Physical and biological characteristics of multi drug resistance (MDR): An integral approach considering pH and drug resistance in cancer.

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Abstract

The role of the Warburg effect in cancer remains to be elucidated with a resurgence in research efforts over the past decade. Why a cancer cell would prefer to use energy inefficient glycolysis, leading to an alteration of pH both inside and outside of the cell, remains to be uncovered. The development of MDR represents a major challenge in the treatment of cancer and it is explained, so far, by the over expression of drug transporters such as the well-known and archetypal P-glycoprotein (Pgp). However, controversies exist regarding the function of Pgp in multi-drug resistance. We suggest here that Pgp-mediated MDR relies fundamentally on pH alterations mediated by the Warburg effect. Furthermore, we propose that the use of proton pump and/or transporters inhibitors (PPIs/PTIs) in cancer are key to controlling both MDR, i.e. sensitize tumors to antineoplastic agents, and drug-related adverse effects.

A lost connection between research fields

Over time, fields of scientific research gain autonomy in proportion to the extent to which they have been freed from economic necessity [1]. They develop their own laws and logics which become field-specific and very often run contrary to those in surrounding fields. They develop increasingly specialized research programmes and these can lead to great achievements. As the classical German social theorist Max Weber observed, 'only by strict specialization can the scientific worker become fully conscious, for once and perhaps never again in his lifetime, that he has achieved something that will endure' [2]. However, Weber also saw the melancholy aspect of ultra-specialization: it leads to the development of research fields that are incommensurable and between which communication is increasingly difficult. It also leads to scientists 'putting the blinkers on' in relation to developments outside their areas of expertise. For example, the somatic mutation theory of cancer together with the "war on cancer" have paved the way to great achievements in molecular biology (e.g. genome project) but their applications to medicine, i.e. oncology, remain minimal since the "magic bullet", i.e. the one gene mutated – one drug concept, that was initially promised is still missing. The constant refining process that accompanies ultra-specialization in scientific fields is comparable to that which occurred in the field of abstract art where, through a process of purification that gradually isolated it from all reference to the wider social world, it became almost entirely propelled by its own inner dialectic [3]. We see here that in its 'purified' state, a field becomes inward-looking.

The results of specialization can be seen in the sub-field of research on MDR in cancer, which suffers from an inherent fundamental paradox. As early as 1973 the drug efflux hypothesis was suggested by Dano Keld [4], which was reinforced in 1976 when Juliano and Ling discovered Pgp in multi drug resistant cells [5]. Since then many works have been carried out to understand the function of Pgp in MDR. However the single use of Pgp to explain MDR in cancer is flawed

as Pgp violates the law of enzyme affinity/specificity on which the entire field of molecular biology is built: '*MDR protein is a very unusual enzyme with extraordinarily broad substrate recognition capabilities; that is, it violates the law of enzyme specificity*' [6]. What is staggering is that even with the presence of a true scientific paradox in Pgp-mediated MDR in cancer, a range of stakeholders, whether economic and market-oriented (Big Pharma), institutional (academia, research organizations) or political (government, pressure groups), have shared (for most are still sharing) many of the same presuppositions about the problem of MDR in cancer and how it might be combatted, although rare attempts exist to suggest changes of strategy in the field of Pgp-mediated MDR [7].

Why is this so? As scientists we know it, because specialist research fields tend to engender in scientists who have been trained in the field, and are thus attuned to its logic, an implicit sense of what is the correct way of doing science and this can inhibit them from gaining insights from other fields [8].

No one would contest the existence of drug efflux mediated by membrane pumps. The question is simply that if membrane pumps exist in MDR cells, how can they work while, at the same time, violating the law of enzyme specificity? Is it really drug transporters that are important or have we overlooked essential components in multi drug resistant cancer cells?

When faced with an apparent paradox it is essential to step out from the discipline and research around how similar issues are dealt with in other fields of enquiry. Understanding the importance of pharmacokinetic / drug delivery is essential to uncover how a drug may or may not cross the bilayer membrane of MDR cells.

To explore the existing connections between MDR in cancer and other fields one will start by recalling concepts used in the field of pharmacokinetics that deals with similar barriers constituted by ATP-ase drug transporters. We shall see that in this context, the Big Pharma industry have focused on determining the optimal biophysical properties of drugs to cross those

barriers (irrespective of drug transporters). Next we shall investigate how those biophysical properties emerge by a clearer understanding of membrane physics. This will allow one to underline a number of studies that have emphasized the important role of the membrane in MDR in cancer. We shall then explain how the notion of specificity or affinity is not required as far as Pgp is involved. Finally, one will demonstrate how the Warburg effect and related changes in pH are involved in changing the membrane in such a way to sustain Pgp activity and MDR.

In conclusion we will discuss about the role of proton pump inhibitors (PPIs) and membrane-bound proton transport inhibitors (PTIs) to circumvent MDR and improve drug efficacy in cancer.

The notion of pharmacokinetics and how it can help in understanding the MDR paradox

The field of pharmacokinetics deals with how drug chemicals are dealt with by complex body systems and as a result how drug chemicals reach their targets. Defining the drug transporters that "cover" biological barriers has been essential for the success of the pharmaceutical industry. The main difference between the field of molecular oncology and pharmacokinetics is that the former works with simple systems (molecules and cells) whereas the later deals with complex body systems. Looking at how the Big Pharma has dealt with biological barriers may yield novel findings that could help to further define MDR.

The' 90s were gloomy years for the pharmaceutical industry with productivity falling below expectations and an average innovation deficit of ~1.3-1.8 for new chemical entities per year [9]. During this period these companies adopted approaches that relied on retrieval of information to determine if a chemical would make a 'likely' drug in advance of costly clinical trials. To this end, Lipinski and collaborators [10] produced a set of rules that attempted to identify the best statistical physico-chemical properties required for an oral compound to

achieve maximum bioavailability, i.e. to cross all biological barriers (where drug transporters are present) before reaching its target. The first of Lipinski's rules is based on the lipophilic index of the drug, the second on the drug's molecular weight (abbreviated "MW" in the remaining text) and the third and fourth rules concern the drug's electrostatic charge properties. These rules are now established drug discovery paradigms and have been largely embraced by the pharmaceutical industry. Of the four rules, the second (MW<500) stands out by way of its apparent simplicity, being unrelated to complex physico-chemical properties of a drug (as is the charge or lipophilic index) but governed solely by a drug's size or volume. This simplicity infers that basic mechanics apply when drugs cross membranes, cells, tissues and biological barriers.

What is worth considering are the following points: (i) The Big Pharma did not focused on drug transporters and Linpinski's rules do not mention drug transporter expression levels when barriers to drugs are considered and; (ii) the drug volume and thus some mechanical properties needs to be considered when drugs cross complex biological barriers.

The next question is why and how biophysics is involved in drug efficacy?

Why is the drug MW so important to cross barriers? An introduction to the biophysics of drug-membrane interactions.

To be (bio)available, drugs must traverse cellular barriers – usually the epithelium or endothelium (e.g. of the gastrointestinal tract, renal tubules or the blood-brain and blood-placenta barriers). To traverse cellular barriers, drugs must cross lipid membranes, and for this Lipinski's 2nd rule postulates that drugs must have a MW<500**.** Therefore, in the sum of energies making up the total activation energy required for a drug to cross cellular membranes a term must exist to underline the role of the membrane. In this case, i.e. when the plasma membrane is considered as a flat object, the physical parameter that best fits such an interaction

is the membrane leaflets' surface tension (σ and unit $[\sigma] = N/m$)[1]. Of course the surface tension parameter needs a proper definition especially in cells. All lipids are amphipathic molecules and as a result optimize their individual surface area in membrane leaflet. This optimization results from the energy balance between steric and/or electrostatic repulsion(s) (related to lipids' head) and the lipid contact with water (related to the hydrophobic aliphatic chain(s)). This balance defines the surface tension. Now**,** when a drug enters a membrane leaflet it will have to "squeeze in" and compress the lipids of the leaflet**,** namely change the surface tension. This impact on the energy balance of lipids composing the leaflet will have a tendency to repulse, i.e. push out, the drug from the leaflet. However this process is not totally rigid as otherwise chemicals would never cross membranes. In fact, lipids are not static as the thermal agitation exists which allows for some flexibility. So if a small enough chemical incorporates into the leaflet and perturbs it in such a way that the resulting membrane energy is below the ambient thermal energy, then the lipids composing the leaflets will not "feel" any difference between the thermal agitation and the incorporation of the drug. So a drug can incorporate a membrane leaflet if it is small enough.

Dimensionally speaking, it follows that a critical cross section for the drug (a_c) can be defined simply by: $a_c \sim k_B T / \sigma$, where $k_B T$ is the thermal energy (k_B is Boltzmann's constant and T the absolute temperature). If the cross section of a drug is lower than the critical value it will incorporate and cross the membrane leaflet, but if it is higher the drug will be blocked.

In bilayer membranes, two types of membrane surface tension can be distinguished, the mean surface tension noted σ_0, which corresponds to the sum of the individual leaflet's surface

[1] Thermodynamically speaking, the physical parameters that are related to spatial dimensions (namely, volume (V), cross section area (a) or line (r)) are the pressure "P": $\delta E = -P.\delta V$, the surface tension "σ": $\delta E = \sigma.\delta a$, and the tension line "γ": $\delta E = \gamma.\delta r$. "δ" is the differential operator and "E" the energy. As far as a membrane is considered, it is the surface tension (and thus the cross section area of the drug) that best describes the mechanical (i.e. physical) interaction and is deduced by posing $\delta E \sim k_B T$.

tension, and the difference in surface tensions $\Delta\sigma$, which corresponds to the difference between individual leaflet's surface tension. Using optical techniques, M. Sheetz and his collaborators have demonstrated that cells have a large reservoir of membrane [11] and an average membrane tension that is remarkably low ($\sigma_0 \sim 0.003 mN/m$) [12]. On the other hand, the difference in surface tensions between leaflets has been demonstrated to be much higher $\Delta\sigma \sim 0.9 mN/m$ [13]. Accordingly, and given the magnitude of this parameter, it is more likely to be involved in impairing the transverse movement of chemicals. The previous equation can thus be refined as follows: $a_c \sim k_B T / \Delta\sigma$. Dealing with a parameter as $\Delta\sigma$ is not intuitive and the last equation needs to be resolved physiologically. A fundamental aspect of the difference in surface tension corresponds to its role in pinocytosis associated with the role of specific lipid flippases maintaining the membrane lipid asymmetry [14]. A direct consequence associated with this asymmetry is a more highly packed inner leaflet as it contains more phospholipids than the outer leaflet resulting in the difference in surface tensions ($\Delta\sigma = \sigma_{out} - \sigma_{\text{int}} \sim 0.9 mN/m$) between the inner (cytosolic) and outer leaflets of the cell plasma membrane. Naturally, bilayer membranes are soft objects and as such, will attempt to release this stored energy. Accordingly, it has been demonstrated that lipid asymmetry corresponds to the physiological motor force that triggers membrane budding, leading to endocytosis (Figure 1) [13, 15, 16]. It is therefore possible to demonstrate that the vesicle radius is written as [13]: $R = 8k_c / h\Delta\sigma$; where k_c is the cell membrane bending modulus and h the membrane thickness. As for drugs small enough that their MW is proportional to their Van der Walls' volume (expressed in $\dot{A}^3$), i.e. $MW \sim V \sim a^{3/2}$, a critical MW (MW_c) can be determined given by:

$$MW_c = (4/3\sqrt{\pi})(hRk_B T / 8k_c)^{3/2} \qquad \text{(Eq.1)}$$

The later relation provides a law with regard to the drugs size (or MW) selectivity on their permeation across cellular membranes: $MW_c \cong 240-250$ at 37°C [17]. As the MW cut off defined by Lipinski's 2nd rule, i.e. $MW_c = 500$, describes the 90th percentile; the former value (i.e. $MW_c \cong 240-250$) is an average in line with Lipinski's rule. Two other important results follow. The first one is that it is also possible to demonstrate that the kinetics of membrane endocytosis is inversely proportional to the vesicle radius [18], i.e.:

$$k_{endo} \sim 1/R \qquad \text{(Eq.2)}$$

And that the kinetics of transverse movement across the membrane is [17]:

$$k_{Drug} \sim \exp\left(A \times MW^{\frac{2}{3}} \times k_{Endo} \right) \qquad \text{(Eq.3)}$$

Where, A, is a constant. It does seem that Lipinski's 2nd rule can be explained by considering simple biophysical arguments and that the membrane plays a key role in this process. But what about drug resistant cancer cells?

Are alterations in the cell membrane observed in MDR and is the drug MW important in multi drug resistant cells?

From what was seen above, if the drug MW is important it is because the membrane is also involved. So changes in the lipid membrane composition and membrane recycling should be expected in drug resistant cells and this seems to clearly be the case. Different **s**tudies have reported changes in membrane composition including neutral lipids, phospholipids, cholesterol and fatty acids [19-24], in some cases related to a change in the lipid metabolism of drug resistant cells [22, 25, 26]. This point has been particularly well underlined when the lipid profile of released exosomes was analysed [27]. Also**,** ultrastructure studies have revealed an increased density of small and large membrane organelles [22, 28-32] and an increase in the kinetics of membrane endocytosis or membrane recycling [29-31, 33, 34] in drug resistant

cells. It is noteworthy that the release of exosomes is also involved in MDR [35].What is perhaps more important is that the MW of a drug itself was also underlined very early (in 1970) in MDR studies in line with the role that the membrane has in delaying a chemicals influx [36, 37]. It is worth noting here that the role of a drugs MW was underlined prior to the discovery of Pgp by Juliano and Ling in 1976 [5]. The connection between membrane endocytosis and the size of a drug chemical with passive influx/uptake of drugs into cells is given by the set of equations described above.

The data points clearly to the membrane as a strong effector of drug resistance but why would the membrane be so central when drug transporting is involved in MDR?

Drug-membrane biophysical interactions to resolve the multi specificity of drug transporters

It is very often suggested that drug transporters work similarly to enzymes in line with the notion of affinity, namely that a drug needs to interact with a transporter to activate the transporter and be expelled. However this view does not work for at least three reasons when focusing on Pgp: (i) the ATP concentration in cells is usually 3-5mM that always exceeds the affinity of Pgp for ATP (K_{mATP}~0.3-1mM) [38, 39], suggesting that the transporter is always "active". (ii) Pgp ATPase activity is relatively independent of the presence of drugs [40], and the affinity of drugs toward transporters is chiefly dependent on their affinity toward the membrane [41]. Finally (iii), the apparent stoichiometry of the hypothesized ATP-coupled active drug transport, i.e. the number of ATP molecules hydrolyzed per drug transported, can be enormous (calculated to be up to ~36000ATP/drug in reconstituted proteo-liposomes) [6, 38]. This suggests that while consuming ATP Pgp does not necessarily lead to drug extrusion. Due to the fact that similar conclusions cannot be drawn for drug transporters other than Pgp due to lack of experimental observations, Pgp remains the archetypal transporter involved in

MDR and it is believed that Pgp is very likely continuously recycling between "open" and "closed" states by over-consuming ATP. This may explain why Pgp and drug resistance are so sensitive to cellular metabolism [42]. It is interesting to note that Pgp activity leads to a parallel acidification of the extracellular medium [43] that, in turn, is thought to be related to initial metastatic steps [44]. Given that the vast majority of metastatic tumours are also multi drug resistant [45], the recycling between open and closed conformations is likely to be essential to explain the *multi* of drug resistance [46].

Here comes an essential point. If Pgp switches between open and closed conformations independently of drugs, what is essential in MDR is that for drugs to be expelled they must remain in the membrane long enough to encounter (or collide with) Pgp. From Eq.3 the kinetics of drug transverse movement is modulated exponentially by two physical parameters related to the biophysical state of the membrane involving the size of the drug (see above) and the kinetics of endocytosis (see below). An increase in the kinetics of membrane endocytosis supporting Pgp function is possible if the Warburg effect and relatively high cytosolic pH are considered.

Cytosolic pH, endocytosis and MDR

Regardless of their origin and genetic background cancer cells and tissues have been found to display an abnormality called "proton reversal" which describes the state by which a cell consists of an interstitial acidic microenvironment secondary to an initial, specific and etiopathogenic intracellular alkalosis [47-53]. A failure to induce intracellular acidification and reverse this phenomenon in cancer tissues has been proposed to be the main factor underlying drug resistance including resistance to the induction of therapeutic apoptosis [54-58]. Also**,** because inner leaflet lipids bear protonable polar heads, pH changes will modify their net charge. In turn this will impact on the sum of electrostatic repulsions and modify membrane

difference in surface tension (i.e. decrease the size of pinocytic vesicles and as a result increase the kinetics of endocytosis) [59].

To consider any effect of the cytosolic pH on lipid packing it is central to understand the notion of packing from a physics standpoint. 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 repulsions that occur mostly through electrostatic effects on the polar heads, and attractions, which concern more the hydrophobic and geometric effects that take place between the aliphatic chain(s). Any changes in this balance are expected to affect the optimal area per lipid (i.e. their packing) and membrane shape. As a non-negligible fraction of the inner leaflet consists of negatively charged lipids, such as phosphatidylserine or PIP_2, for example [60] a slight increase in proton concentration around neutrality (e.g. decrease in cytosolic pH) will eliminate or shield these negative charges and decrease the electrostatic repulsion between polar groups. Although such an electrostatic counterion effect might in principle be generalized to intracellular cations, it is obvious that exchangeable protons will have a more pronounced effect on negatively charged lipids. As a final result, a low cytosolic pH 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 [61-63]. A similar result was also obtained on living cells [64]. Conversely, when the cytosolic pH increases (i.e. when cells become reliant on the Warburg effect), 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. As a result, the changes in cytosolic pH observed when cells switch their state of resistance is an important clue for understanding the observed alterations of intracellular accumulation of drugs as a function of their size. This way of thinking has permitted the theoretical corroboration of the connection between the cytosolic pH (linked to Warburg effect), the membrane biophysical properties and the MDR levels in several cell types [59] (see figure 2). The interaction between the membrane and the cytosolic pH can explain why PPIs overcome the Pgp-mediated MDR [65].

Beyond the cell membrane

Using arguments and results developed by us and others the general view is that drug sensitivity or drug resistance can only be understood if one steps outside of a Pgp-centred view to engage with a holistic approach of cancer. This true and fundamental scientific approach is equivalent of saying that what has been exposed in this review needs to be duly criticized as well to push the boundary that it creates under the form of a new research field. In the context of drug sensitivity (or drug resistance or drug refractoriness) in cancer it is essential to underline the fact that many interactions between the various cellular compartments exist that underlines the complexity of the disease that, in turn, may provide fundamental clues as to how MDR progresses. An illuminating study performed in resinless ultrathin EM sections has shown that a staggering network of interconnected cytoskeletal filaments does exist between polyribosomes, mitochondria and a myriad of unidentified small structures attached to the cytoskeleton [66]. Using the same technique, the nuclear space appears as a complex network of core filaments connecting with the nuclear lamina, and the chromosomes appear attached to spindle fibers, which are in turn interconnected through several thin filaments. None of these structures are visible using conventional resin embedding technique. This introduces the

concept that the cell has to be considered as a whole, and that this whole is not entirely known also because of the compartmentalization of the research approaches; and this is true for MDR as well. In general the membrane to cytoskeleton connection is entirely deranged in cancer cells, determining an aberrant cell polarization in turn related to the metastatic behaviour [67]. Research has been carried out showing that Pgp is linked to actin through ERM and that this connection is key for MDR in human tumor cells [68, 69]. How such interaction can be understood in the framework provided by the membrane is unclear but it underlines that fact that cells should be considered in a holistic way, also because cancer cells are independent and behave as an unicellular microorganism committed to survive in a very hostile environment [70].

Conclusion: From bench to bedside

While MDR remains linked to drug transporters, alterations in pH gradient resulting from the Warburg effect across the cell membrane or organelles is well known to impact on the biophysical properties of the cancer cell membrane sustaining drug transporter activity. Therefore it is in theory possible to improve drug uptake by cells by normalizing the pH using PPIs. This point was demonstrated recently in tumor sarcospheres [71]. Furthermore the same study demonstrated that tumor sarcospheres were becoming more sensitive to lower drug doses of anticancer agents raising hope that adverse effects linked to the administration of chemotherapy could, one day, be reduced or controlled in patients [71]. PPIs are amongst the most commonly prescribed drugs in human medicine and have gone through the process of rigorous safety testing and monitoring. Very few clinical side effects are seen even at higher doses and as such it seems easy to justify the continued investigation into the use of this class of drug for the treatment of cancer in companion animals [71-74] and humans [75-78]. They may provide an alternative or additional source of therapy to animals and humans which could

result in lower treatment costs, greater availability and safer handling compared to current cytotoxic protocols. PPIs and PTIs could potentially form part of a universal treatment which may have direct benefits in treating a number of different cancer types while combating problems associated with chemotherapy such as drug resistance, severe side-effects and even death secondary to present day chemotherapy**.**

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553 Legends

554

555 Figure 1: (A) Lipid asymmetry at the vesicular scale: Given the small size of vesicles, the radius
556 and membrane thickness are relatively close together ($R/h \sim 10$). Thus, the outer leaflet of a
557 vesicle (S_{out}) has significantly more lipids than the inner leaflet (S_{in}). As the vesicle is
558 spherical, noting $S_0 = 4\pi R^2$ the neutral surface area namely the surface area between the outer
559 and inner leaflets, it follows at the first order that $S_{out} = 4\pi(R + h/2)^2 \sim S_0(1 + h/R)$ and
560 $S_{in} = 4\pi(R - h/2)^2 \sim S_0(1 - h/R)$. Thus $S_{out} - S_{in} \sim S_0 \cdot h/R$. (B) Sketch representing the
561 current model linking fluid phase endocytosis to the membrane phospholipid number
562 asymmetry [14]. In the left panel, the translocation of dark-headed lipids into the inner leaflet
563 induces a differential packing of lipids between leaflets leading to membrane bending and
564 vesiculation [13, 15]. Note the membrane recycling that occurs in cells (right panel), i.e. the
565 exocytosis of vesicles with a size similar to endocytic vesicles, allows the maintenance of lipid
566 asymmetry and thus the maintenance of the differential packing of leaflets at the level of the
567 plasmalemma. Accordingly, the lipid number asymmetry has been experimentally deduced
568 from studies on drug sensitive cells (K562) with a value $\Delta N / N_0 = 4\%$ providing a ~35nm
569 vesicle radius [13]. (C) Representation of the different energy barriers (noted together $U(x)$)
570 and involved when a drug traverses the bilayer cellular membrane. Two leaflets have been
571 represented with an inner leaflet containing more phospholipids related to the increase in the
572 difference in surface tensions (upper graph). Energy profiles of lipid packing in both leaflet
573 (plain curve-middle graph) and hydrophobic core of membrane (dashed curve-middle graph)
574 are both involved in providing penalty energies with regard to the transbilayer movement of
575 drugs. As the inner leaflet is packed, drugs crossing the membrane will be trapped in this leaflet
576 which will delay and impair their flow into the cytosol [79]. The latter effect will be dependent

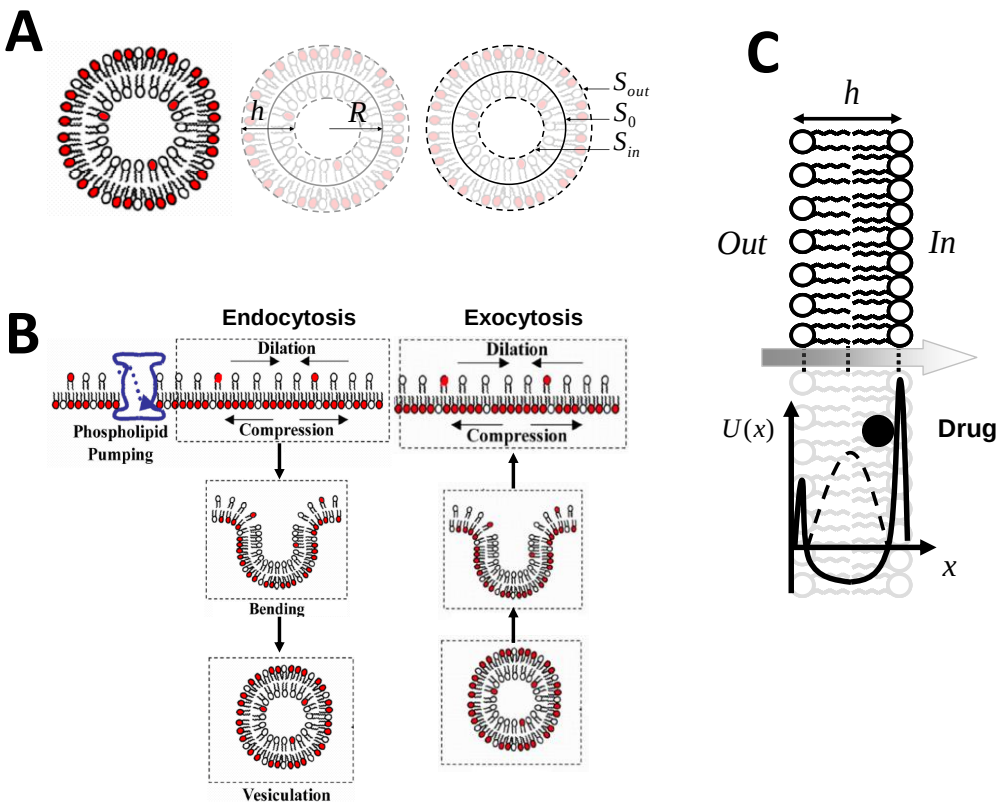
577 on the size of drugs as bigger drugs will “feel more strongly” this mechanical barrier. In the
578 present paper, this effect is supposed to be central for the high levels of cross resistance to
579 drugs.

580

581 Figure 2: (A) Comparison between experimentally measured doxorubicin resistance levels
582 obtained in cells (blanked circles) and the theory (filled circles). The open circles
583 corresponding to SW1573 (lung derived cancer cells), K562R (leukemic cancer cells) and
584 MCF-7R (breast derived cancer cells) are indicated with arrows and labels. Finally the straight
585 line is the linear regression of experimental data which agrees very well with the theory.

586 Figure 1

587



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590 Figure 2

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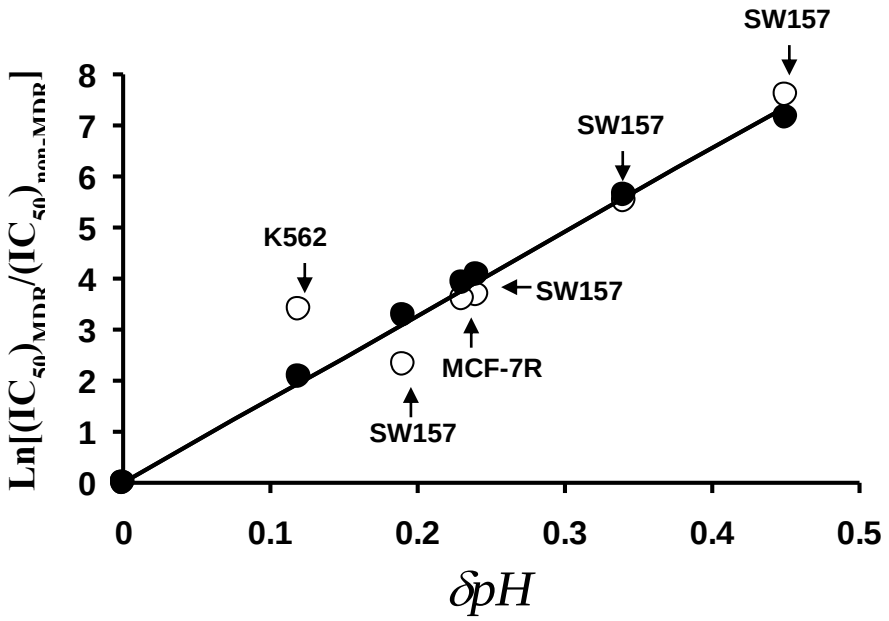

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