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ARTICLE

Multi drug resistance-dependent ''vacuum cleaner'' functionality potentially driven by the interactions between endocytosis, drug size and Pgp-like transporters surface density

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Abstract In cells, multi drug resistance (MDR) is associated with Pgp-like transporters expression extruding drugs from cellular membranes. MDR is efficiently generated with a relatively small fraction of membrane transporters. As the insertion of drugs into cellular membranes is widespread, there are no reasons why a drug should incorporate the membrane in the vicinity of a transporter. As a result a further elusive hypothesis is usually invoked: these transporters act like ''vacuum cleaners'' of drugs embedded in the membrane. Nonetheless, how these transporters attract drugs remains obscure. To clarify the ''vacuum cleaner'' notion, we suggest that during its residency time in cellular membranes, the lateral movement of drugs from their point of insertion to transporters is governed by Brownian's diffusion, which allows the drugs/transporters interaction. Taking into account the functionality of Pgp-like transporters, namely the extrusion of drugs from the plasma membrane inner leaflet, we characterize how the state of drug resistance is triggered involving: membrane endocytosis, drug physico-chemical properties and the surface density of Pgp-like transporters. In addition, the theory developed provides for the first time a theoretical proof of Lipinski's second rule with regard to drugs' size (or MW) selectivity on their permeation across cellular membranes.

List of symbols

a	drug cross section area
D	membrane diffusion coefficient
$f(K)$	non-recurring step number of a two dimensional random walk
ΔG	drug dehydration energy
h	membrane thickness
k_c	bending modulus of the membrane
k or k_0	altered or control kinetics of endocytosis
K	step number of a two dimensional random walk
N_{Pgp}	number of Pgps in the outer cellular surface
p_{Pgp}	meeting probability between a drug and a Pgp
$\tilde{p}_{\mathrm{Pgp}}$	drug extrusion probability by Pgp
r_{MDR}, $r_{\text{non-MDR}}$	escape rate (i.e. probability per unit of time) into the cytoplasm of drugs in the membrane of drug resistant (''MDR'') and drug sensitive (''non-MDR'') cells
R	vesicle radius
S_{cell}	cellular surface area

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S_{Pgp}	cross section area of Pgps in the cellular surface
t_0	drug residency time in the membrane
U	membrane barrier potential
ρ_{Pgp}	fraction of the cellular surface covered by Pgp transporters
ρ^c_{Pgp}	critical surface area covered by Pgp-like transporters leading to drug resistance
σ_{in}	inner leaflet surface tension
σ_{out}	outer and inner leaflet surface tension
$\Delta\sigma = \sigma_{in} - \sigma_{out}$	difference of surface tension between the inner and outer leaflets
χ_{MDR}, $\chi_{non\text{-}MDR}$	ratio between the endocytosis kinetics and the escape rate into the cytoplasm of drugs initially in the plasma membrane of resistant (''MDR'') and sensitive (''non-MDR'') cells

Introduction

To overcome the cellular barriers and ultimately interfere with their specific intracellular targets, therapeutics/drugs rely on their affinity with their targets as well as their ability to cross cellular membranes. Among transmembrane proteins impairing the drug transbilayer movement in bacteria and cells, are the MDR transporters such as the well known P-glycoprotein (Pgp). Pgp-like transporters are known to impair the accumulation of drugs within the cytoplasm of cells by extruding drugs at a rapid rate from the plasma membrane inner leaflet, before they reach the cytosol (Ambudkar et al. 1999; Bornmann and Roepe 1994; Eytan 2005; Germann 1996; Nielsen et al. 1995; Ramu et al. 1989; Raviv et al. 1990; Sharom 1997; Sirotnak et al. 1986; Spoelstra et al. 1992; Stein et al. 1994). The ability of Pgp-like transporters to extrude various compounds relies on a poor transport substrate selectivity (Ferte 2000).

Although the function whereby these transporters extrude drugs from the inner leaflet of biomembranes is well defined once a drug meets a Pgp-like transporter in the inner leaflet, a further hypothesis is usually invoked, namely, that these transporters act as ''vacuum cleaners''. Such an assumption is usually invoked as therapeutic drugs partition efficiently into cellular membranes and their insertion into membranes is likely to be widespread; therefore there are no reasons why a drug should insert into the membrane in the vicinity of a transporter. Indeed, as MDR upon Pgp transfection occurs at a low level of Pgp expression, representing ~0.4% of membrane proteins (Mao and Scarborough 1997), and the cellular location of these transporters is principally in intracellular compartments (~70% of them) (Kim et al. 1997); the ''vacuum cleaner'' effect is cited, affirming that drugs will inevitably meet Pgp-like transporters. It follows that the efficiency of Pgp-like transporters to mediate MDR relies both on the transporters ability to extrude drugs but also, and fundamentally, on the transport of drugs from their initial incorporation in plasma membranes to the transporters prior to extrusion. As a result, it may well be that MDR results from different and synergic processes.

The possibility that different processes might act synergistically in cells expressing Pgp-like transporters is neither new nor a mystery. For example, attempts to impede the activity of transporters in order to increase the intracellular accumulation of drugs, is possible by the use of agents known as drug resistance modulators. Although some modulators alter the activity of transporters through direct physical interactions (Rao et al. 1998; Wang et al. 2004), modulators are also commonly known to affect the membrane lipid bilayer fluidity, i.e. its biophysical properties, and thus permeability to drugs (Callaghan et al. 1993; Drori et al. 1995; Dudeja et al. 1995; Frezard and Garnier-Suillerot 1998; Liang and Huang 2002; Ramu et al. 1983; Regev et al. 1999). Indeed, increasing the accumulation of drugs and cytotoxicity can also be obtained by modulators in drug sensitive cells (Cano-Gauci and Riordan 1987; Cass et al. 1989; Horton et al. 1993). These results suggest that both specific cellular membrane biophysical properties and Pgp-like transporters are fundamental in MDR. Accordingly, a comparison of kinetics of the transbilayer movement of drugs mediated by drugs/biomembranes interactions and the outward pumping of drugs mediated by Pgp-like transporters, has suggested that the time required for the drugs to traverse the plasma membrane is a central factor in MDR (Eytan 2005).

Taking into account that the transbilayer movement of drugs is not spontaneous, during their time of residency in the plasma membrane, drugs are not static but likely to diffuse laterally. The lateral diffusion of drugs might highlight the ''vacuum cleaner'' effect if one considers that the longer the diffusive lateral path, the higher the probability of a drug meeting a transporter. However, since the extrusion of drugs mediated by Pgp-like transporters takes place in the inner leaflet, it follows that part of the membrane barrier (i.e. part of

the activation energy needed for a drug to traverse the membrane) resides within this leaflet, preventing drugs from leaving the membrane before being extruded.

The membrane fluidity refers to the lipids packing within a layer, involving both notions of surface pressure and surface tension. Accordingly, changes have been noted regarding the cellular membrane of MDR cells, and in particular, higher rates of endocytosis have been measured (Altan et al. 1999; Sehested et al. 1987a, b). Interestingly, the local membrane budding which leads to membrane vesiculation and controls the endocytosis kinetics, has been associated with an endogenous higher compression of the cellular membrane inner leaflet (Devaux et al. 1986; Farge 1995; Farge et al. 1999; Rauch and Farge 2000). Thus, a possible mechanism involved in the membrane barrier of MDR cells, which could play a role in the inner leaflet, may well be linked to membrane endocytosis.

Using these assumptions, we decided to address theoretically, step by step, whether the elusive ''vacuum cleaner'' hypothesis can be replaced by another taking into account endocytosis, the drug size and polarity, the surface density of transporters and the drug lateral diffusion. Thus, after having proposed a model of MDR in this context, several predictions will be deduced and discussed.

Model: Part I

Residency time of drugs in the plasma membrane linked to the plasma membrane difference of surface tension and the dehydration energy of the drug

Considering a drug composed of two parts, hydrophilic/polar and hydrophobic, at a very low concentration in an aqueous medium, considered as the outer cellular medium. Since the hydrophobic part of the drug provides an energy penalty in the outer aqueous medium, the drug will have a tendency to incorporate cellular membranes by inserting its non-polar part into the hydrophobic core of bilayer membranes, which allows it to minimize its own energy. Nonetheless, when the drug is incorporated in the outer leaflet of the cellular membrane, a lateral compression ΔS_{out} of the leaflet equivalent to the cross section area, a, of the drug once inserted is generated such that $\Delta S_{\text{out}} = -a$. As a result, the energy of the drug increases by a factor:

$$U^0_{\text{out}} = \Delta S_{\text{out}} \sigma_{\text{out}} = -a\sigma_{\text{out}} \quad (1)$$

where σ_{out} is the outer leaflet surface tension. Equation 1 is valid only if the insertion of drugs does not alter the cellular outer surface tension, i.e. that the concentration of drugs in the outer medium is low. This is supported by the facts that: (1) a low physiological concentration of drugs is administrated orally to avoid cytotoxicity (Yamanaka et al. 1979), (2) a large amount of drugs administered are metabolized by specific enzymes impairing their activity before reaching their targets (Kivisto et al. 2004) and (3) only a small fraction of oral drugs pass through the intestinal barrier (Chan et al. 2004).

Once incorporated into cellular membranes, studies have demonstrated that classical anti cancer drugs that have an affinity with Pgp-like transporters, such as anthracycline derivates, have a relatively slow transbilayer diffusion ~10 s (Regev et al. 2005). With a membrane thickness ~5 nm one gets an apparent diffusion coefficient through biomembranes ~10^{-6} $\mu m^2/s$. However, based on Stokes–Einstein's relation, these anthracycline derivates have a diffusion coefficient in water ~ 10^2 $\mu m^2/s$ (Cirilli et al. 1993; Heijn et al. 1999), which gives a ratio in the diffusion coefficients of eight orders of magnitude. Such a difference can be explained either by the intrinsic membrane viscosity and/or a membrane barrier potential linked to the activation energy needed for a drug to traverse the membrane. Assuming the simple case of a homogenous membrane of a given diffusion coefficient (~10^{-6} $\mu m^2/s$), and that the time needed for a drug to cross the membrane (~10 s) only depends on the membrane viscosity, in this context the drug's lateral length of diffusion should also be ~1 nm, as the square displacement of the drug is proportional to the diffusion coefficient multiplied by the residency time. Consequently, when cells are resistant to drugs, as extrusion of drugs occurs via Pgp related mechanisms, this would imply that the surface density of Pgp should be ~1 nm^2. This result contradicts biological data showing that drug resistance occurs when Pgp-like transporters only represent ~0.4% of total membrane proteins (Mao and Scarborough 1997). As a consequence, not only the membrane viscosity but also the membrane barrier potential, are likely to intervene in slowing down the transbilayer movement. In this context, the connection existing between the activation energy needed to cross the plasma membrane and the drug residency time is (see supplementary material (SM) for full analytical development):

$$t_0 \cong \frac{h^2}{2D}\left[\frac{1}{h}\int_0^h e^{\left[U(x)-U^0_{out}\right]/k_B T} dx\right] \quad (2)$$

where t_0, D, h, k_B, T U^0_{out} and $U(x)$ represent the drug residency time in the membrane, the average value of the membrane diffusion coefficient, the membrane thickness, the Boltzmann's constant, the absolute temperature, the drug's energy in the outer leaflet and within the membrane, respectively (see SM or Fig. 1a).

$U(x)$ is a complex function of the set of interactions between a drug and the membrane components, which varies over the membrane thickness. Initially in the outer leaflet, in order to traverse the bilayer the drug must transfer its hydrophilic (polar) part into the hydrophobic core of the membrane, and compensate the potential difference of surface tension existing between leaflets (Fig. 1a). This will inevitably increase the drug's energy. For the sake of simplicity, one will assume that both the drug's dehydration energy, ΔG, and the outer and inner leaflets surface tension, σ_{out} and σ_{in} respectively, are constant, i.e. are average values over the membrane thickness. Using these assumptions, it results that the drug's energy when crossing the membrane is

$$U_{\text{out}} \cong \Delta G - a\sigma_{\text{out}} \tag{3}$$

in the outer leaflet and

$$U_{\text{in}} \cong \Delta G - a\sigma_{\text{in}} \tag{4}$$

in the inner leaflet. Recalling (2), the integration can be split in two, firstly over a distance varying between 0 and $h/2$ (outer leaflet) and secondly over a distance varying between $h/2$ and h (inner leaflet):

$$t_0 \cong \frac{h^2}{2D}\left[\frac{1}{h}\left(\int_0^{h/2} e^{\left[U-U^0_{\text{out}}\right]/k_BT}dx + \int_{h/2}^{h} e^{\left[U-U^0_{\text{out}}\right]/k_BT}dx\right)\right] \tag{5}$$

Inserting (3) and (4) into (5) it follows:

$$t_0 \cong \frac{h^2}{4D} e^{\Delta G/k_BT}\left(1 + e^{-a\Delta\sigma/k_BT}\right) \tag{6}$$

where $\Delta\sigma = (\sigma_{\text{in}} - \sigma_{\text{out}})$ corresponds to the difference of surface tension between the inner and outer leaflet respectively. The difference of surface tension has been shown to be the causation of membrane budding leading to the creation of intracellular vesicles of radius R, written under the form $R = -8k_c/h\Delta\sigma$, where k_c is the bending modulus of the membrane (Rauch and Farge 2000). Thus (6) can also be rewritten as

$$t_0 \cong \frac{h^2}{4D} e^{\frac{\Delta G}{k_BT}}\left(1 + e^{8\frac{k_c a}{k_BThR}}\right) \tag{7}$$

Since the membrane barrier energy will prolong the residency time of the drugs in the membrane, the drugs are expected to diffuse laterally, which may in turn increase the probability that they meet and are extruded by Pgp-like transporters.

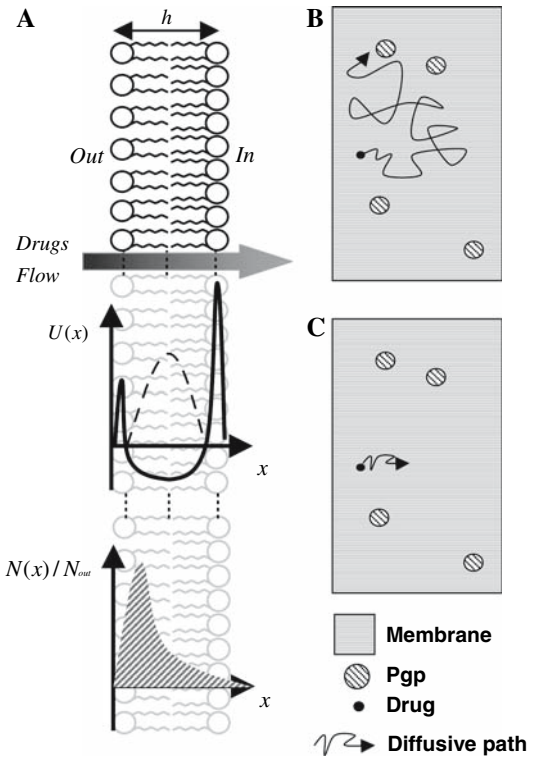

Fig. 1 **a** Representation of the different energy barriers involved when a drug traverses the bilayer cellular membrane. Two leaflets have been represented with an inner leaflet containing more phospholipids related to the increase in the difference of surface tension (*upper graph*). Energy profiles linked to the surface tension in leaflets (*plain curve-middle graph*) (Cantor 1999) and the hydrophobic core of the membrane (*dashed curve-middle graph*) (Ulander and Haymet 2003) are both involved in providing penalty energy with regard to the drug transbilayer movement. For the sake of simplicity, and to provide literal expressions, only averaged values of these profiles have been taken into consideration. The dashed area (*lower graph*) represents the probability density of drugs in the membrane. In effect, due to the membrane barrier energy, more drugs are found in the outer leaflet than the inner leaflet (see supplementary material). **b** Effect of the residency time on drug/Pgp interaction. During its residency time in the membrane, we assume that the drug diffuses laterally over a length that is related to the membrane barrier energy to bypass. As a result a drug may encounter a Pgp. **c** Effect expected on drug/Pgp interaction when the drug lateral path is shortened. A decrease of the lateral path length is likely to decrease the probability of a drug meeting and being extruded by Pgp, which in turn may result in a higher intracellular drugs accumulation

Model: Part II

Relation between the 2D random walk of drugs and Pgp-like transporters mediating MDR

Assuming that: (1) The number of Pgps on the cellular surface of MDR cells remains globally constant; (2) due to their large transmembrane structure the lateral diffusion time of Pgp-like transporters can be neglected compared to the lateral diffusion time of drugs; (3) the number of drugs in the membrane and the outward pumping kinetics are respectively low and fast enough, such that the probability that two drugs meet a given transporter at the same time is negligible.

As a result, transporters can be considered as static with a probability of presence on the cellular surface given by $\rho_{\mathrm{Pgp}} = N_{\mathrm{Pgp}}\, S_{\mathrm{Pgp}}/S_{\mathrm{cell}}$, where N_{Pgp}, S_{Pgp} and S_{cell} represent the number of transporters, the cross section area of transporters and the cellular surface, respectively. During its residency time in the cellular membrane, a drug is expected to follow a two dimensional random walk, with a formal condition restricted to time scales greater than the single transverse diffusion time, i.e. $h^2/4D$. Consequently, the number of steps performed is $K = t_0/(h^2/4D)$. Nonetheless, the probability of a drug and a transporter meeting will depend on non-recurring walks and for K large (i.e. $t_0 > h^2/4D$) the number of non-recurring walks, $f(K)$, is (Rudnick and Gaspari 2004):

$$f(K) \cong \pi K/\ln(K) \tag{8}$$

As a result, the probability of the event "drug meeting a Pgp" in the membrane, p_{Pgp}, is equivalent to the probability of meeting one transporter, i.e. ρ_{Pgp}, multiplied by the number of non-recurring steps, i.e. $f(K)$. Using (6)–(8) it follows:

$$p_{\mathrm{Pgp}} = \rho_{\mathrm{Pgp}} f(K) \cong \rho_{\mathrm{Pgp}} \pi \frac{e^{\frac{\Delta G}{k_B T}}\left(1 + e^{8\frac{k_c a}{hRk_B T}}\right)}{\ln\left[e^{\frac{\Delta G}{k_B T}}\left(1 + e^{8\frac{k_c a}{hRk_B T}}\right)\right]} \tag{9}$$

Nonetheless, a single meeting between a drug and a transporter is not enough to generate extrusion, since this process requires the location of the drug in the inner leaflet of the plasma membrane. Figure 1a clearly shows that the dehydration energy of the drug plays a central role in the middle of the membrane when the drug is entering into the inner leaflet, and thus one will legitimately consider that the energy required from the drug must be higher than the dehydration energy. In addition, as the drug remains in the outer leaflet of the cellular membrane for a much longer time than the single transverse diffusion time, i.e. $t_0 > h^2/4D$, the drug residency time in the outer leaflet can be considered as an infinite time in first approximation, and thus the probability that a drug enters the inner leaflet can be approached by Maxwell–Boltzmann's distribution, written as

$$\frac{\int_{\Delta G}^{+\infty} e^{-E/k_B T} dE}{\int_{0}^{+\infty} e^{-E/k_B T} dE} = e^{-\Delta G/k_B T} \tag{10}$$

Assuming that when entering the inner leaflet and meeting a transporter a drug is extruded, using (9) and (10) the probability, $\tilde{p}_{\mathrm{Pgp}}$, that a drug is effectively expelled from the membrane by a transporter is

$$\tilde{p}_{\mathrm{Pgp}} \cong e^{-\frac{\Delta G}{k_B T}} \rho_{\mathrm{Pgp}} \pi \frac{e^{\frac{\Delta G}{k_B T}}\left(1 + e^{8\frac{k_c a}{hRk_B T}}\right)}{\ln\left[e^{\frac{\Delta G}{k_B T}}\left(1 + e^{8\frac{k_c a}{hRk_B T}}\right)\right]} \tag{11}$$

From (11), different predictions can now be addressed.

Predictions

Prediction 1: Drugs size or MW selectivity on their transbilayer movement

From (7) and (11) it can be deduced that the time required for the transbilayer movement, and thus the probability that a drug is extruded by a Pgp-like transporter, is a function of the cross section area, a, of the drug which may become involved in MDR if

$$a > a_c = hR\frac{k_B T}{8k_c} \tag{12}$$

where a_c indicates the critical cross section area of the drug, beyond which the difference of surface tension is likely to affect its transbilayer movement. A numerical value of a_c can be determined; considering a membrane thickness h~5 nm, a vesicle radius R~35 nm determined for drug sensitive cells (Rauch and Farge 2000) and a membrane bending modulus, $k_c \sim 2.10^{-19}$ J (Bloom et al. 1991), it follows a_c~0.4 nm^2 at 37°C (Fig. 2a). Furthermore, assuming that the MW of the drug is proportional to its Van der Walls volume (expressed in $\dot{A}^3$), a critical MW can also be deduced $\mathrm{MW}_c = (4/3\sqrt{\pi})(hRk_B T/8k_c)^{3/2} \sim 240$ (Fig. 2b), providing a law with regard to the drugs' size (or MW) selectivity on their permeation across cellular membranes.

Prediction 2: Membrane endocytosis involvement in Pgp-mediated MDR

The possible involvement of endocytosis in MDR can be determined using (12) since it has been experimentally shown that the kinetics of endocytosis is proportional to the membrane phospholipid number asymmetry, i.e. inversely proportional to the vesicle radius (Farge et al. 1999). Assuming that endocytosis is altered in drug resistant cells, (12) can therefore be rewritten as

$$\frac{8k_c a}{hRk_BT} = \frac{8k_c a}{hR_0k_BT}\frac{R_0}{R} = \frac{a}{a_c}\frac{k}{k_0} \tag{13}$$

where R_0, k_0 and R, k are the vesicle radius and the rate of endocytosis when cells are sensitive (subscript "0") and resistant to drugs respectively. To determine the magnitude of (13), using for example the value of the cross section area of the drug idarubicin $a \approx 0.75$ nm^2 (Heywang et al. 1998), it follows that $a/a_c \sim 1.8$. As the kinetics of endocytosis can be increased up to four fold (Farge et al. 1999) and that MDR cells display a high vesiculation rate (Altan et al. 1999; Sehested et al. 1987a, b), the magnitude of $a/a_c{\cdot}k/k_0$ can range up to ~ 8. Thus, exp $(a/a_c{\cdot}k/k_0) > > 1$ and using (13) it follows that (11) can also be written as

$$\tilde{p}_{\mathrm{Pgp}} \cong \rho_{\mathrm{Pgp}}\pi\frac{e^{\frac{a}{a_c}\frac{k}{k_0}}}{\frac{\Delta G}{k_BT}+\frac{a}{a_c}\frac{k}{k_0}} \tag{14}$$

From (14) it can be seen that any increase in the endocytosis kinetics will increase the probability of a drug being extruded by Pgps. Conversely, any decrease of endocytosis kinetics may impair the extrusion of drugs mediated by Pgps (Fig. 1b, c).

Prediction 3: Drugs sensitivity versus drugs resistance function of both Pgps number in plasma membrane, drug size and endocytosis

From (14) and when $\tilde{p}_{\mathrm{Pgp}} = 1$, it can be deduced that the minimal condition regarding the critical cellular surface area covered by Pgp-like transporters, ρ^c_{Pgp}, triggering the extrusion of drugs that is

$$\rho^c_{\mathrm{Pgp}} \cong \frac{1}{\pi}\left(\frac{\Delta G}{k_BT}+\frac{a}{a_c}\frac{k}{k_0}\right)e^{-\frac{a}{a_c}\frac{k}{k_0}} \tag{15}$$

Equation (15) plotted in Fig. 2c provides the following information: (1) two areas with regard to both drug resistance and sensitivity can be differentiated, and (2) the probability of a drug escaping Pgps tends exponentially toward zero when the membrane barrier energy, linked to the drug's size and to endocytosis, increases. Using $a/a_c \sim 1.8$, $k/k_0 \sim 4$ and $\Delta G/k_B\,T \sim 10$ ($\Delta G \sim 10k_BT$ corresponds to the carboxylic group dehydration energy (Ulander and Haymet 2003)) it follows $\rho^c_{\mathrm{Pgp}} \cong 0.5\%$. Thus, the critical fraction of the cellular surface area covered by Pgp and triggering the extrusion of drugs can be extremely low. In addition, using the previous definition: $\rho^c_{\mathrm{Pgp}}{=}N^c_{\mathrm{Pgp}}\,S_{\mathrm{Pgp}}/S_{\mathrm{cell}}$, with a cell surface ~100 μm^2 (cell radius ~5 μm) and a Pgp surface area ~75.10^{-6} μm^2 (Lee et al. 2002), it follows that $N^c_{\mathrm{Pgp}} \sim 5{,}000$. This result suggests that the coupling between Pgps-like transporters and the slow transbilayer movement of drugs associated with the difference of surface tension is potentially a very efficient mechanism in MDR. Thus it is therefore important to determine the escape rate into the cytoplasm, of drugs initially located in the membrane of MDR cells.

Prediction 4: Drugs escape rates from the membrane into the cytoplasm of MDR cells

The flow of drugs within the cytoplasm is modulated both by a slow transbilayer movement and the presence of Pgp-like transporters. In this context, the probability per unit of time that a drug escapes the membrane to go into the cytoplasm, noted r_{MDR}, can be expressed as the probability that a drug is not extruded by Pgp, i.e. $1-\tilde{p}_{\mathrm{Pgp}}$ (14), divided by the drug residency time within the membrane t_0 (7), which using (15) leads to:

$$r_{\mathrm{MDR}} = \frac{\exp\left[-\left(\frac{\Delta G}{k_BT}+\frac{a}{a_c}\frac{k}{k_0}\right)\right]}{h^2/4D}\left(1-\frac{\rho_{\mathrm{Pgp}}}{\rho^c_{\mathrm{Pgp}}}\right) \tag{16}$$

Assuming that the membrane diffusion coefficient remains identical between drug sensitive and resistant cells, (16) can also be used to describe the escape rate of a drug from the membrane for sensitive cells, noted as $r_{\text{non-MDR}}$, by posing $\rho_{\mathrm{Pgp}} = 0$ and $k = k_0$. Thus, the ratio between escape rates in drug resistant and sensitive cells can therefore be deduced:

$$\frac{r_{\mathrm{MDR}}}{r_{\mathrm{non-MDR}}} \cong e^{-\frac{a}{a_c}\left(\frac{k}{k_0}-1\right)}\left(1-\frac{\rho_{\mathrm{Pgp}}}{\rho^c_{\mathrm{Pgp}}}\right) \tag{17}$$

Equation (17) shows that although the inhibition of the transmembrane flow of drugs going into the cellular cytoplasm can be linearly performed through the expression of Pgp-like transporters, i.e. when $\rho_{\mathrm{Pgp}} = \rho^c_{\mathrm{Pgp}}$, the transmembrane flow of drugs can also be reduced

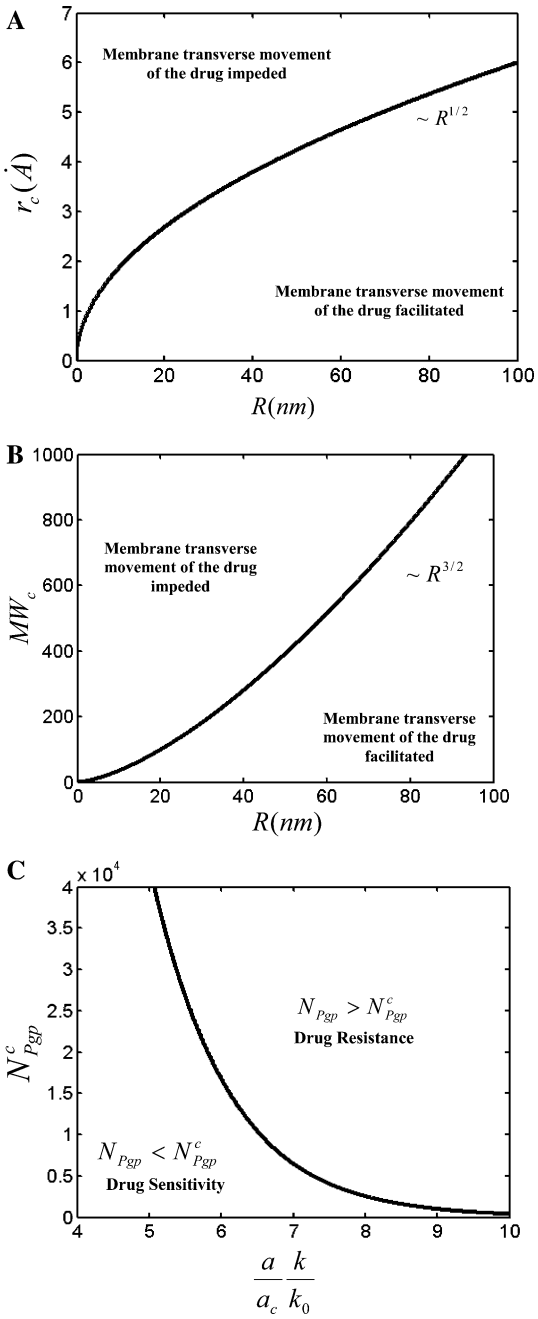

Fig. 2 **a** Relation existing between the drugs' Van der Walls radius, $r_c(\dot{A})$ and their ability to bypass the membrane barrier as a function of vesicles radius, $R(nm)$ (i.e. function of the difference of surface tension), scaling as $r_c \sim R^{1/2}$[exactly: $r_c \cong 0.6 \quad R^{1/2}$ using constants seen in the text]. **b** Relation existing between the drugs' MW and their ability to bypass the membrane barrier as a function of vesicles radius $R(nm)$, scaling as $MW_c \sim r_c^3 \sim R^{3/2}$ [exactly: $MW_c = 4\pi\ r_c^3/3 \cong 1.1\ R^{3/2}$ using constants seen in the text]. **c** Representation of the critical number of Pgp-like transporters to trigger drug resistance as a function of the relation between the cross section area of the drug and the endocytosis kinetics (15). The plot (performed using $\Delta G = 10 k_B\ T$) allows the definition of two areas delimiting drug resistance and sensitivity

exponentially as a function of the kinetics of endocytosis. For example, assuming that the Pgps expression verifies $\rho_{Pgp} = 0.99\rho^c_{Pgp}$, using the value of the cross section area of the anthracycline drug idarubicin, and assuming that the kinetics of endocytosis is increased four fold in drug resistant cells, the ratio between the escape rates is ~4.10^{-5}. In other words, assuming a transbilayer characteristic time of about 10 s in drug sensitive cells, it follows that the same characteristic time in drug resistant cells should be ~2.5 days. However, over such a long time, a drug is unlikely to remain in the membrane, but likely to be internalized via endocytosis.

Prediction 5: Final location of drugs, cytoplasm versus internal compartments

Although resistant cells display low levels of free cytoplasmic drugs, they clearly display an increase of drugs trapped within intracellular compartments, which remains a major feature in MDR. As a result, drugs that have failed to cross the membrane and enter the cytoplasm are likely to follow membrane endocytosis, which results in their being trapped within internal endocytic compartments. This point is crucial, since a large fraction of Pgps (~70%) is located in the membrane of endosomes, and it is expected that once drugs have reached these organelles, Pgps in these compartments alter the transbilayer movement of drugs (Kim et al. 1997). Considering a drug in the membrane initially, the important physical parameter determining the probable future location of the drug will depend on the ratio between the kinetics of endocytosis, previously noted k, and the escape rate of the drug from the membrane into the cytoplasm, previously noted r. Noting χ_{MDR} as the ratio when cells are resistant, it follows that when $\chi_{MDR}>1$ or $\chi_{MDR}<1$, drugs will be principally located in the endosomes or the cytoplasm, respectively. As χ_{MDR} can also be written as

$$\chi_{MDR} = \frac{k}{r_{MDR}} = \frac{k_0}{r_{non-MDR}}\frac{k}{k_0}\frac{r_{non-MDR}}{r_{MDR}} \tag{18}$$

noting $\frac{k_0}{r_{non-MDR}} = \chi_{non-MDR}$ as the same ratio when cells are sensitive and inserting (17) it follows:

$$\chi_{MDR} = \chi_{non-MDR}\frac{k}{k_0}\frac{e^{\frac{a}{a_c}\left(\frac{k}{k_0}-1\right)}}{1-\frac{\rho_{Pgp}}{\rho^c_{Pgp}}} \tag{19}$$

Equation (19) shows that without changes in the kinetics of endocytosis ($k_0 = k$), drugs will fill endocytic

compartments when ρ_{Pgp} is close to ρ^c_{Pgp}. For example, in drug resistant cells displaying an outer surface fraction of Pgp such as $\rho_{Pgp} = 0.99\rho^c_{Pgp}$, the probability that a drug, initially in the outer cellular surface of the membrane, ends up in endosomes, is 10^2 times higher than in sensitive cells. Considering furthermore that the kinetics of endocytosis is increased four fold, using the cross section area of idarubicin, the same probability is now ~$8{\cdot}10^5$ times higher than in sensitive cells (Fig. 3). Thus, both Pgp and altered endocytosis can potentially take account of drugs trapped within intracellular compartments.

Discussion

Although the biological activity of Pgp-like transporters is recognized in MDR, the associated ''vacuum cleaner'' effect remains unclear and does not seem to be related to drugs' chemical structure, as their extrusion is done with similar efficiency. Furthermore, an increasing number of studies point out that the size of drugs, or MW, might well be determinant in their transmembrane movement or MDR, which invokes possible physical/mechanical effects (Lipinski et al. 2001; Mitragotri et al. 1999; Zamora et al. 1988). As a result, we have investigated how cellular membrane mechanical properties may be involved in drug resistance. In this context, a simple and linear model is proposed, taking into account

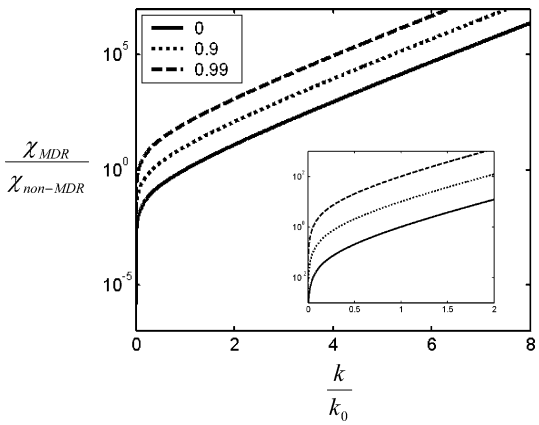

Fig. 3 Plot of $\chi_{MDR}/\chi_{non\text{-}MDR}$ for idarubicin ($a/a_c \approx 1.8$) as a function of the endocytosis kinetics and of the cellular surface area covered by Pgp when $\rho_{Pgp} = 0$ (*bold line*), $\rho_{Pgp} = 0.9$ (*dotted line*) or $\rho_{Pgp} = 0.99$ (*dashed line*). For sensitive cells it is expected that both organelles and cytoplasm will be filled by drugs, in which case $\chi_{non\text{-}MDR} \sim 1$ and thus $\chi_{MDR}/\chi_{non\text{-}MDR} \sim \chi_{MDR}$. In this condition, the inset shows that at a low or moderate surface expression of Pgp, increases or decreases in endocytosis kinetics can strongly affect the location of idarubicin within MDR cells

both membrane endocytosis and drugs' lateral diffusion in the plasma membrane, assuming a low concentration of drugs avoiding changes in the cellular membrane difference of surface tension. To clarify this point, we do not reject that a high concentration of compounds may alter endocytosis, however concentrations required for such an effect are within the range of ~100 to 1,000 μM (Raucher and Sheetz 1999), which are not physiological likely to generate strong cytotoxicity.

Can the ''vacuum cleaner'' hypothesis be explained by a ''two dimensional random walk'' hypothesis?

As already stated, the Pgp mediated ''vacuum cleaner'' activity today remains elusive. Nonetheless, this assertion helps to represent the fact that the drugs will inevitably interact with transporters. In this work, we suggest that the movement of drugs in the plasma membrane is driven by Brownian's motion. In this context, the ''vacuum cleaner'' hypothesis can be replaced by a two dimensional random walk, which seems to be self-sufficient, since MDR occurs with a fraction of Pgp covering the cellular surface as low as ~0.1%. In addition, taking endocytosis into consideration, drugs that may have escaped from the Pgps located in the plasma membrane are likely to be internalized via endocytosis into endosomes where a large fraction of Pgps are present. Note that the present model does not exclude that drugs potentially escaping the plasma membrane and membrane endocytosis to go into the cytosol, may also be pumped into the lumen of organelles by Pgp-like transporters localized in the membranes of those organelles. However, this point has not been treated since the level of drugs into the cytoplasm is likely to be low (Fig. 3). Overall, the model strongly suggests that endocytosis may well be a key factor in MDR, and that a diminution of the difference of surface tension is likely to increase the accumulation of drugs within MDR cells (Fig. 1b, c), by: (1) altering the probability between a Pgp and a drug meeting (15, Fig. 2c), (2) changing the critical MW altering the membrane transverse movement of drugs (12, Fig. 2b) and (3) decreasing the kinetics of membrane endocytosis, transporting drugs toward endosomes (19, Fig. 3).

Can endocytosis be the foundation of Lipinski's second rule?

Today, although the design of drugs often provides efficient pharmacology, their bioavailability (adsorption and biodistribution within tissues) represents a

major challenge for the pharmaceutical industry. The limit of efficient therapeutics is no longer set by the chemistry or the design against biological targets, but instead by the ability of drugs to cross all the membrane barriers before reaching their target. In this context, experimental and computational approaches have been used to estimate what physico-chemical characteristics should have the ''best'' drug (Lipinski et al. 2001). From these studies five rules have emerged (''the rule of 5''), defining drug properties for maximum efficiency. Among them is the second rule stating that a drug must have a MW equal or lower than 500, relatively close to the model prediction MW_c~240 for a vesicle radius ~35 nm (MW_c~500 is achieved for a rational vesicle radius ~50 nm). It is therefore tempting to take on board Lipinski's second rule as an indirect and potential proof of the role played by the cellular membrane difference of surface tension limiting the transbilayer movement of drugs.

What putative origin or cause is responsible for an increase in the kinetics of endocytosis in MDR cells?

We have presumed that membrane endocytosis is fundamental in MDR. As Pgp-like transporters have been shown to function as inverted flippases, it is therefore unlikely that they promote an increase in surface tension/endocytosis by locating phospholipids into the inner leaflets of resistant cells. Thus, the origin of the increase in endocytosis/membrane recycling kinetics is likely to arise from another, certainly related, mechanism. Interestingly, Pgp-like transporters belong to the ionic channel transporters family altering the membrane electrical potential and/or cytosolic pH (Harguindey et al. 2005), and changes in the state of drug resistance have been shown to occur in parallel to cytoplasmic pH changes. In particular, drug sensitive cells display a low cytoplasmic pH (pH~6.5), compared to drug resistant cells (pH~7.4) (Altan et al. 1998), which seems to rely on a Pgp-dependant mechanism (Harguindey et al. 2005). Both clathrin and fluid phase endocytosis are known to be altered in this condition of low cytosolic pH, and in particular an increase in the radius of non-coated/fluid phase vesicles has been observed (a fivefold increase at pH ~6) (Davoust et al. 1987; Lindgren et al. 1997; Sandvig et al. 1987, 1988; Sandvig and van Deurs 1994). As the membrane budding radius is inversely related to the difference of surface tension, this suggests that a low cytosolic pH may trigger, either directly or indirectly, a decrease in the difference of surface tension, which is expected to affect the cellular membrane difference of surface tension. In this case, the accumulation of drugs in sensitive cells could be facilitated when the cytosolic pH is low (pH~6.5) and abrogated when the cytosolic pH is neutral (pH~7.4) and Pgps are expressed in MDR cells.

Potential experimental verification of the model

The verification of this model must be performed, not by high resolution microscopy such as classical confocal microscopy, but through high content photon analysis, e.g. fluorescence correlation spectroscopy (FCS), allowing the use of a very low concentration of drugs (~1 nM). With such a technique, the experimental direction to undertake is to change in the kinetics of endocytosis of resistant cells via changes in either membrane phospholipid composition, membrane electrical potential or cytoplasmic acidity, all known to alter endocytosis (Davoust et al. 1987; Farge 1995; Lindgren et al. 1997; Rauch and Farge 2000; Sandvig et al. 1987, 1988; Sandvig and van Deurs 1994). Subsequently, changes occurring in the drugs/membrane interaction and the location of drugs into cells might be determined.

Conclusion

In conclusion, we suggest or confirm that: (1) the mechanism of drug resistance may be related to more than one biological process and that these subsequent mechanisms may act synergistically in MDR cells; (2) beyond a critical cross sectional area, the difference of surface tension causing endocytosis is likely to impair the passive transbilayer movement of drugs, increasing their probability of being extruded by Pgp-like transporters; (3) the phospholipid metabolism and the associated cellular membrane physical properties are likely to give important insights into drug delivery or resistance.

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