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Chapter 3

Cell Membranes, Cytosolic pH and Drug Transport in Cancer and MDR: Physics, Biochemistry and Molecular Biology

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Abstract

Pleiotropic resistance (or multidrug resistance, or MDR) to anticancer drugs in malignant diseases takes place either primarily or secondarily in the treatment of cancer. The first report suggesting that MDR could be the result of active drug extrusion was published in 1973 by Dano [1]. A few years later, P-glycoprotein (Pgp) was identified by Juliano and Ling as a membrane protein that actively extrudes membrane embedded drugs. Biochemistry is a "science of contacts" between at least two biochemical elements (e.g. a drug and a transporter). These elements have to interact in order for a reaction to occur (e.g. drug extrusion). Taking into account that drugs do not necessarily incorporate into the membrane in the vicinity of a drug transporter, these transporters have been thought to exhibit a "vacuum cleaner" like-activity on membrane embedded drugs. Accordingly, understanding this activity is essential; as the movement of membrane embedded drugs, or otherwise, movement of the transporters must occur if extrusion is to take place. These activities should be considered as targets for anticancer drugs. Drug movement occurs by lateral diffusion (Brownian movement) in the membrane and the physical conditions required for drug extrusion by membrane transporters is known as the "2D random walk" theory. A deeper understanding is now possible, because there is enough literature enabling understanding of: (a) why the membrane is central to Pgp-

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mediated drug extrusion; (b) why the drug's molecular weight is an essential determinant in MDR, and; (c) why an alkaline cytosolic pH is essential in MDR.

After recalling the importance of pH in many aspects of cancer**,** including MDR, we will focus on the reason why a better understanding of the "vacuum cleaner" hypothesis is needed to clarify the subsequent processes involved in MDR. This will lead us to consider the cell membrane's mechanical properties and cytosolic pH alkalinization as important factors in the etiopathogenesis and understanding of MDR.

Introduction

A. The Role of pH in Cancer and MDR: An Overview

It appears that we have now reached a deadlock in cancer chemotherapy [2]. This seems to be related to the fact that most of the antitumor agents used in the clinical situation are based upon the old "anti DNA" paradigm which, over the last 60 years, has attempted to induce an antitumor effect by targeting DNA synthesis or cell division and proliferation. It has recently been proposed that the modest progress achieved in this area, and the lack of selectivity of current antitumor agents, suggests that the "anti DNA" framework is either conceptually poor or at least too simplistic for such a complex disease [2]. Some of these authors have proposed that the limitations of the present-day approaches can be responsible for the fact that some therapies can even exacerbate the original malignant phenotype, induce suppression of apoptosis and negatively affect the progression of the disease [2-4]. As an alternative to this approach, a proton-related mechanism underlying the initiation and progression of the neoplastic process has recently been described [5-8].

This model is based upon the fact that regardless of their origin and genetic background, all cancer cells and tissues have a pivotal energetic and homeostatic disturbance of their metabolism that is completely different to all normal tissues: an aberrant regulation of hydrogen ion dynamics that leads to a reversal of the pH gradient in cancer cells and tissues [5-8]. This causes an interstitial acid microenvironment secondary to an initial, specific and etiopathogenic intracellular alkalosis, which is the opposite of the normal pH gradient. This specific abnormality of cancer cells and tissues, called "proton reversal or H^+-reversal, is increasingly considered to be one of the most differential hallmarks of cancer [7, 9]. It is remarkable that at the intracellular level, an elevated intracellular pH (pH_i) plays a direct and specific role in transformation and tumor development [6-8, 10, 11].

The basic abnormality in the relationship between intracellular and extracellular dynamics of the hydrogen ion allows for the creation of a unifying view of several of the most important aspects of cancer research. These include diverse topics such as etiopathogenesis, cancer cell metabolism and neovascularisation, and multiple drug resistance (MDR), selective apoptosis, the metastatic process, cancer chemotherapy and even the spontaneous regression of cancer (SRC) [5, 7, 12, 13]. There is now ample data demonstrating that this reversed proton gradient is driven by a variety of proton (export) extrusion mechanisms that underlie the initiation and progression of the neoplastic process, as well as resistance to treatment [14]**.** This means that therapeutic targeting of the transporters that are more active in cancer cells

could be selective for malignancy. These findings are likely to lead to the development of more specific and less toxic therapeutic measures for malignant diseases.

B. pH_i and Multiple Drug Resistance (MDR) to Anticancer Drugs

A direct cause and effect relationship between the degree of MDR and the elevation of tumor pH_i has been reported by several laboratories [15-22]. The following conclusions can be reached based on the following lines of experimental evidence:

1. A failure to induce intracellular acidification and reverse the altered and specific proton reversal of cancer tissues has been proposed to be the main factor underlying drug resistance, and including resistance to the induction of therapeutic apoptosis, in both highly alkaline cancer cells and in malignant cells with a normal or slightly elevated pH_i, [23-27].
2. The alkalinity characteristic of many, or possibly all, kinds of malignant cells is also known to decrease the retention of anticancer drugs such as vinblastine, adriamycin, cisplatin, paclitaxel, and campthothecin [20, 27-34].
3. In a parallel manner, the accumulation of some drugs in the cytosol increases with cellular acidification and decreases with an alkaline shift. This feature is mediated by overexpression of the Na^+/H^+ antiporter, or other proton transporters [21, 35, 36].

To exemplify points 1, 2 and 3, one of the most representative and seminal findings in this area has been the observation that pH alkalization from pH_i 7.0 to pH_i 7.4 triggers a ~2000-fold increase in adriamycin resistance in human lung cancer cells [15]. This level of resistance can be reversed by cellular acidification via administration of verapamil [15]. In parallel studies, the intracellular concentration of adriamycin was shown to be increased by more than 100-fold after inducing cellular acidification, an acid-base energetic change that behaves on its own as a "chemosensitizing agent" [18].

4. At the same time, no matter how paradoxical it may seem, some drugs, mainly weak bases, can be sequestered away from the cytosol within highly acidic organelles, such as lysosomes, endosomes, and the Golgi network of otherwise alkalinized cells, and in this way are inactivated (the so called protonation effect or ion trapping effect) [23, 29, 37, 38].
5. There is a large body of work suggesting that the main function of P-glycoprotein (P-gp) is to catalyze ATP-dependent proton extrusion. P-gp expressing cells exhibit cytoplasmatic alkalinization [22, 39]. In addition, the overexpression of specific H^+-ATPases leads to a increase in pH_i which has a positive correlation with drug resistance [21, 32, 33, 40], and inhibition of apoptosis [29, 41]. In support of these observations, specific inhibitors of H^+-ATPase, like Bafilomyicin A_1, induce the intracellular accumulation of drugs by inhibiting their efflux via an acidifying mechanism [40, 42-44].

6. Furthermore, extracellular acidity, secondary to intracellular alkalinization and H^+-reversal, has a positive effect on P-glycoprotein expression and also decreases the cytotoxicity of some chemotherapeutic drugs [45-47].
7. Lastly, findings directly relating cytosolic alkalinization with abnormalities in membrane depolarization in MDR-positive drug resistant cancer cells have been published [18, 24, 28, 35, 48, 49]. The energetic alterations of cell membrane depolarization, low transmembrane potential, pH_i elevations and the perturbations in cellular ion transport are considered to be responsible for most of the characteristics of multidrug-resistant cells.

In conclusion, a large set of available data in the literature indicates that any mechanism that leads to an elevation of pH_i, either through overexpression/activation of the NHE1 following activation of Na^+-K^+-ATPase [6, 50]; via hyperactivity of vacuolar H^+-ATPases, or by other mechanisms contribute to the onset and/or maintenance of MDR, which inhibits tumor cell death induced by anticancer drugs [3, 18, 21, 36, 51-56]**.**

C. Weighing the Role of Drug Handling and Extrusion by Transporters against Changes in Cytosolic pH

There have also been reports suggesting that pH changes, either extracellular or intracellular, may have no significant effect on drug extrusion by P-glycoprotein [57-59]. However, it has been widely accepted that Pgp is a transporter that binds to and extrudes drugs.

These two hypotheses describing MDR may appear to be deadlocked. On one hand**,** it is argued that cytosolic pH alkalinization is central to MDR and can be mediated by Pgp expression levels. On the other hand, it is suggested that drugs are directly extruded by Pgp-like transporters. Although these hypotheses are different, they are not necessarily mutually exclusive. It is possible to envisage MDR as resulting from a drug handling mechanism *and* a high cytosolic pH. It follows that the contribution of each of these two processes could be measured and their respective involvement in MDR could be elucidated.

This exercise would be possible if the “vacuum cleaner” hypothesis that has been invoked to explain how drugs interact with transporters prior to extrusion is re-examined.

D. The “Vacuum Cleaner” Hypothesis: A Model in Need of Clarification

Pgp-like transporters are presumed to impair the intracellular accumulation of drugs by extruding them at a rapid rate from the plasma membrane inner leaflet, even before they reach the cytosol [60-70]. Although the function whereby these transporters extrude drugs from the inner leaflet is well defined once a drug binds to a Pgp-like transporter, a further hypothesis is usually invoked, namely, that these transporters act as “vacuum cleaners” that vacuum membrane-embedded drugs. This assumption is usually invoked as therapeutic drugs partition efficiently into cellular membranes and their insertion into

membranes is widespread. Therefore, there is no reason to conceive of why a drug should insert itself into the membrane in the vicinity of a transporter. It follows that the efficiency of Pgp-like transporters to mediate MDR should rely on the transporters' ability to extrude drugs, and also fundamentally, on the transport of drugs from their initial incorporation in plasma membranes to the transporters *prior* to extrusion. Given this naïve standpoint, it was suggested that MDR results from different and synergic processes.

Impeding the activity of transporters in order to increase the intracellular accumulation of drugs, is possible by the use of drug resistance modulators. Although some modulators alter the activity of certain transporters through direct physical interactions [71, 72], modulators are also commonly known to affect the membrane's biophysical properties, and thus permeability to drugs [73-78]. These data indicate that specific cellular membrane biophysical properties and Pgp-like transporters are fundamental in the pathogenesis of MDR.

Transbilayer movement of drugs is not spontaneous; it occurs via lateral diffusion. The lateral diffusion of drugs might explain how they come to interact with transporters. The longer the diffusive lateral path, the higher the probability of a drug binding to a transporter. However, as the binding of drugs mediated by Pgp-like transporters takes place in the inner leaflet, it follows that part of the activation energy needed for a drug to traverse the membrane completely resides within this leaflet. Thus, drugs are prevented from moving in the reverse direction, and are extruded.

The ability of a drug to traverse the cell membrane has been shown to be dependent on specific biophysical properties of lipid bilayers. One of these is fluidity, which refers to the packing of lipids within a layer. Parameters include surface pressure and surface tension. Accordingly, changes have been noted regarding the cellular membrane of MDR cells. In particular, higher rates of endocytosis have been measured [79, 80]. It is worth noting that the local membrane budding which leads to membrane vesiculation and controls the kinetics of endocytosis has been associated with an endogenous higher compression of the cellular membrane inner leaflet [81-84]. Thus, a possible mechanism involved in the membrane barrier of MDR cells that could play a role in the inner leaflet, may be linked to membrane endocytosis itself and also may be related to the membranes' mechanical properties.

Given the need to add additional dimensions to the "vacuum cleaner" model, it was decided to address theoretically, whether it can be further developed by exploring additional factors related to the membrane physical properties, endocytosis, and the relationship between intracellular and extracellular pH.

I. The Physical Biology of MDR

A. The Role of Physical, Chemical, and Mechanical Factors Affecting the Transverse Movement of Drugs Across the Cell Membrane

Once they are incorporated into cellular membranes, studies have demonstrated that classical anticancer drugs that have an affinity with Pgp-like transporters, have a relatively slow transbilayer diffusion ~10s [85]. With a membrane thickness ~5nm, an apparent diffusion coefficient through biomembranes ~$10^{-6}\mu m^2/s$ can be obtained. However, based on the Stokes-Einstein relation, these therapeutics have a diffusion coefficient in water of ~$10^2\mu m^2/s$ [86, 87], 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 activation energy needed for a drug to traverse the membrane. In this context, it can be demonstrated that the connection that exists between the activation energy needed to cross the plasma membrane and the drug residency time is:

$$t_0 \cong \frac{h^2}{2D}\left[\frac{1}{h}\int_0^h e^{\left[U(x)-U_{out}^0\right]/k_BT}\,dx\right] \tag{1}$$

where t_0 is the drug residency time in the membrane, D is the average value of the membrane diffusion coefficient, h is the membrane thickness, k_B is Boltzmann's constant, T is the abolsute temperature, and U_{out}^0 is the drug's energy in the outer leaflet and within the membrane (Figure 1B). $U(x)$ is usually a very 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 [88], and compensate the potential difference in surface tensions existing between leaflets (Figure 1A). This will inevitably increase the drug's energy. 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. Under these conditions, the above formula can be reduced to:

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

where $\Delta\sigma=\left(\sigma_{in}-\sigma_{out}\right)$ corresponds to the difference in surface tensions between the inner and outer leaflet respectively and a is the drug's cross section area once intercalated in the cell membrane. The difference in surface tensions 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 (Figure 1A) [84]. Thus, Eq.6 can also be rewritten as:

$$t_0 \cong \frac{h^2}{4D}e^{\frac{\Delta G}{k_BT}}\left(1+e^{8\frac{k_ca}{k_BThR}}\right) \tag{3}$$

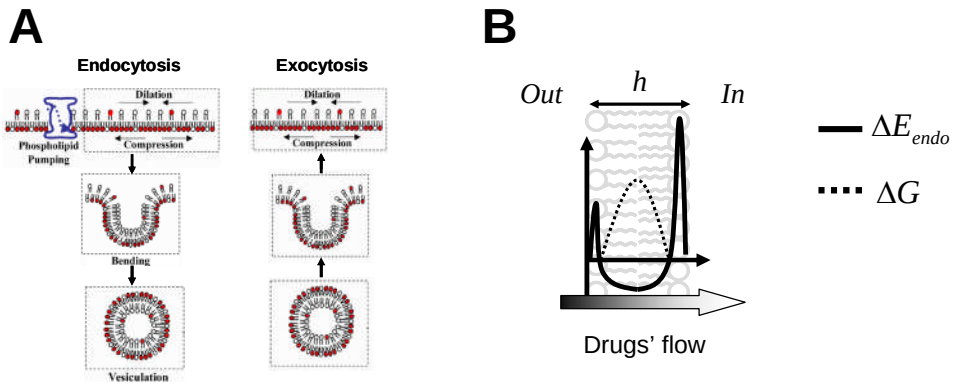

Figure 1: (A) The lipid number asymmetry induced fluid phase endocytosis (FPE) model: Sketch representing the current model that has been applied in living cells linking FPE and the membrane phospholipid number asymmetry thought to be maintained by aminophospholipid translocase. In the left panel, the translocation of lipids into the inner leaflet (shaded heads) induces a differential lipid packing between leaflets (namely a difference in surface tensions) leading to membrane bending and vesiculation [83, 84]. 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 existing 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 ~35nm vesicle radius [84]. **(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. Energy profiles (noted U(x) in the text) of both surface tension in leaflets (solid curve) and hydrophobic core of membrane (dashed curve) are involved in providing a penalty energy with regard to the transbilayer movement of drugs.

It is worth noting that the latter equation contains three physical parameters that are likely to be involved in affecting the transverse movement of drugs. The first one already mentioned is the membrane viscosity (hidden in the diffusion coefficient), the second one has a physico-chemical origin and is classically related to the drug dehydration energy, and the last one, related to the difference in surface tension, states that specific mechanical constraints exist when drugs cross the cell membrane and that these constraints are directly related to membrane endocytosis (Figure 1A&B).

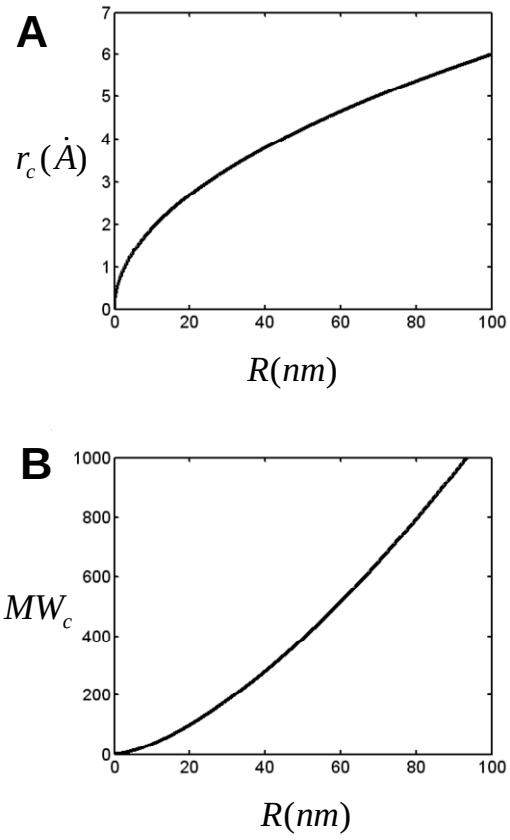

Figure 2: (A) Relationship existing between the drugs' Van der Waals radius, $r_c(\dot{A})$ and their ability to bypass the membrane barrier as a function of vesicular radius, $R(nm)$ (i.e. function of the difference in surface tension), scaling as $r_c \sim R^{1/2}$ [exactly: $r_c \cong 0.6\ R^{1/2}$]. (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: $MW_c = 4\pi r_c^3/3 \cong 1.1\ R^{3/2}$].

From Eq.3, it follows that the time required for the transbilayer movement of drugs is a function of the cross sectional area of the drug which is expected to become involved in MDR if $a > a_c = hRk_BT/8k_c$, where a_c indicates a critical cross sectional area beyond which the difference in surface tensions is likely to affect its transbilayer movement (namely beyond which the exponential function will be numerically high). A numerical value of a_c can be determined; considering a membrane thickness $h \sim 5nm$, a vesicle radius $R \sim 35nm$ determined for drug sensitive cells [84] and a membrane bending modulus, $k_c \sim 2.10^{-19}\ J$ [89], it follows $a_c \sim 0.4nm^2$ at 37ºC (Figure 2A). Assuming legitimately that the MW of the drug is proportional to its Van der Waals volume (expressed in $\dot{A}^3$), a critical MW can also be deduced $MW_c = (4/3\sqrt{\pi})(hRk_BT/8k_c)^{3/2} \sim 240$ (Figure 2B), providing a law

with regard to the drug size (or MW) selectivity on its permeation across the cell membrane. As endocytosis is altered in drug resistant cells and the kinetics of endocytosis is inversely proportional to vesicle radius [83], it follows that the mechanical interaction between a drug and the membrane can be re-written as:

$$8k_c a / hRk_B T = 8k_c a / hR_0 k_B T \cdot R_0 / R = a / a_c \cdot k / k_0 = (MW / MW_c)^{2/3} \cdot k / k_0 \quad (4)$$

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. Finally, the drug residency time can be rewritten as:

$$t_0 \cong \frac{h^2}{4D} e^{\frac{\Delta G}{k_B T}} \left(1 + e^{\frac{k}{k_{0c}} \cdot \left(\frac{MW}{MW_c} \right)^{2/3}} \right) \quad (5)$$

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

So far we have determined how the duration of time of drugs are in the cell membrane can be affected by the membrane's physical properties. We need now to determine how the duration of time within the membrane will favor interaction with drug transporters.

B. The Critical Relationship between The Two Dimensional (2D) Random Walk Of Membrane –Embedded Drugs and Pgp-Like Transporter Surface Density in Mediating MDR

The previous section may be summarized as follows: (i) the number of Pgps on the cellular surface of MDR cells remains globally constant; (ii) due to their large transmembrane structure the lateral diffusion time of Pgp-like transporters can be considered negligible when compared to the lateral diffusion time of drugs; and (iii) the number of drugs in the membrane and the outward pumping kinetics are respectively low and fast enough, such that the probability of two drugs interacting with 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 $\rho_{Pgp} = N_{Pgp} S_{Pgp} / S_{cell}$, 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 time within the cellular membrane, a drug is expected to follow a 2D 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 a number of steps large enough (i.e.

$t_0 > h^2 / 4D$) it is possible to demonstrate that the probability of the event "drug meeting a Pgp" in the membrane, p_{Pgp}, is [90]:

$$p_{Pgp} \cong \rho_{Pgp} \pi e^{\frac{\Delta G}{k_B T}} \left(1 + e^{\frac{k}{k_0} \cdot \left(\frac{MW}{MW_c} \right)^{2/3}} \right) \Bigg/ \ln \left[e^{\frac{\Delta G}{k_B T}} \left(1 + e^{\frac{k}{k_0} \cdot \left(\frac{MW}{MW_c} \right)^{2/3}} \right) \right] \tag{6}$$

However, meeting a Pgp is not enough and Pgp-mediated drug extrusion requires the location of the drug in the inner leaflet of the plasma membrane. For that the probability that a drug enters the inner leaflet can be approached by Maxwell-Boltzmann's distribution, written as:

$$\int_{\Delta G}^{+\infty} e^{-E / k_B T} dE \Bigg/ \int_{0}^{+\infty} e^{-E / k_B T} dE = e^{-\Delta G / k_B T} \tag{7}$$

Assuming that when entering the inner leaflet and meeting a transporter a drug is extruded. The probability, $\tilde{p}_{Pgp}$, that a drug is effectively expelled from the membrane by a transporter becomes finally:

$$\tilde{p}_{Pgp} \cong e^{-\frac{\Delta G}{k_B T}} p_{Pgp} \tag{8}$$

From the later equation, it follows that a membrane-embedded drug will inevitably meet a drug transporter only if $\tilde{p}_{Pgp} = 1$. With this condition and by using Eq.6, it is thus possible to determine the critical surface density of transporters ρ_{Pgp}^c to generate MDR:

$$\rho_{Pgp}^c = 1/\pi \cdot \ln \left[e^{\frac{\Delta G}{k_B T}} \left(1 + e^{\frac{k}{k_0} \cdot \left(\frac{MW}{MW_c} \right)^{2/3}} \right) \right] \Bigg/ \left(1 + e^{\frac{k}{k_0} \cdot \left(\frac{MW}{MW_c} \right)^{2/3}} \right) \tag{9}$$

It is worth noting that when the mechanical effect becomes dominant, namely when the drug MW is large (MW>240) or when the kinetics of endocytosis is steadily increased as cells become resistant to drugs, then the surface density of Pgp-like transporters needed to trigger drug resistance is predicted to be low. Formally, this regime is best described by

$$\rho_{Pgp}^c \sim e^{-\frac{k}{k_0} \cdot \left(\frac{MW}{MW_c} \right)^{2/3}}$$

Doxorubicin with a MW of 545 can be used as an example. Assuming that the kinetics of endocytosis is increased 4-fold in the MDR state (which remains a realistic value, reviewed in [79, 91, 92]), it follows that the surface density of Pgp required would only represent ~0.1% of the cellular surface area. This means that as the kinetics of endocytosis increases then the number of P-gp transporter with a drug handling activity required decreases in an exponential way. Thus, if the main activity of P-gp is to handle drugs, then most of them would be functionally ineffective.

Given that MDR assays measure the effective dose (ED), defined as the ability of drugs to kill 50% of a cell population (that is related to drug influx), we need to model how P-gp transporter and endocytosis interact to affect drug influx.

C. Determination of the Drug Influx into MDR Cells

The ability of any classical drug to kill a cell is dependent on its ability to cross the membrane. The drug's influx is modulated both by their 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. $1-\tilde{p}_{Pgp}$ (Eq.8), divided by the drug residency time within the membrane t_0 (Eq.5), which using Eq.9 leads to:

$$r_{MDR} = \frac{e^{-\left(\frac{\Delta G}{k_B T}+\frac{k}{k_0}\cdot\left(\frac{MW}{MW_c}\right)^{2/3}\right)}}{h^2/4D}\left(1-\frac{\rho_{Pgp}}{\rho_{Pgp}^c}\right) \tag{10}$$

Assuming that the membrane diffusion coefficient remains identical between drug sensitive and resistant cells, Eq.10 can also be used to describe the escape rate of a drug from the membrane of sensitive cells, noted as $r_{non-MDR}$, by posing $\rho_{Pgp}=0$ and $k=k_0$. Thus, the ratio between escape rates in drug resistant and sensitive cells can therefore be deduced:

$$\frac{r_{MDR}}{r_{non-MDR}} \cong e^{-\left(\frac{MW}{MW_c}\right)^{2/3}\cdot\left(\frac{k}{k_0}-1\right)}\left(1-\frac{\rho_{Pgp}}{\rho_{Pgp}^c}\right) \tag{11}$$

Eq.11 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 $\rho_{Pgp}=\rho_{Pgp}^c$, the transmembrane flow of drugs can also be reduced exponentially as a function of the kinetics of endocytosis.

Finally, by taking the logarithm of Eq.11 it emerges a universal power law (i.e. 2/3) that describes the drug/membrane mechanical interaction, and is directly related to the MW of the drug used:

$$\ln\left(r_{MDR} / r_{non-MDR}\right) \cong -\left(MW / MW_c\right)^{2/3} \cdot \left(k / k_0 - 1\right) + \ln\left(1 - \rho_{Pgp} / \rho_{Pgp}^c\right) \qquad (12)$$

The right member of Eq.12 is a function of three parameters. These parameters are never measured simultaneously in MDR studies. However, what has been measured is the relationship between MDR levels and the drug MW. Eq.12 demonstrates that if the theory presented here is correct, then the ability of drugs to cross the cell membrane should follow a power law that is a function of their MW. Thus, it should be possible to test the validity of the theory proposed.

II. The Role of Drug Size in MDR

Bielder and Riehm's Seminal 1970's Study: Proof That the Membrane's Mechanical Properties Are Central to MDR

Eq.9 explains MDR when both drug properties (MW and dehydration energy) and transporter surface density are fixed. A problem immediately arises when cross resistance to drugs is taken into consideration. In this case the theoretical relationship discussed above (Eq.9) may not be verified. In particular, the surface density of transporters may not be adequate to expel another drug with different physical properties. The latter scenario would be the case if a smaller drug (i.e. a drug with a lower MW) than the one used to select the resistant phenotype is used, as it would cross the membrane more easily, which would decrease the probability of interaction between a drug and a transporter. If this scenario occurs, the theory suggests that the influx of drugs into the cytosol would be chiefly dependent on the mechanical interaction between the difference in surface tension and the MW/volume of drugs (see the exponential dependency in Eq.11).

The theory suggested in the last paragraph should be able to predict the levels of cross resistance to drugs observed; at least by giving the "power law" of the relationship between the MDR levels and the MW/volume of drugs; at best by matching published data on the subject.

The role of drug sizes involved in MDR was initially discovered in 1970 [93] and confirmed later [94]. The authors of this seminal study generated a drug resistant cell line (DC3F lung-derived cell line) and studied the concept of cross resistance. As a first step they generated a resistant cell line by selective incubation with actinomycin D (MW=1255) for several months, which led to expression of Pgp transporters [95]. As a second step they measured cross resistance levels using several other drugs (MW<1255). They looked at how both resistant and sensitive cell lines would respond to various drugs.

As they used smaller drugs, the main expected effect opposing drug entry should be driven by the drug/membrane mechanical interaction (and not transporters). In this case, the amount of a drug entering cells should follow:

$$\ln\left(r_{MDR} / r_{non-MDR}\right) \cong -\left(MW / MW_c\right)^{2/3} \cdot \left(k / k_0 - 1\right) \qquad (13)$$

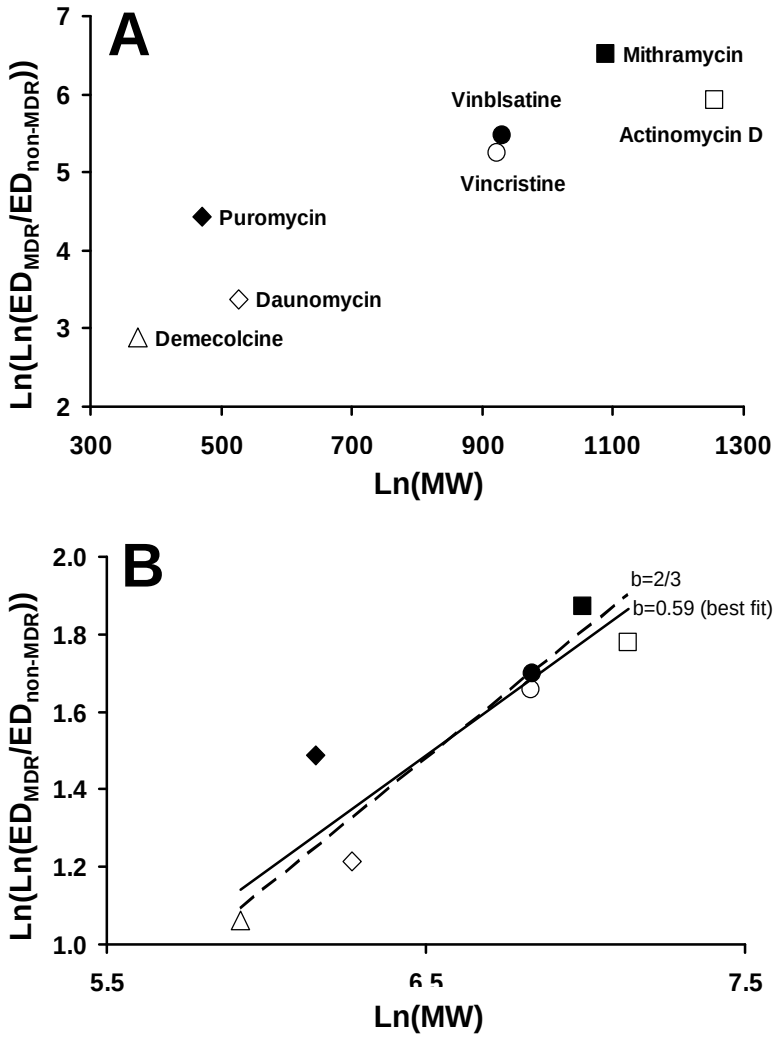

Figure 3: (A) Data from [93]. The Neperian logarithm of relative resistance levels is plotted against the MW of drugs. Drug's name is also given. (B) Same graph as in (A) where the power law predicted by Eq.14 (b=2/3, dashed curve) including the power law deduced from the best fit (b=0.59, plain curve) are plotted together and compared against the experimental data.

Given that there exists an inverse relationship between the ability of drugs to cross the membrane of cells and the dose needed to kill cells, it follows that by noting ED, the effective dose, the ratio between the EDs in drug resistant and sensitive states should be:

$$\ln\left(ED_{MDR} / ED_{non-MDR}\right) \cong \left(MW / MW_c\right)^{2/3} \cdot \left(k / k_0 - 1\right) \tag{14}$$

The numerical data from Bielder and Riehm's study are plotted in Figure 3A. In order to determine whether a power law exists, a double Neperian logarithm was applied to Bielder and Riehm's data (Figure 3A) and it was found that, indeed, a power law ~0.59±0.11 can be

determined, which is similar to the one suggested by the drug/membrane mechanical interaction ~0.66 (2/3~0.66) (Figure 3B).

Thus, we know that the drug/membrane mechanical interaction is fundamental regarding the ability of drugs to cross the membrane and kill cells. A central underpinning, in the general case, is identification of the biological mechanism responsible for the increase in the kinetics of endocytosis in MDR cells. Cytosolic alkalization seems the most likely candidate for this.

III. The Role of pH_i in MDR

A. Why Would the pH_i be an Important Factor in a Cell Membrane's Mechanical Properties?

Given the importance of pH_i in drug resistance, as drug movement across the cell membrane is affected by the differential packing of lipids, it may well be that this differential packing is governed by pH changes. To consider any effect of the pH_i on lipid packing it is central to understand the notion of packing from a physics stand point. 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 lipid 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 fraction (~30%) of the inner leaflet lipids are negatively charged, such as phosphatidylserine**,** for example [96]; a slight increase in positive ion concentration (e.g. decrease in pH_i) is expected to interact with lipids, "masking" their negative charges and decreasing the electrostatic repulsion between them. Given the size of hydrogen ions (i.e. exchangeable protons) they will have a more pronounced effect on negatively charged lipids than any other cation. As a final result, a low pH_i is more likely to be central in abolishing the physical repulsion between lipids, and thus decreases the surface tension (i.e. the lipid packing of the cytosolic leaflet. Note that both 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 [97-99]. Conversely, when the cytosolic 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 repulsions and thus their packing. Thus, this higher lipid packing would increase the surface tension of the leaflet in contact with the milieu of elevated cellular pH in the case of drug resistant cells. So, if the pH affects the packing of lipids, and the packing of lipids affects the intracellular accumulation of drugs, it follows that the cytosolic pH should affect the intracellular accumulation of those drugs.

Finally, the changes in pH_i 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.

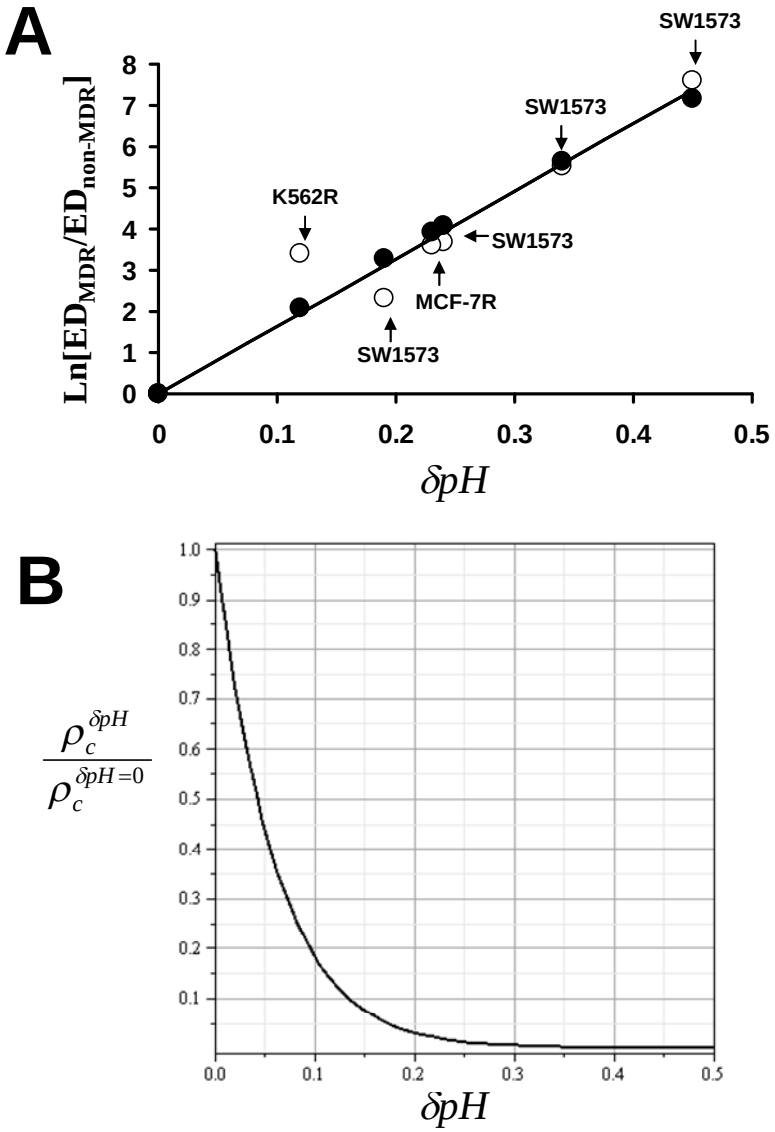

Figure 4. (A) Comparison between experimental (blanked circles) doxorubicin resistance levels obtained in cells and the theory (filled circles- Eq.16) with $\delta N / N_0 = 2.27\%$ (note that this value concerning the lipid asymmetry falls within the error margins defined previously $\delta N / N_0 = 2 \pm 0.5\%$ [84]. The open circles corresponding to SW1573 (lung derived cancer cells), K562R (leukemic cancer cells) and MCF-7R (breast derived cancer cells) are indicated with arrows and labels. Finally the straight line is the linear regression of experimental data which agrees very well with the theory. (B) Effect of pH changes (cytosolic alkalization) on the surface density of Pgp transporters needed to trigger drug resistance. As seen in the figure the number of Pgp can decrease exponentially when the pH increases: the membrane takes over.

B. Fundamental Relationship between pH and MDR Levels

Given Eq.14, it is possible to determine the effect of pH on the EDs ratio. It is possible to demonstrate that if the pH is involved, then the effective doses should be written as follows:

$$\ln\left(\frac{ED_{MDR}}{ED_{non-MDR}}\right) \cong \left(\frac{MW}{MW_c}\right)^{2/3} \cdot \left[\frac{\left(1+\left(\frac{1+\gamma}{1+10^{-\delta pH}}\right)^2\right)^{1/2}-1}{2\frac{\delta N}{N_0}}\right] - \ln\left(1-\frac{\rho_{Pgp}}{\rho_{Pgp}^c}\right) \quad \text{(Eq.15)}$$

In Eq.15, $\delta pH = pH_{MDR} - pH_{non-MDR}$ is the cytosolic pH difference between the MDR and non MDR states, and $\gamma \cong 1.5$ is a physical constant that describes the probability that a hydrogen ion interacts with a negatively charged lipid in the sensitive state.

From Eq.15, the effect of Pgp mediated drug extrusion is only a Neperian logarithm of the surface density of transporters. This indicates that this term will be dominant only if $\rho_{Pgp} \sim \rho_{Pgp}^c$ (in which case the Neperian logarithm becomes infinite). Note however that when the EDs are determined, the amount of drug added to cells is necessarily higher than the concentration of the same drug in culture condition that maintains the resistant phenotype. Therefore, full drug resistance is never reached when the effective doses are determined. It means therefore that when the effective doses are determined: $\rho_{Pgp} < \rho_{Pgp}^c$ and that in the last term can be omitted. In this condition, it follows that:

$$\ln\left(\frac{ED_{MDR}}{ED_{non-MDR}}\right) \cong \left(\frac{MW}{MW_c}\right)^{2/3} \cdot \left[\left(1+\left(\frac{1+\gamma}{1+10^{-\delta pH}}\right)^2\right)^{1/2}-1\right] \Big/ 2\frac{\delta N}{N_0} \quad \text{(Eq.16)}$$

Eq.16 can be plotted against experimental data issued from numerous studies performed in the field of cancer. Accordingly, it is possible to demonstrate the correlation between Eq.16 and the experimental data obtained with doxorubicin (Figure 4A). In turn, this suggests that the effective doses needed to kill cells are not dependent on a drug handling activity by Pgp-like transporters, but on the cytosolic pH changes only.

C. Drug Handling Versus Cytosolic pH: Relative Involvement of Each

At the cellular level, both Pgp expression levels and cytosolic alkalization are correlated with MDR levels. Figure 4A demonstrates that a drug handling activity is not needed to interpret the results. The reason can be traced back to Eq.9. It was then stated that when the kinetic of endocytosis increases, the surface density of Pgp needed to trigger full drug

resistance can decrease exponentially. This is because when the kinetic of endocytosis increase, the inner leaflet of the membrane is further packed (Figure 1A & Eq.4) and, as a result, membrane-embedded drugs will remain in the membrane for longer times and diffuse over very long distances. As they diffuse over long distances, there is no need for lots of membrane Pgps. This means that although Pgps can indeed handle drugs, there efficiency and involvement when cytosolic pH changes are taken into consideration, is likely to be low as the interaction between drug size and the membrane's mechanical properties (i.e. the packing of lipids) take over (Figure 4B).

Conclusion

In summary, drug resistance has been attributed to an increase in pH_i. Furthermore, a large variety of MDR modifiers known to be able to revert resistance to chemotherapeutic drugs have been shown to exert their cellular effects through a pH-acidifying mechanism. Conversely, the alkalinizing effect of other pH_i regulatory transporters, like the electrogenic vacuolar H^+-ATPases (V-ATPase), for example, have been shown to be involved not only in the etiology of malignant generation, but also in drug extrusion and resistance to chemotherapy.

No matter what we have learned of the MDR phenomenon in recent years, this is still a complex problem involving multiple and interrelated synergisms. In this chapter, we have summarized how drug transporters, membrane mechanical properties, and cytosolic pH can interact together. A key point that was reached is that even if a drug handling activity is involved, its efficiency is likely to be low. In this line, it is demonstrated that the pumping out of anticancer drugs from the cell, if any, would be relatively inefficient compared to the effect of pH on the membrane. Although other more complex biological mechanisms involved in drug resistance, e.g. anti-apoptotic stimuli linked to cytosolic alkalization [100] might take place, and are certainly not ruled out, the theory presented in this chapter is supported by experimental evidence.

The consistent features reviewed here are of paramount importance in any strategy directed at counteracting and overcoming MDR and, in any attempt to improve the chemotherapeutic approach to anticancer treatment. In summary, the bulk of existent evidence indicates that future attempts to override MDR, no matter what different approaches are considered, should be approached from the perspective afforded by homeostatic alterations of the hydrogen ion. This proposal comes from the fact that MDR reversion can be achieved by lowering pH_i specific to cancer cells and tissues in most, if not in all, kinds of solid tumor cells and leukemias. It is therefore very much worth considering that targeting the cytosolic pH with drugs through a concerted action of different proton transport inhibitors could help to overcome MDR in different clinical situations in oncology.

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