes the transverse movement of drugs as a function of their size (Eq. 3). **c** The set of energies involved will allow the drug to remain in the membrane and to diffuse randomly and meet transporters. Note that this 2D random walk model which explains in simple term how drugs meet transporters, has also been suggested and put forward to replace the "vacuum cleaner model" (see (Rauch and Pluen 2007)). **d** In the presence of transporters, it is expected that the setting of these energies (see (B)) will trap the drug in the inner leaflet allowing drug and transporter to meet for any extrusion to take place

more pronounced effect on negatively charged lipids than any other cation. As a final result, a low cytosolic pH is more likely to be central in collapsing the physical repulsion between lipids, and thus to decrease the surface tension (i.e. the lipid packing of the cytosolic leaflet as the lipid packing and surface tension are proportional to each other). 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 will be available to mask the lipids charge, which in turn is expected to increase their repulsion and thus their packing. Thus, this higher lipid packing would increase the surface tension of the leaflet in contact with the milieu of elevated pH, namely the cytosol in the case of drug resistant cells.

Finally, the changes in pH observed when cells switch their state of resistance may be directly responsible for altering the intracellular accumulation of drugs as a function of their sizes, thus impairing their activity. In due course, this could provide a strong argument for the unification of the fields of drug bioavailability and drug resistance.

I will endeavor to provide: a short review of endocytosis which is mediated by the differential lipid packing between

leaflets, and how this differential lipid packing is involved in selecting drugs membrane permeability as a function of their sizes (similar to Lipinski's 2nd rule); a model linking lipid packing and cytosolic pH will be proposed. Finally, a comparison between published data and the theory proposed will be given.

Accordingly the main conclusions will be:

1. Cytosolic alkalinization allows cells to put in place a membrane mechanical barrier that impairs the transverse movement of chemicals as a function of their sizes.
2. Contrary to changes in the membrane potential which *stops pulling* cationic drugs into cells when they become resistant to drugs, the mechanical barrier *opposes drug entry* into cells irrespectively of their charges.
3. In Pgp expressing cells, high levels of doxorubicin resistance are unlikely to be related to drug handling activity.
4. A mechanical control of drug delivery can now be properly envisioned.

From Lipinski's 2nd rule to a Law regarding drugs' size selectivity on their permeation across cellular membranes

To traverse cellular barriers, drugs must cross lipid membranes, and for this Lipinski's 2nd rule postulates that drugs must have, among other properties, a MW lower than 500.[2] As stated in the introduction, this rule suggests then that drug volume is thus a limiting parameter when cell membranes are traversed. In turn, this suggests a basic mechanical interaction between a drug and the cell membrane. Therefore, in the sum of energies making up the total activation energy required for a drug to cross cellular membranes, there must be an energy term that is a specific function of the drug's dimension so that the drug/membrane interaction yields an energy $\geq k_B T$ (where k_B is Boltzmann's constant and T the temperature in Kelvin). In this case, i.e. when the plasma membrane is considered, the physical parameter that best fits such an interaction is the leaflets' surface pressure, σ.[3] However, in cells, two types of membrane tension can be distinguished, the mean surface tension noted σ_0, which corresponds to the sum of individual leaflet's surface tension, and the difference in surface tensions $\Delta\sigma$, which corresponds to the difference between individual leaflet's surface tension namely between the inner and outer leaflet. However, 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, it follows that the magnitude of the drug critical cross section, a_c, can be defined by:

$$a_c = -k_B T \,/\, \Delta\sigma \tag{1}$$

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 associated with this inward pumping is a constantly more highly packed inner leaflet as it contains more phospholipids than the outer leaflet (Fig. 1a). 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) and that the vesicle radius, R, can be expressed as (Fig. 1a) (Rauch and Farge 2000):

$$R = -8k_c/h\Delta\sigma \tag{2}$$

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 Walls' 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):

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

Equation 3 provides a law with regard to the drugs' size (or MW) selectivity on their permeation across cellular membranes. Using the numerical values of physical constants or biological parameters from drug sensitive cells, it follows that $MW_c \cong 240$ at 37°C, which is remarkably close to the value given by Lipinski's 2nd rule. In addition, the enthalpy energy, ΔH, required to traverse the membrane is the sum of two terms linked to the drug dehydration and size, given by (Rauch 2008; Rauch and Pluen 2007):

$$\Delta H = \Delta G + k_B T \cdot (MW/MW_c)^{2/3} \tag{4}$$

where ΔG is the dehydration energy of a drug crossing the membrane (Fig. 1b). Note that Eq. 4 demonstrates that the

[2] Lipinski's rules defines the 90th percentile of physico-chemical properties drugs should have to achieve the greatest bioavailability.

[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.

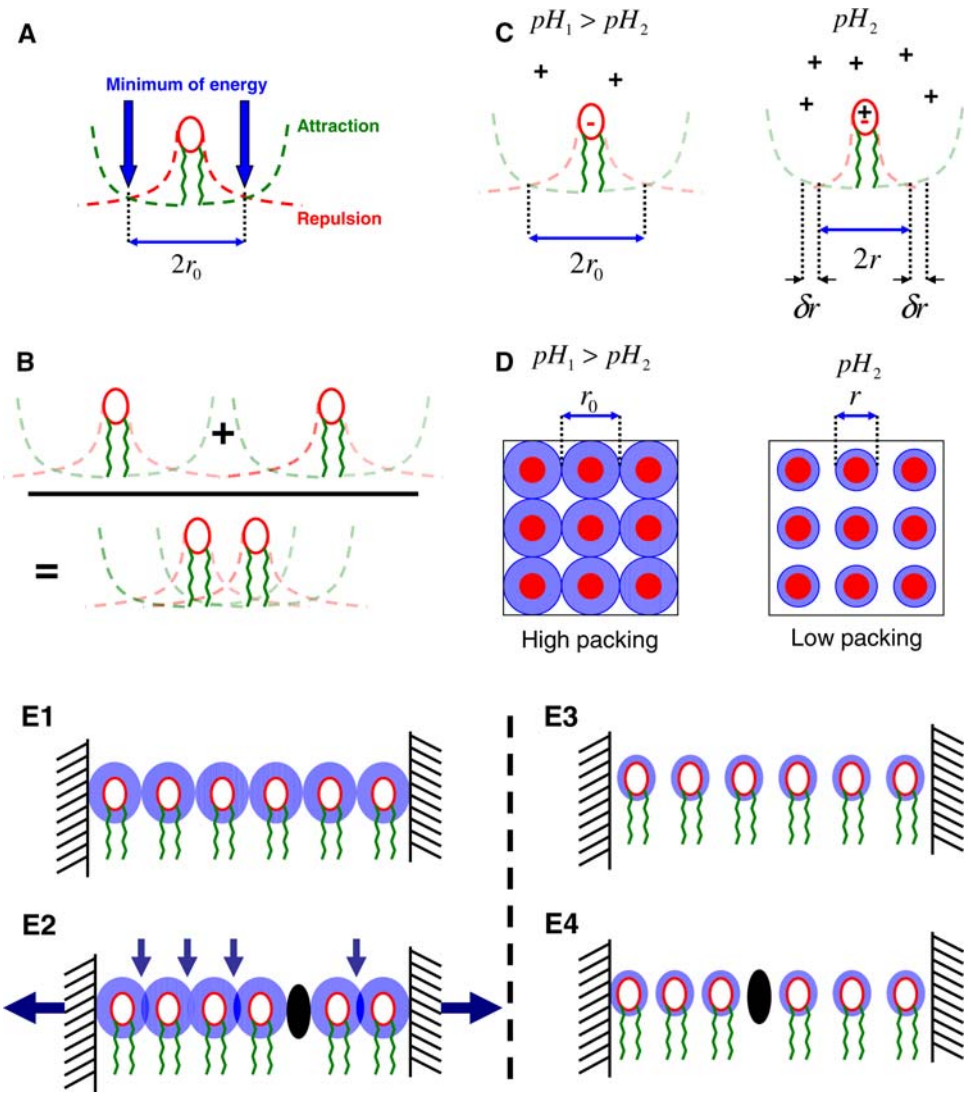

Fig. 2 Effect of pH on lipids packing. **a** Assuming a leaflet composed of charged lipids. The optimal area per lipid is determined by the competition between an energy that reflects lipids attraction linked to their hydrophobic tails, and a repulsion energy which we will assume to be linked to a net charge carried by all the lipids. The competition between these two terms defines a minimum of energy. Note that in the figures r_0 corresponds to the optimal distance between adjacent lipid heads. **b** Thus the minimum of energy provides the optimal distance between lipids including their optimal area in the monolayer. Note that the packing of lipids is not always defined by hardcore contact and that, accordingly, there is room to change this packing. **c** With regard to negatively charged lipids, an increase in the concentration of hydrogen ions allows more hydrogen ions to interact with lipids' head. Thus by masking their negative charge the long range repulsion between lipids is disturbed. The resulting effect will be an alteration of the positioning of the minimum of energy, which will be closer to lipids. **d** Top view of a portion of membrane. The lipid's hardcore head is colored in red and the optimal area per lipid driven by repulsive/attractive interactions is drawn in blue. Changes in pH are expected to redefine the optimal area per lipid and thus their packing. In the figure a decrease in the pH is represented, i.e. $pH_2 < pH_1$ (pH_2 would stand for the drug sensitive state). In conclusion, a low cytosolic pH is expected to decrease the surface area per lipid. Lipids should have more room thus decreasing their packing. However, in our case one is initially dealing with an inner/cytosolic leaflet that is initially more packed (see Fig. 1a). Changing the pH is thus expected to affect the packing of inner leaflet lipids. To conclude, it is important to note that the packing of lipids can vary even though the number of lipids is unchanged. In this case, pH-driven alteration in the lipids repulsion causes this change. Accordingly, this change will affect the transverse movement of drugs across the membrane and thus their efficacy. This point is represented in Figs. e1–e4. **(e1)** Assuming a lipid leaflet and that the leaflet has an optimized configuration. This means that the resulting mechanical packing is low. **(e2)** A drug incorporating the leaflet will have to "squeeze" the leaflet mechanically leading to lipid energies overlapping (*vertical arrows*), which in turn will lead to high surface tensions (*horizontal arrows*). **(e3)** If on the contrary the lipids have a lower packing than in E1, a drug can cross the leaflet easily without being disturbed by the mechanical packing of lipids **(e4)**. This sketch demonstrates how the changes in cytosolic pH may have a very strong effect on drug permeability

critical MW given by Eq. 3 will impact strongly on the ability of a drug to cross the membrane as a function of its MW.

It is important to recall that this enthalpy energy is central for any transporter mediated drug extrusion. Not only will this energy barrier allow drugs to remain in the membrane for longer, thus permitting drugs to diffuse randomly toward transporters (Fig. 1c) but also, by blocking drugs in the inner leaflet this process is supposed to favor the pumping and extrusion from the inner leaflet (Fig. 1d) (Rauch and Pluen 2007; Shapiro et al. 1997; Shapiro and Ling 1997).

The next parts will address how the cytosolic pH affects the difference in surface tensions and vesicle radius, which, in drug resistant cells, will have an impact on the critical molecular weight defined by Eq. 3.

Overview of the model

Before starting writing equations it is important to underline the physical principles that will be used, for that a "toy model" is best to understand the main principles.

The optimal area per lipid is given by the competition between lipids repulsion and attraction. The attraction between lipids is dominated by aliphatic chains that are hydrophobic and consequently, lipids will tend to attract each other (green curve in Fig. 1a). On the other hand, lipids cannot get very close to each other because the hydrophobic attraction is balanced by both steric and electrostatic repulsions that are caused by the composition of their head (red curve in Fig. 2a). As a result, the competition between these two terms of energy defines a minimum of energy (Fig. 2a). In turn this means that if two lipids are left to interact with each other in aqueous solution, they will tend to be in the minimum of energy of each other (Fig. 2b). It is worth noting that there is no need for lipids to touch each other (hard core notion) to define the "packing".

If we now assume that lipids are initially negatively charged (Fig. 2c), this means that the main contribution to the repulsion is linked to the charge of lipids. In this scenario, it is expected that the number of hydrogen ions that will interact with negatively charged lipids will be a function of the pH of the solution. Therefore, at low pH (pH_2 in Fig. 2c), more hydrogen ions will interact with negatively charged lipids. In turn this will affect the repulsion between lipids, changing the optimal area per lipid and thus their packing. At a constant surface area the packing of lipids is expected to decrease (Fig. 2d). In our case, one is initially dealing with an inner leaflet that is more packed that drives endocytosis (Fig. 1a). It follows that a low cytosolic pH should decrease the packing of lipids which, in turn, is expected to decrease the difference in surface tensions between leaflets (packing and surface tension are proportional to each other). Given Eq. 2, this should increase the fluid phase vesicle radius and thus allow bigger drugs to cross the membrane (see Eq. 3). Conversely, high cytosolic pH should increase the packing of lipids and block the transverse movement of drugs across the cell membrane.

To conclude, the cytosolic pH would put in place a mechanical barrier (or mechanical activation energy) impeding drugs transverse movement across the plasmalemma of cells.

It is now important to fully address this problem in physical terms to get predictions.

Model part I: determination of energies involved

To determine and model how the pH may alter the mechanical properties of the cell membrane, we shall consider the thermodynamic equilibrium of an ideal leaflet, namely a surface S, composed of N identical lipids. The optimal area per lipid in the monolayer, a_0, can be determined by optimizing the contribution of different energies arising from the structural properties of lipids, of which the main interactions are hydrophobic, steric and electrostatics. The electrostatic interactions between lipids can be subdivided into charge–charge, charge–dipole and dipole–dipole interactions. However, the magnitudes of charge–dipole and dipole–dipole interactions are much weaker and at a relatively short range compared to strong and long range charge–charge interaction ($\gg k_B T$, where k_B and T are Boltzmann's constant and the temperature in Kelvin) (Gershel 1995).

Therefore, the hydrophobic interaction will be represented by a single term of energy (E_1); the membrane energy resulting from steric, charge–dipole and dipole–dipole interactions (both short ranges and relatively weak interactions $\sim k_B T$) will be represented by a single energy term (E_2). Finally, E_3 will characterize the charge–charge interactions ($\gg k_B T$).

The first energy (E_1) is linked to the non polar (hydrophobic) part of the lipids, which increases as the surface area per lipid increases (due to contact with water). As a result, this term is positive and proportional to the non optimized area per lipid, a, written under the form:

$$E_1 = KNa \tag{5}$$

where K has the dimension of a tension (i.e. a 2D-elastic modulus).

The second energy (E_2) describes short range and weak interactions between lipids. This energy is proportional to the lipid density (N/S) and to the number of close neighborhoods, z, located in the vicinity of each lipid. Consider a 2D-lattice where each site is occupied by a lipid. In this lattice, the presence probability of a given lipid is $\sim (N/S)\theta^2$

where the characteristic length that defines the lattice mesh is θ, that is expected to be numerically close to the lipid head radius assuming a rod-like shape for lipids. As one considers weak interactions only, the interaction energy per lipid is $\sim z\tilde{\varepsilon}(N/S)\theta^2$, where the number of close neighborhoods is z and $\tilde{\varepsilon}$ is the typical energy involved in pair-interaction between lipids. Repeating this operation for each lipid of the monolayer it follows that the total interaction energy is $\sim z\tilde{\varepsilon}(N/S)\theta^2 N/2$, where the factor 1/2 is present to avoid counting the same pair-interaction twice. Finally, noting $z\tilde{\varepsilon}\theta^2 = v \cdot k_B T$, where v is similar to the second virial coefficient of a 2D polar head gas, it follows that this energy can be written as $E_2 = Nk_B Tv/2 \cdot (N/S)$. Given that $N/S = 1/a$ it follows:

$$E_2 = \frac{1}{2} N k_B T v \frac{1}{a} \tag{6}$$

The third energy of interest (E_3) is the energy between charged lipids. One will assume an homogenous distribution of charged lipids in the membrane and that due to the presence of free cytosolic electrolytes, the net charge of the lipid is screened over a critical lateral length l_c, which is Debye's length (Nguyen et al. 2005). Note that l_c is the square root of the sum of square ionic concentrations and any changes in the membrane potential reflected by a change in ionic concentrations, would affect l_c (see "Discussion"). One will note p_0 the probability that a given lipid in the monolayer is charged, i.e. p_0 is the ratio between the number of charged lipids and the total number of lipids. Under such conditions, a given charged lipid can only influence another charged lipid if the latter is within the surface area defined by the critical length l_c and expressed as πl_c^2. Thus the probability that a given charged lipid interacts with another is $p_0 \pi l_c^2/a$, where $\pi l_c^2/a$ is the number of lipids in the surface area defined by l_c, and p_0 the probability that the lipid is charged. It follows that the interaction energy between a given charged lipid and another in the monolayer is $\bar{\varepsilon}(l_c) p_0 \pi l_c^2/a$, where $\bar{\varepsilon}(l_c)$ is the interaction energy that is function of the critical length l_c. Repeating the same operation over each charged lipid composing the monolayer without counting twice the same pair-interaction it follows:

$$E_3 = \frac{1}{2} N \bar{\varepsilon}(l_c) p_0^2 \pi l_c^2 \frac{1}{a} \tag{7}$$

In Eq. 7, a literal expression of $\bar{\varepsilon}(l_c)$ must be given. As a mean field approach has been considered so far, $\bar{\varepsilon}(l_c)$ represents the characteristic energy linked to electrostatic interactions between two charges, i.e. $\bar{\varepsilon}(l_c) \sim q^2/Dl_c$, where q and D are the mono-valent lipid charge and the dielectric constant of water, respectively (Nguyen et al. 2005). Posing $\bar{\varepsilon}(l_c) = \bar{\varepsilon}_0/l_c$ where $\bar{\varepsilon}_0$ is a function of the charge and the dielectric constant it follows:

$$E_3 = \frac{1}{2} N \bar{\varepsilon}_0 p_0^2 \pi l_c \frac{1}{a} \tag{8}$$

Given the set of energies, the optimization of the area per lipid can be performed.

Model Part II: lipid area optimization

As it is assumed that the monolayer is composed of a constant number of lipids N(i.e. $S = Na$), the optimization of the monolayer energy is given by optimizing the surface area per lipid:

$$\left[\partial_a \sum_{i=1,2,3} E_i\right]_{a=a_0} = 0 \tag{9}$$

It follows:

$$a_0^2 = \frac{k_B T v}{2K}\left[1 + \frac{\bar{\varepsilon}_0}{k_B T} p_0^2 \frac{\pi l_c}{v}\right] \tag{10}$$

The term in brackets is the ratio E_3/E_2, namely long versus short range interactions. This ratio is equal to one if $p_0 \to p_0^*$ with $(p_0^*)^2 \sim k_B T v/\pi l_c \bar{\varepsilon}_0$. Given the strong reactivity of hydrogen ions with negatively charged lipids, only changes in pH will be considered here and accordingly the parameter l_c will be presumed constant (see "Discussion"). In this case, the optimal area per lipid is determined by the short range interactions with a numerical value $p_0^* \sim 0.6$ (Appendix A). From Eq. 10, the surface tension of a monolayer can then be deduced.

Model Part III: determination of the new difference in the surface tension between the leaflets of the cell membrane

In the development above only charge screening was considered to alter the area per lipid in the monolayer. A lipid number asymmetry between leaflets will now be considered (Rauch and Farge 2000). Given that the membrane mechanical moduli are not significantly affected by the physiological variations of cytosolic pH considered here (Zhou and Raphael 2007); noting δN the number of extra lipids added or removed from a leaflet (via the flippase activity), and δa the change in the surface area per lipids linked to the lipid charge being masked by a hydrogen ion, it follows that the surface tension of leaflets are:

$$\begin{aligned}\sigma_{in} &= -K\frac{(N_0+\delta N)(a_0+\delta a)-N_0 a_0}{S_0} \cong -K\left(\frac{\delta N}{N_0}+\frac{\delta a}{a_0}\right)\\ \sigma_{ex} &= -K\frac{(N_0-\delta N)(a_0+\delta a)-N_0 a_0}{S_0} \cong -K\left(-\frac{\delta N}{N_0}+\frac{\delta a}{a_0}\right)\end{aligned} \tag{11}$$

In the above equation, N_0 is the average number of lipids in a monolayer prior to adding or removing lipids from the

internal/cytosolic (subscript "in") or external (subscript "ex") leaflets, respectively. Inserting Eq. 7 into Eq. 8 and using the expression of p_0^* it follows:

$$\sigma_{\text{in}} \cong -K\left[\frac{\delta N}{N_0} + \left(\frac{1+(p/p_0^*)^2}{1+(p_0/p_0^*)^2}\right)_{\text{in}}^{1/2} - 1\right] \tag{12a}$$

$$\sigma_{\text{ex}} \cong -K\left[-\frac{\delta N}{N_0} + \left(\frac{1+(p/p_0^*)^2}{1+(p_0/p_0^*)^2}\right)_{\text{ex}}^{1/2} - 1\right] \tag{12b}$$

In Eqs. 12, p and p_0 are the probabilities of finding a negatively charged lipid in the membrane of MDR and drug sensitive cells, respectively. The drug sensitive state will be taken as initial state. To compare the theoretical data to experimental data, one will assume that the external pH does not vary or, equivalently, that negatively charged lipids are chiefly found in the inner leaflet. As a result, $\sigma_{\text{ex}} \cong K\delta N/N_0$ (i.e. $p = p_0$ in Eq. 12b). Thus the difference in surface tensions, $\Delta\sigma = \sigma_{\text{in}} - \sigma_{\text{ex}}$, can be written as follow:

$$\Delta\sigma \cong -2K\frac{\delta N}{N_0} - K\left[\left(\frac{1+(p/p_0^*)^2}{1+(p_0/p_0^*)^2}\right)^{1/2} - 1\right] \tag{13}$$

In Eq. 13 the subscript "in" has been removed for clarity. In Eq. 13 the first term in the right member describes the lipid packing associated with their asymmetry in number, the second term describes the influence of electrostatic repulsion between charged lipids on their individual area (i.e. packing—see Fig. 2d). The drug sensitive state depends on two probabilities, namely p_0 and p_0^*, their relative importance needs to be discussed.

Assuming firstly that $p_0/p_0^* \ll 1$, namely that the long range repulsion between lipids is "over screened" in the drug sensitive state (i.e. $E_3/E_2 \ll 1$). This means that when cells switch their state from sensitive to resistant via alkalization of their cytosolic pH, they should still be sensitive to drugs with a sensitivity similar to their initial sensitivity up to a point where $p_0/p_0^* \sim 1$. In this case, resistance to drugs should come as a "threshold of resistance" and not as a monotonous increase in drug resistance linked to an increase in the cytosolic pH. However, experimental data has demonstrated that the intracellular pH and drug resistance are linked together in a monotonous way (Keizer and Joenje 1989), which suggests that the regime described by $p_0/p_0^* \ll 1$ can be excluded from the present study.

Assuming secondly that $p_0/p_0^* \gg 1$. This would mean that the electrostatic repulsion between lipids is already strong in the drug sensitive state, namely that the long range repulsion between lipids is "dominant" (i.e. $E_3/E_2 \gg 1$). Accordingly, this would mean that small relative changes in pH would not affect the difference in surface tensions very much and thus drug resistance. Thus, the regime described by $p_0/p_0^* \gg 1$ can also be excluded from the present study.

Therefore, the cytosolic pH involved in the drug resistant states is more likely to verify the relation $p_0/p_0^* \sim 1$. This assumption suggests that drug resistance would correspond to a switch in the electrostatic interaction between charged lipids. More specifically, drug sensitivity would result from the fact that charged lipids do not see each other (electrostatically) at acidic pH because their charge is masked by hydrogen ion and that, when the masks are removed (at higher pH) then lipids can see each other that is, in turn, involved in drug resistance (namely it impairs the transverse movement of drugs across the membrane—see thereafter). Thus Eq. 13 can be rewritten as:

$$\Delta\sigma \cong -2K\frac{\delta N}{N_0} - K\left[\left(\frac{1+(p/p_0)^2}{2}\right)^{1/2} - 1\right] \quad \text{if } p_0/p_0^* \sim 1 \tag{14}$$

The probability p/p_0 needs now to be expressed as a function of the intracellular pH.

Model Part IV: determination of the relationship between p/p_0 and the cytosolic pH

An adsorption model as previously described in Diu et al. 1997 will be used to model the interaction between hydrogen ions and negatively charged membrane lipids (see Appendix B). Noting e_0 the magnitude of the electrostatic interaction energy between a negatively charged lipid and hydrogen ion, and μ and μ_0 the chemical potentials of hydrogen ions in the cytosol of drug resistant and sensitive cells, respectively, the ratio of probabilities p/p_0 is written as:

$$\frac{p}{p_0} = \frac{1+e^{(e_0+\mu_0)/k_BT}}{1+e^{(e_0+\mu)/k_BT}} \tag{15}$$

In Eq. 15, the chemical potentials are the sum of two terms. The first term summarizes the interaction energies between hydrogen ions and the solvent (i.e. water) and other cytosolic proteins. The second term is of entropic origin, written as $k_BT \ln [H^+]$, and describes the cost incurred if all hydrogen ions were to lose their degree of freedom to interact with proteins or lipids. Using $[H^+] = 10^{-\text{pH}}$, Eq. 15 can be rewritten as:

$$\frac{p}{p_0} = \frac{\gamma+1}{\gamma+10^{-\delta\text{pH}}} \tag{16}$$

where $\delta\text{pH} = \text{pH} - \text{pH}_0$ (the subscript "0" stands for the sensitive state) and $\gamma = e^{-(e_0+\mu_0)/k_BT}$ the Boltzmann's probability that a hydrogen ion interacts with a negatively charged lipid in the initial drug-sensitive state. It is possible to demonstrate that $\gamma \sim 1.5$ (Appendix B).

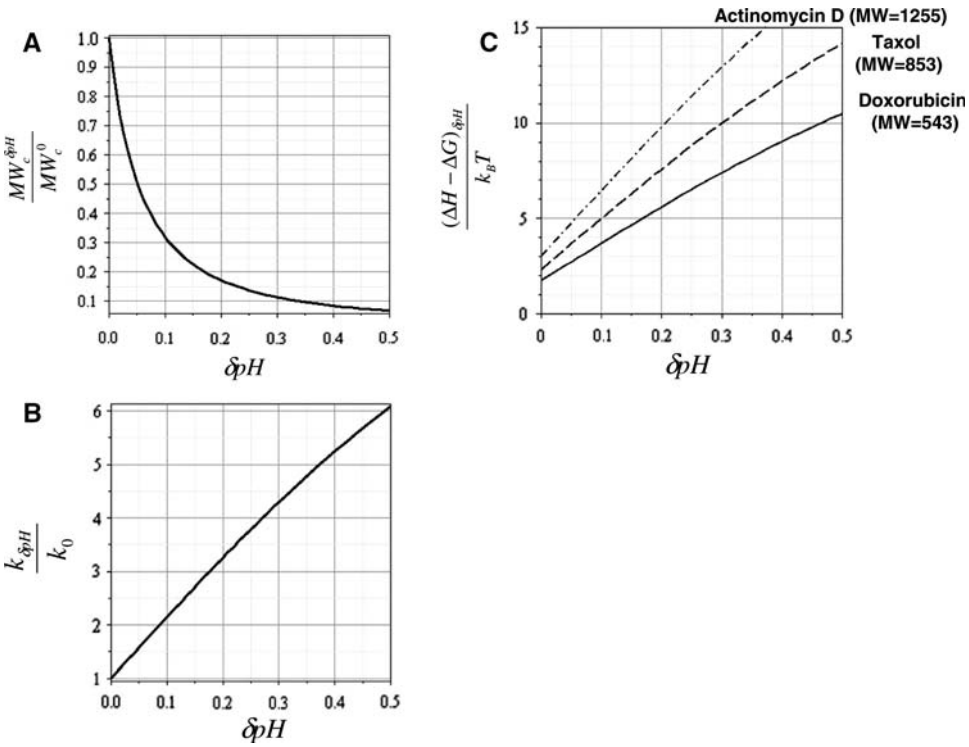

Fig. 3 For each figure we used $\gamma = 1.5$. A lipid asymmetry $\delta N/N_0 = 2\%$ has been used. **a** Plot of Eq. 18 representing the effect of a variation in the intracellular pH on size selectivity of drug membrane permeation (i.e. similar to Lipinski's 2nd rule). **b** Plot of Eq. 19 representing the effect of a variation in the intracellular pH on the kinetic rates of endocytosis between sensitive and resistant cells. **c** Variation of the enthalpy energy as a function of the pH difference for three anticancer drugs with different molecular weights. It is noteworthy that at the enthalpy energy becomes significant ($>k_BT$) as soon as the pH increases

Finally, noting $\Delta\sigma_0 = -2K\delta N/N_0$ the difference in surface tensions associated with the "true" lipid number asymmetry between membrane leaflets (Fig. 1), inserting Eq. 16 into Eq. 14, it follows:

$$\frac{\Delta\sigma_{\delta\mathrm{pH}}}{\Delta\sigma_0} \cong 1 + \frac{\left[\left(\frac{1+\left((\gamma+1)/\left(\gamma+10^{-\delta\mathrm{pH}}\right)\right)^2}{2}\right)^{1/2} - 1\right]}{2\delta N/N_0} \quad \text{if } p_0/p_0^* \sim 1 \tag{17}$$

In Eq. 17 the subscript "δpH" has been added to emphasize the fact that the pH is now taken into consideration. Given a lipid number asymmetry $\delta N/N_0 \sim 2 \pm 0.5\%$ (Fig. 1a) (Rauch and Farge 2000) and $\gamma \sim 1.5$, predictions can be determined.

Prediction 1: effect of the intracellular pH on Lipinski's 2nd rule

We have previously seen that Eq. 1 determines a critical MW cut off (Rauch and Pluen 2007). Re-noting MW_c^0 this critical molecular weight when $\delta\mathrm{pH} \cong 0$ and $\mathrm{MW}_c^{\delta\mathrm{pH}}$ when $\delta\mathrm{pH} \neq 0$, then it follows that this critical MW must vary as:

$$\left(\frac{\mathrm{MW}_c^{\delta\mathrm{pH}}}{\mathrm{MW}_c^0}\right)^{2/3} = \frac{\Delta\sigma_0}{\Delta\sigma_{\delta\mathrm{pH}}} \tag{18}$$

In Fig. 3a the effect of an increase in the intracellular pH on the critical MW determined by Eq. 18 is represented. To summarize, an increase of $\sim$0.2 unit in the intracellular pH diminishes the critical molecular weight MW_c^0 by 75–80%. This range of difference between cytosolic pHs of sensitive and resistant cancer cells is classically measured (Belhoussine et al. 1999; Keizer and Joenje 1989). As a result, a slight increase in the cytosolic pH can elevate the mechanical activation energy a drug has to bypass to cross cells membrane.

Prediction 2: effect of the intracellular pH on the kinetic rate of fluid phase endocytosis

Membrane endocytosis is altered in drug resistant cells and, in particular, is increased $\sim$2- to $\sim$10-fold depending on the cell type (reviewed in Ferte (2000), see also (Altan et al. 1999; Colin et al. 1996)). As it has been experimentally shown that the kinetic rate of endocytosis is proportional to the difference in surface tensions between the membrane leaflets (Farge et al. 1999), using Eq. 17 it is possible to predict how membrane endocytosis will be affected by pH changes:

$$\frac{k_{\delta\mathrm{pH}}}{k_0} = \frac{\Delta\sigma_{\delta\mathrm{pH}}}{\Delta\sigma_0} \tag{19}$$

where k_0 and $k_{\delta\mathrm{pH}}$ are the kinetic rates of endocytosis when cells are sensitive (subscript "0") and resistant (subscript "δpH") to drugs, respectively. In Fig. 3b the effect of an increase in the intracellular pH on the kinetic rate of endocytosis is represented. It is found that an increase of

$\sim$0.2 unit in the intracellular pH measured in drug resistant cells (Belhoussine et al. 1999), increases the kinetic rate of endocytosis by a factor of $\sim$3.

Prediction 3: effect of the intracellular pH on the enthalpy energy

Using Eqs. 19 and 4 it is possible to predict the variation of the mechanical enthalpy energy as a function of the difference in pH and molecular weight of drugs only:

$$\begin{aligned}(\Delta H - \Delta G)_{\delta \mathrm{pH}} &= k_{\mathrm{B}}T \cdot \left(\mathrm{MW}/\mathrm{MW}_{\mathrm{c}}^{\delta \mathrm{pH}}\right)^{2/3} \\ &= k_{\mathrm{B}}T \cdot \left(\mathrm{MW}/\mathrm{MW}_{\mathrm{c}}^{0}\right)^{2/3} \cdot \Delta\sigma_{\delta \mathrm{pH}}/\Delta\sigma_{0}\end{aligned} \tag{20}$$

This equation represented in Fig. 3c demonstrates that the enthalpy energy is sensitive to both pH and the molecular weight of drugs used.

Prediction 4: drug resistance levels versus intracellular pH

Based on these previous results which, at least theoretically, predict a strong effect of the cytosolic pH on drug permeation across the cell membrane, it was also decided to compare the predictions of pH changes using published data from in vivo studies.

The relationship between the levels of resistance to doxorubicin (dox) and the intracellular pH has been determined in several Pgp-expressing cell lines including: SW1573 lung cancer cells, MCF-7R breast derived cancer cells and K562R erythroleukemia cancer cells (see Table 1 legend and Fig. 4). There are three main reasons why these cell lines have been chosen: (a) Comparatively, between the aforementioned cells, the cytosolic pH of sensitive cell lines from which the resistant cells are derived are all $\sim$6.9 (see Table 1). This suggests that if increases in cytosolic pH are involved in the resistance to drugs, all these cells should respond similarly to these increases. (b) This value of pH ($\sim$6.9) for these drug sensitive cells is the lowest which has been recorded in the literature to date (at the best of my knowledge). Given that Eq. 17 assumes that drug sensitivity results from the fact that the cytosolic pH is low enough to screen out the electrostatic repulsion between charged lipids, it is expected that this should be the case at this pH $\sim$ 6.9. Note however that, as seen earlier, two regimes can be considered, the "over screened" regime given by $p_0/p_0^* \ll 1$, or the "screened" regime given by $p_0/p_0^* \sim 1$. The choice regarding the regime is given by the last argument (c): Experimental data have demonstrated that when the cytosolic pH of sensitive cells is $\sim$6.9, the intracellular pH and drug resistance are linked together in a monotonous way (Keizer and Joenje 1989). All things considered and assuming that all the cells chosen above have more or less the same type and number of lipid composing their membrane, this suggests that the regime described by $p_0/p_0^* \ll 1$ can be excluded from the present study (otherwise, as previously stated, the resistance to drugs should come has a threshold of resistance). Accordingly we are left with the $p_0/p_0^* \sim 1$ regime on which Eq. 17 is based.

Table 1 Numerical values of cytosolic pHs versus resistance levels (IC_{50} s) for the drug doxorubicin (dox) determined experimentally in different cell types (Belhoussine et al. 1999; Keizer and Joenje 1989)

Cell line	Cytosolic pH		$\mathrm{Ln}[(IC_{50})_{MDR}/(IC_{50})_{non\text{-}MDR}]$		$\rho_{Pgp}/\rho^{c}_{Pgp}$ [a]
	Sensitive	Resistant	Experimental	Theory[b]	
SW1573	6.93	7.12	2.30	3.47	–1.62
SW1573	6.93	7.17	3.68	4.33	–0.47
SW1573	6.93	7.27	5.52	5.95	–0.09
SW1573	6.93	7.38	7.60	7.56	0.38
MCF-7R	6.91	7.14	3.68	4.16	0.73
K562R	6.94	7.06	3.40	2.21	–0.27

The resistance levels have been selected following the incubation of dox at different concentrations. The cells types are: squamous cell carcinoma of the lung/SW1573 selected via incubation of Dox (Keizer and Joenje 1989), MFC-7R originating from a human breast-adenocarcinoma cell line (MCF-7) (Altenberg et al. 1993; Belhoussine et al. 1999; Boscoboinik et al. 1990; Fairchild et al. 1987), and K562R cells originating from a human erythroleukemic cell line (K562) established from a patient with chronic myelogenous leukemia in blast transformation (Lozzio and Lozzio 1975; Tsuruo et al. 1986)

[a] Further to the non-linear regression analysis, the relative surface density of drug transporters was determined to determine the systematic bias (see text). These data are plotted in Fig. 4b

[b] This column corresponds to the theoretical prediction following the non-linear regression analysis of the "experimental" column, using Eq. 22 and by posing $\rho_{Pgp} = 0$. These data are plotted in Fig. 4a

The cellular sensitivity or resistance to drugs is usually determined using the IC_{50} 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} 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. Naturally, the drug's target is still presumed to be present in the resistant state. Accordingly, the higher the IC_{50} the lower the sensitivity, and thus there exists an inverse relationship between the ability of drugs to cross the membrane of cells and the IC_{50}. 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):

$$\frac{r_{MDR}}{r_{non-MDR}} \cong e^{-\frac{MW^{2/3}}{MW_c^{2/3}}\left(\frac{\Delta\sigma_{\delta pH}}{\Delta\sigma_0}-1\right)}\left(1-\frac{\rho_{Pgp}}{\rho^c_{Pcp}}\right) \quad (21)$$

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 and; 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 $\rho_{Pgp} \rightarrow \rho^c_{Pcp}$ then $r_{MDR}/r_{non\text{-}MDR} \rightarrow 0$, i.e. the kinetic of membrane permeation is null.

Importantly, in Eq. 21 the exponential term describes the residency time of drugs in the membrane that, subsequently, allows them to meet and be extruded by Pgp-like transporters (given by the non exponential term in Eq. 21) (Fig. 1c). If drug resistance occurs without the expression of transporters then the second term must be omitted by posing $\rho_{Pgp} = 0$. Note however that when transporters are expressed the first term *cannot* be omitted as it represents the "vacuum cleaner" effect upon which transporters activity rely. Finally, it is worth mentioning that the drug dehydration energy does not appear as it is cancelled upon division of kinetic rates.

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

$$\ln\left(\frac{(IC50)_{MDR}}{(IC50)_{non-MDR}}\right) \cong \frac{MW^{2/3}}{MW_c^{2/3}}\left(\frac{\Delta\sigma_{\delta pH}}{\Delta\sigma_0}-1\right) - \ln\left(1-\frac{\rho_{Pgp}}{\rho^c_{Pgp}}\right) \quad (22)$$

Equation 22 states that drug resistance levels would result from the interaction between the intracellular alkaline pH (in the resistant state), the lipid packing, the drug size (or MW) and the surface density of drug transporters.

The experimental data obtained from cells SW1573 (Keizer and Joenje 1989), K562R and MCF-7R (Belhoussine et al. 1999) demonstrate that the level of resistance to doxorubicin (Table 1) is more or less a linear function of the difference in the cytosolic pH between the resistant and sensitive states (Fig. 4a). Note however that none of the authors have determined the levels of transporter expression in these cell lines, although they have been correlated to drug resistance levels (Chen and Simon 2000).

Thus, in order to test the model, namely to determine the relative effects of pH and drug transporters in MDR cells, a two steps analysis has been performed.

In the first step, given Eq. 17, $\gamma \sim 1.5$ and $MW_{dox}/MW_c \sim 2.28$, the model (i.e. Eq. 22) is challenged against the experimental data in Table 1 by posing $\rho_{Pgp} = 0$, and by using a non-linear regression determining whether the model can provide the numerical value of the lipid number asymmetry, which has been determined previously: $\delta N/N_0 = 2 \pm 0.5\%$ (Rauch and Farge 2000). Note that one should get an incorrect or unrealistic numerical value for this parameter if the $\rho_{Pgp} = 0$ assumption is wrong, or if the model linking the membrane to pH, as proposed in this paper, is false. If, on the contrary, the numerical value of the lipid asymmetry deduced is sound, this does not rule out the potential role of drug transporters. However, to determine whether they are involved a second step is needed.

In this second step, I will analyze the statistical systematic bias that should occur due to the expression and drug-handling activity of drug transporters in these cells. Simply, if drug transporters (with a drug handling activity) are involved, and further explain MDR levels in these cell lines on the top of the interaction between membrane and pH, the determination of the statistical residues should provide this information. For that, I will perform the difference between the experimental data (Table 1) and the theory (Table 1—issued from the first step i.e. with the assumption $\rho_{Pgp} = 0$) and using Eq. 22, deduce the relative surface density of transporters (i.e. ρ_{Pgp}/ρ^c_{Pgp}) as a function of doxorubicin resistance levels. Overall, a statistical trend, namely the Pgp-related systematic bias, should emerge that would provide the signature of Pgps' drug-handling activity. If there is no statistical trend or correlation between the resistance levels and the relative surface density of transporters, one will have to question the efficiency of any drug-handling activity.[4]

First step: I found that by posing $\rho_{Pgp} = 0$, Eq. 22 fits the best the experimental data (Table 1) if $\delta N/N_0 = 2.27 \pm 0.14\%$ (Fig. 4a, $R^2 = 0.95$, T test $= 15.81$, p value $< 10^{-4}$, F test $= 60.29$). The later numerical value determined statistically corresponds remarkably well to the lipid number asymmetry already determined experimentally elsewhere, in drug sensitive cells, with the value $\delta N/N_0 = 2 \pm 0.5\%$ (Rauch and Farge 2000). In Fig. 4a,

[4] Again, one could argue that this analysis is fallacious as it is a one way analysis and that, instead of doing as described above, one could also assume that there is no membrane-related effect and deduce the surface density of transporters only as a function of resistance levels. This could be possible by posing that the first term of the right hand side of Eq. 22 is null. Note however that, by doing so, we would get mathematical values regarding Pgp-like transporters surface densities, but no mechanisms whereby membrane embedded drugs meet transporters. This would put back the field of drug resistance into its "dark ages" when it was assumed that a "vacuum cleaning" effect had to take place to represent how drug and Pgp interact (see discussion in this paper or discussions in Rauch and Pluen, 2007 or Rauch, 2008). Thus the analysis exposed here is not fallacious but the only one that can be performed given the data published on this subject and that does not take for granted the fact that a drug and transporter assemble together.

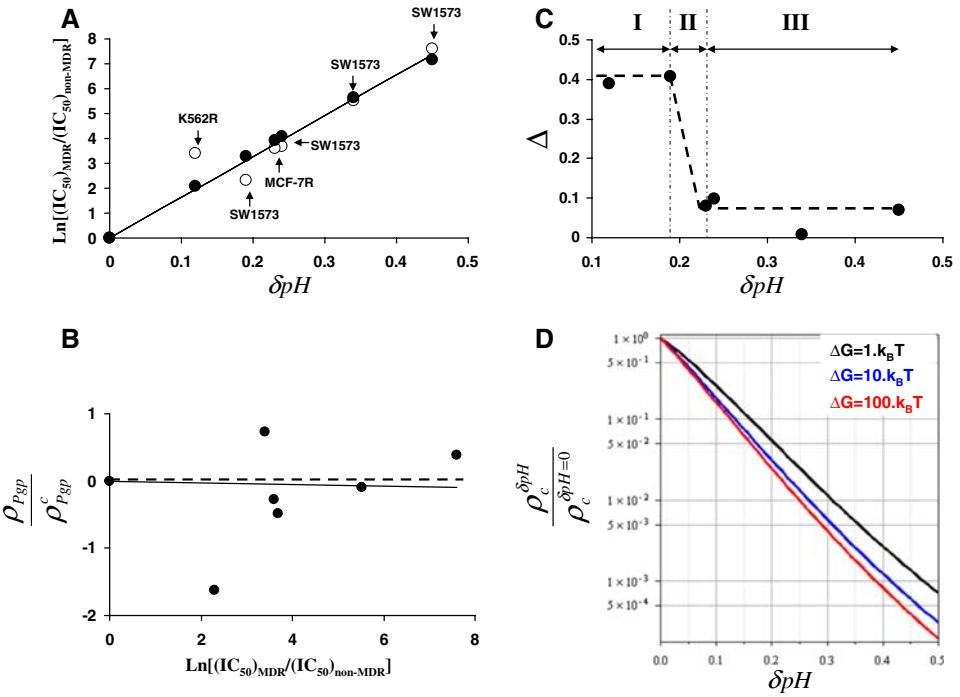

Fig. 4 **a** Comparison between experimental data (*blanked circles*—Table 1) and theoretical (*filled circles*—Eq. 22) with $\delta N/N_0 = 2.27\%$. The blank circles corresponding to SW1573, K562R and MCF-7R are pointed by an *arrow* and named. Finally the straight line is the linear regression of experimental data which clearly agrees with the theory. **b** Given Eq. 22, the difference between the theoretical predictions and experimental data have been calculated to determine any correlative effects related to the surface density of drug transporter with a drug-handling type activity. The plain line is the best linear fit of the supposed systematic bias due to Pgp surface density and supposed activity. The later straight line can be compared to the dashed line, which corresponds to the absence of correlation. **c** Absolute relative difference between the experimental points (*blank points* in **a**) and the theory (*black points* in **a**), noted Δ, plotted against the variations of the cytosolic pH. This graph indicates the presence of three regimes. A regime of low pH changes and low levels of resistance (regime I) in which the theory may not be totally adequate (higher Δ values) to describe the drug resistance levels given the initial assumptions, a transition regime centred around δpH $\sim$ 0.2 (regime II), and a final regime in which, the high levels of resistance and thus high pH changes display a good agreement with the theory (regime III). **d** Effect of pH changes (cytosolic alkalization) on the surface density of Pgp transporters needed to trigger fully drug resistance, $\rho_c^{\delta pH}$, compared to the surface density of Pgp transporters when no pH changes are taken into consideration, $\rho_c^{\delta pH=0}$. As seen in the figure the number of Pgp transporters can decrease exponentially when the pH increases: the pH-driven mechanical enthalpy takes over. The three curves represent the amount of Pgp required for different drugs with specific dehydration energy (namely 1.k_BT, 10.k_BT or 100.k_BT)

the black dots correspond to the theoretical prediction and the blank dots correspond to the experimental data (Table 1). The straight line corresponds to the best linear fit of experimental data. Note that if a linear fit was to be plotted against the theoretical predictions (black dots), the difference between the two slopes would differ by less than 0.1% (data not shown). As a result, this first analysis suggests strongly that pH and membrane are both involved in MDR and define drug resistance levels.

Second step: In order to determine the effect of drug transporters, I have subtracted the theoretical prediction (black dots) to the experimental data (blank dots) and transform these data to get $\rho_{pgp}/\rho^{c}_{Pgp}$ (see last column in Table 1). Note again that if there is any effect concerning Pgp expression that is correlated to a drug handling activity in MDR levels, a systematic bias should appear that would prove its involvement in drug resistance. Remarkably, I found that this analysis does not give any obvious correlation between drug resistance levels and surface density of transporters (Fig. 4b). The best straight line that passes through the origin of axis[5] has a slope of −0.012 ($R^2 = 0.07$, T test = –0.178, p value = 0.864, F test = –0.401), which is similar to the absence of correlation given by the dashed straight line in Fig. 4b.

It seems therefore that pH, membrane and Lipinski's 2nd rule, together, can give an important insight into doxorubicin resistance levels in cancer cells and that, a drug handling activity is, statistically speaking, unlikely in this case.

[5] This is a constraint that presumes naturally that when Pgp is not expressed: $(IC50)_{MDR} = (IC50)_{non\text{-}MDR}$.

Prediction 5: δpH induced high doxorubicin resistance regime

From Fig. 4a, it can be seen that doxorubicin resistance levels are better matched by the theory if the cytosolic pH changes appreciably. To determine the regime of cytosolic pH changes in which the model is likely to be fully valid, the absolute relative difference between the experimental points (blank points) and the theory (black points), noted Δ in Fig. 4c, were plotted against the variations of the cytosolic pH. Figure 4c indicates the presence of three regimes. A regime of low pH changes and low levels of resistance (regime I), in which the theory may not be totally adequate to describe the drug resistance levels given the assumptions made initially, a transition regime centred around δpH $\sim$ 0.2 (regime II), and a final regime to which correspond the high levels of resistance and thus high pH changes that display good agreement with the theory. Accordingly, it can be postulated that pH is central to drug resistance when its value reaches a threshold value $\sim$0.2. This value of pH difference leads to a mechanical enthalpy energy $\sim 5k_BT$ for doxorubicin (Fig. 3c), which becomes significant regarding the transverse movement of the drug.

Prediction 6: weighing drug handling *versus* pH mediated membrane/drug mechanical interaction in MDR

As already stated and seen in Fig. 4b, a drug handling activity does not seem to be required to explain MDR levels in cancer cells. In fact this result can be traced back to Eq. 15 previously published by (Rauch and Pluen 2007). In the paper cited, this equation determined the surface density of Pgp-like transporters required with a drug handling activity to trigger full drug resistance, given the mechanical interaction between drugs and the cell membrane. It was found that the amount of membrane Pgp required for any drug handling could decrease exponentially when the mechanical interaction between a drug and the cell membrane are taken into consideration. Using Eq. 15 from (Rauch and Pluen 2007), and Eq. 17 and Eq. 19 from the present study, it is possible to plot the effect of cytosolic alkalinization on the amount of Pgp theoretically required, if drug handling is taken into consideration (Fig. 4d). It can be demonstrated that the initial amount of Pgp required can be divided by $\sim$20–50 when the cytosolic pH is increased by a factor $\sim$0.2. This means that as the pH increases Pgp activity can decrease and that pH and Pgp-mediated drug handling are inversely related. Note that this point is independent of the drug dehydration energy. Therefore, drug resistance can occur independently of Pgp activity or expression so long that pH requirements are met.

Discussion

pH, membrane mechanical properties and drug resistance

The single consideration of Pgp transporters expression extruding drugs as a floppase (Higgins and Gottesman 1992) as the unique cause of drug resistance, has been demonstrated to be too restrictive. Consequently, more needs to be understood about the physiology of drug resistance. As drug resistance seems to result from synergisms taking place at the cellular level, there is a real need to merge what we know about the underlying mechanisms involved.

Previous works have suggested that the membrane and its mechanical properties of drug resistant cells is key in understanding drug resistance (Ferte 2000; Rauch and Pluen 2007; Rauch 2008), whereas others have suggested that the intracellular pH is central (Altan et al. 1999; Belhoussine et al. 1999; Harguindey et al. 2005; Hoffman et al. 1996; Keizer and Joenje 1989; Raghunand et al. 1999; Roepe et al. 1996; Simon et al. 1994).

I demonstrate here that membrane mechanical properties and pH can interact together and has, potentially, a strong effect on the transverse movement of drugs. The present model proposes that impaired intracellular accumulation of drugs observed in drug resistance may correspond to a switch in the electrostatic interaction between charged lipids. More specifically, drug sensitivity would result from the fact that charged lipids do not see each other (electrostatically) at acidic pH because their charge is masked by hydrogen ion and that, when the masks are removed (at higher pH) then lipids can see each other, which affects the differential lipid packing between leaflets. Thus, it is suggested that this effect is involved in drug resistance, namely that it impairs mechanically the transverse movement of drugs across the membrane.

It is important to note that although drug electrostatic properties, pH and membrane potentials have often been cited to explain drug resistance (Wadkins and Roepe 1997), the model presented does not deal with the electrostatic interactions between drugs and the membrane potential, but with the mechanical impediment of drugs transverse movement.

Although I certainly do not rule out other complex biological mechanisms involved in drug resistance, e.g. anti-apoptotic stimuli linked to cytosolic alkalization (Lagadic-Gossmann et al. 2004) nonetheless, the theory presented in this present work is reasonably well validated experimentally for doxorubicin when drug sensitive cells have an acidic cytosolic pH $\sim$ 6.9 (see Table 1 and Fig. 4). Note again that a starting cytosolic pH $\sim$ 6.9 for sensitive cancer cells fulfills all the criterions that are

needed for the full validity of the theory. Note that the fact that sensitive cancer cells may display a different cytosolic pH does not rule out the theory, it simply suggests that further parameters have to be taken into consideration, e.g. the membrane potential, and that Eq. 17 has to be modified accordingly.[6]

It is noteworthy that the model proposed in this paper does not consider Pgp's drug handling activity and that, unless these membrane transporters are also involved in changing the cytosolic pH, the theory does not make any use of them directly to explain high levels of doxorubicin resistance in cancer cells. As a result, cells lacking Pgp expression would still be resistant so long that the cytosolic pH changes according to the theory.

As the present work focuses on doxorubicin, the natural question is: Do similar conclusions apply to other drugs? Given that the theory is focused on the MW of the drug (whatever the drug type) and the intracellular pH, a similar conclusion should apply to other drugs. Unlike doxorubicin that has been the *tarte a la crème*[7] of drug resistance studies in the past; I have not been able to uncover systematic studies linking cytosolic pH to other drugs ability to kill cells.

It is important to recall that although the present theory demonstrates the mechanical interaction between drug MW and the cytosolic pH in MDR; pH is not the only parameter that modulates the packing of lipids on which the mechanical enthalpy is based (Fig. 3c, d). For example, alteration in the metabolism of lipids observed in MDR cell lines may be involved as well (see (Rauch 2008) and references therein). Therefore, although I support a direct role of pH in MDR, I would not be surprised if a drug resistant cell line had other strategies to alter the packing of membrane lipids.

pH, membrane mechanical properties, membrane potential and drug resistance

The membrane potential is clearly involved in drug resistance. However, contrary to high levels of resistance that seem to be driven by cytosolic alkalization, the membrane potential seems to express relatively low levels of resistance (cf. the notion of "true transfectant") (Wadkins and Roepe 1997). As a result, it has been suggested that the way the membrane potential is fundamentally involved in drug resistance is related to the driving force it applies to cationic drugs across the membrane (or affect the thermodynamic balance of drugs between either sides of the membrane).

The electrostatic property of drugs has not been developed in this paper as my aim was to understand how the membrane mechanical properties are involved in high levels of MDR. However, it is important to note that in the present model the "membrane potential" appears indirectly, namely not as the classical Nernst's Law, in Debye's length (see Eq. 7 and paragraph above), as Debye's length corresponds to the sum of square concentrations. With regard to the membrane mechanical properties, if I have assumed that the membrane potential is unlikely to be involved, namely that Debye's length does not change between the sensitive and resistant states, it is because: hydrogen ions are smaller than any other ions and can thus interact more easily with negative charges and that cells have to remain electrostatically neutral and thus the sum of square concentrations is unlikely to be significantly altered. I acknowledge that there is a real need to merge all the observations performed regarding drug resistance and that more needs to be done to understand fully MDR levels in living cells. Future work will address the influence of drug charge and membrane mechanical properties in MDR.

For the time being, it is important to recall and underline that the involvement of the membrane potential in drug resistance is somewhat different from the mechanical packing exposed in this paper. Indeed, it was demonstrated that the membrane potential pulls cationic drugs into drug sensitive cells. As the membrane potential increases (i.e. decreases in absolute value) when cells become resistant to drugs, it is expected that the force pulling drugs into cells initially vanishes. If one considers a membrane potential $\sim$–70 mV the drop in energy when a cationic drug crosses the membrane would be $\sim 3k_BT$ (Roepe et al. 1993). This order of magnitude corresponds to the order of magnitudes given by Fig. 3c when δpH is below $\sim$0.2 units. However, contrary to the "drug-pulling" mechanism mediated by the membrane potential, the packing of inner leaflet lipids opposes drug entry (based on MW) once the cytosol becomes alkaline. Thus these two forces are essentially opposed. As a result, there is likely to be a crossover regarding the order of magnitudes between the "drug-pulling" and lipid packing mechanisms. The fact that as the pH increases the "drug-pulling" mechanism decreases (Roepe et al. 1993), could explain why the agreement between the theory and the data is better once δpH $\sim$ 0.2 or above (Fig. 4c).

Endocytosis and resistance to doxorubicin

A recurrent feature of doxorubicin resistance is the fact that this drug is trapped in the acidified compartments. How this drug (and related weak bases) ends in these compartments has been a long standing question. Here again, the mechanical properties of the cell membrane (i.e. the

[6] If the present theory has to be re-used in biological studies, I recommend to be contacted to make sure that the initial hypotheses are adequate.

[7] Translate as "strong focus".

differential lipid packing) and endocytosis may explain fully this feature.

It was demonstrated that the increase in the pH gradient between the organelles and cytosol of drug resistant cells allow the trapping of weak base drugs, such as doxorubicin, in acidified organelles (Altan et al. 1998, 1999; Raghunand et al. 1999; Schindler et al. 1996), which are then supposedly expelled via exocytosis (Martinez-Zaguilan et al. 1999; Raghunand et al. 1999). Although these works were the first to link the cytosolic alkalization and the alteration of membrane recycling which is simultaneously observed when cells become resistant to drugs (Sehested et al. 1987a, b; Seidel et al. 1995; Sognier et al. 1994), they also assumed that weak base drugs can cross the membrane to diffuse toward acidified organelles that surround nuclei and get trapped (Raghunand et al. 1999). Nonetheless, drug resistant cells that are also adherent cells (like MCF-7 cells for example) have a large proportion of the apical part of their nucleus that is not protected by acidified organelles, as these surround the nuclei laterally. For example see Fig. 1 in Altan et al. (1998). Therefore, if weak base drugs can cross the membrane to diffuse inside cells, these drugs could reach the nucleus of these cells and, ultimately kill them. Accordingly and to avoid this, i.e. for cells to be resistant to drugs, drugs need to be trapped at the membrane level prior to entering cells. In the present model, this would be linked to the difference in surface tensions between leaflets that is steadily increased because of the cytosolic alkalization. Accordingly, drugs would follow the membrane recycling and be endocytosed. This would explain why drugs (like doxorubicin) always reach acidified organelles and get trapped.

On the antagonism between the "vacuum cleaner" and "drug handling" hypotheses

Pgp is assumed to have a floppase, i.e. drug handling, activity (Higgins and Gottesman 1992). Although few problems supporting this view have been highlighted when basic enzymatic chemistry is considered in drug-pumping-mediated MDR (Wadkins and Roepe 1997), a question that has remained open is how membrane embedded-drugs reach these membrane transporters. To solve this question it was postulated initially that these transporters "vacuum clean" membrane-embedded drugs. It is important to recall that this assumption has been put forward to certify (and not to explain) that any membrane-embedded drug has to reach transporters and be extruded. In order to clarify this hypothesis a model has been suggested previously whereby, instead of considering "vacuum cleaning", the lateral diffusion of membrane-embedded drugs is considered (known as the "2D random walk model"). This model allowed for reconsideration of Pgp mediated drug resistance using a different angle and it became clear that, in this context, drug resistance is a two step process.

The first step controls, and ensures, that any membrane embedded drug meet a transporter and the second is related to the floppase activity of drug transporters. Moreover, it was demonstrated that the first stage is driven by drug/membrane interactions and that this stage would control totally Pgp-mediated drug extrusion and efficiency. Again, this last result comes from the simple fact that the first step has to take place prior to any pumping activity, given that there is no reason why a drug incorporates the cell's membrane in the vicinity of a Pgp membrane protein.

I demonstrate in this present paper that the process whereby a drug meets a transporter, and which relies totally on drug/membrane interaction, previously elusively called the "vacuum cleaning" effect, seems to be sufficient to explain high levels of doxorubicin resistance, and that a pumping/drug handling activity, although possible, is not needed as the drug/membrane interaction is necessary and sufficient (see Fig. 4d).

Lipinski's 2nd rule and implications: toward a mechanical control of drug delivery?

As already stated, the membrane has always been considered as an important parameter in drug delivery and bioavailability. During the 1990s, the pharmaceutical industry noticed that too many compounds were terminated in clinical development because of unsatisfactory pharmacokinetics. As a result, experimental and computational approaches have been used to estimate what physico-chemical properties should have the "best" drug for maximum efficiency. From this study, rules have emerged named after their discoverer: "Lipinski's rules" (Lipinski et al. 2001). The first rule is related to the partitioning of the drug between the octanol and water phases (which is an indicator of the drug miscibility with biomembranes); the second rule is related to the molecular weight of the drug and the third and fourth rules are related to the charge state of the drug which are important to predict how the drug is likely to behave in acidic or basic mediums (which reflects in part the acidity in the gastro-intestinal tract). These rules clearly work and are used daily by the pharmaceutical industry. However, they remain difficult to "pin down" scientifically as they have been chiefly deduced from large scale statistical analysis. As a result, these rules are considered and used as paradigms in drugs development. Nonetheless, there is a real need to clarify these rules if one wants to increase drugs' bioavailability and understand the barriers to drugs delivery.

It comes that a theoretical justification of these rules (namely transforming these rules into laws) is likely to give important information in the drugs delivery field, for example providing new targets.

It is now acknowledged that there is a critical MW beyond which the transverse movement of drugs across the membrane will be altered. Remarkably, the theoretical value deduced from in vitro studies is very similar to the value deduced from Lipinski's study (Rauch and Pluen 2007). Although more studies need to be done on this subject, it would be unexpected that a factor chance only explains this similitude. Based on the idea that drug MW is central to its delivery, I demonstrate here that such critical MW is likely to be dependent on the intracellular pH. As a result, I propose (or confirm) that by changing the intracellular pH or the membrane mechanical lipid asymmetry in an adequate way, it should be possible to change the critical MW, increasing drug bioavailability. Finally, I would like to recall that if drug size is involved, then basic membrane mechanics has to be involved as well. In turn this suggests that drug bioavailability is necessarily mechanically controllable.

Conclusion

I have:

1. demonstrated that drug resistance levels can be simply explained by the rise in the intracellular pH via the mechanical interaction between the cell membrane and drug size.
2. opened the way to the concept that drug delivery is very likely mechanically controllable.

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Appendix A: numerical determination of p_0^*:

From $\bar{\varepsilon}(l_c) = \bar{\varepsilon}_0/l_c$, $\bar{\varepsilon}_0$ can be rewritten as $\bar{\varepsilon}_0 = k_B T l_B$ with Bjerrum's length defined as $l_B = q^2/Dk_B T \sim 7$ Å (the dielectric constant of hydrated polar head is assumed to be close to the one of water (Peitzsch et al. 1995)). Given the iso-osmotic condition, i.e. an intracellular concentration in electrolytes $\sim$0.1 M, it follows $l_c \sim 9.6$ Å (using Debye's length as described in Nguyen et al. (2005)). With a lipid cross section area $\pi\theta^2 \sim 50$ Å^2 it follows for a classical 2D hexagonal lattice ($z = 6$): $p_0^* \sim \sqrt{z\pi\theta^2/\pi^2 l_c l_B} \sim 0.6$.

Appendix B: determination of the hydrogen ion-free lipid probability:

Assuming that a hydrogen ion and a negatively charged lipid interact together with a resulting energy $-e_0$ ($e_0 > 0$ is the magnitude of the interaction). In this case, each negatively charged lipids can be in two states, occupied (i.e. interacting with hydrogen ion) or non-occupied. It follows that the partition function of a negatively charged lipid is $\zeta = 1 + e^{(e_0+\mu)/k_B T}$ (μ is the chemical potential of hydrogen ion in solution). Therefore, the probability that a negatively charged lipid is free of hydrogen ion is $p = \left[1 + e^{(e_0+\mu)/k_B T}\right]^{-1}$. It follows that when $p_0/p_0^* \sim 1$, where $p_0^* \sim 0.6$ (Appendix A), $p_o = \left[1 + e^{(e_0+\mu_0)/k_B T}\right]^{-1}$ can be rewritten as $p_0^* = 0.6 = [1 + 1/\gamma]^{-1}$ with $1/\gamma = e^{(e_0+\mu_0)/k_B T}$. It follows that $\gamma \cong 1.5$.

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