Review

The role of proton dynamics in the development and maintenance of multidrug resistance in cancer

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1. Multi-drug resistance (MDR)

In order to introduce the subject we will, firstly, discuss the current representation regarding MDR mediated by active membrane drug pumps and, in a second part, review the many hidden paradoxes behind the single notion of drug pumping to explain MDR. This will allow a smooth and natural introduction of other tumourregenic elements such as the pH gradient across the membrane and the alteration of the physical properties of the membrane.

1.1. MDR mediated by drug pumps

Multi-drug resistance (MDR) is characterised by the development of resistance to an anticancer drug, which is then accompanied by resistance to other structurally and pharmacokinetically unrelated drugs. Ultimately, MDR describes the failure of a diverse range of drugs to reach and/or act on their targets, which include DNA drugs. Ultimately, MDR describes the failure of a diverse range of resistance to other structurally and pharmacokinetically unrelated of resistance to an anticancer drug, which is then accompanied by resistance to other structurally and pharmacokinetically unrelated.

With a projected 382.4 per 100,000 people expected to suffer from some form of malignant neoplasm in 2015, improving treatment is an essential focus of cancer research today. Multi-drug resistance (MDR) is the leading cause of chemotherapeutic failure in the treatment of cancer, the term denoting a characteristic of the disease-causing agent to avoid damage by drugs designed to bring about their destruction. MDR is also characterised by a reversal of the pH gradient across cell membranes leading to an acidification of the outer milieu and an alkalinisation of the cytosol that is maintained by the proton pump vacuolar-type ATPase (V-ATPase) and the proton transporters: Na+/H+ exchanger (NHE1), Monocarboxylate Transporters (MCTs), Carbonic anhydrases (CAS) (mainly CA-IX), adenosinetriphosphate synthase, Na+/HCO3 −/HCO3 −co-transporter and the Cl−/HCO3 −exchanger. This review aims to give an introduction to MDR. It will begin with an explanation for what MDR actually is and go on to look at the proposed mechanisms by which a state of drug resistance is achieved. The role of proton-pumps in creating an acidic extracellular pH and alkaline cytosol, as well as key biomechanical processes within the cell membrane itself, will be used to explain how drug resistance can be sustained.

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ABSTRACT

With a projected 382.4 per 100,000 people expected to suffer from some form of malignant neoplasm in 2015, improving treatment is an essential focus of cancer research today. Multi-drug resistance (MDR) is the leading cause of chemotherapeutic failure in the treatment of cancer, the term denoting a characteristic of the disease-causing agent to avoid damage by drugs designed to bring about their destruction. MDR is also characterised by a reversal of the pH gradient across cell membranes leading to an acidification of the outer milieu and an alkalinisation of the cytosol that is maintained by the proton pump vacuolar-type ATPase (V-ATPase) and the proton transporters: Na+/H+ exchanger (NHE1), Monocarboxylate Transporters (MCTs), Carbonic anhydrases (CAS) (mainly CA-IX), adenosinetriphosphate synthase, Na+/HCO3 −/HCO3 −co-transporter and the Cl−/HCO3 −exchanger. This review aims to give an introduction to MDR. It will begin with an explanation for what MDR actually is and go on to look at the proposed mechanisms by which a state of drug resistance is achieved. The role of proton-pumps in creating an acidic extracellular pH and alkaline cytosol, as well as key biomechanical processes within the cell membrane itself, will be used to explain how drug resistance can be sustained.

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drugs'. Although the evidence for the presence and role of drug efflux pumps in many cases of MDR is irrefutable, it has been shown that an intracellular alkaline shift alone is sufficient for the failure of accumulation of intracellular chemotherapeutic agents within the appropriate compartments of cells [15] accompanied by increased drug efflux and decreased cytosolic accumulation [16]. These data have resulted in a shift of the concepts used and related therapeutic goal to combine targeting both Pgp function and pH changes in cancer. In addition, the introduction of pH in MDR has opened the door to new “synthetic theories” aiming at understanding MDR as a whole and not focused only on Pgp-like drug transporters.

A quick review of paradoxes behind the single use of Pgp theory in MDR will now highlight the need for new synthetic theories involving other tumourogenic parameters, especially the pH.

1.2. Paradox one: the drug-pumped-to-ATP-consumed ratio

It was in 1973 that Dano Keld suggested that the mechanism of resistance was due to an outward efflux [17]. This hypothesis clearly gained credance when three years later P-glycoprotein (Pgp) was identified by Juliano and Ling as the membrane protein over-expressed in MDR cancer cells that actively extrude membrane amphiphatic drugs [18]. Since then many biological, biochemical and structural studies have been carried out on this family of ABC transporters. To summarize, a conformational change in the structure of Pgp upon ATP binding allows access from the lipid bilayer inner leaflet to the internal cavity of volume ~6000 Å³ [19–23]. Drug binding to Pgp is more sensitive to ATP binding rather than hydrolysis, and two ATP molecules need to be bound on Pgp to allow its full activation [23–28]. The use of crystallography methods and basic biology found that the turnover rate of Pgp ATPase is in the range of ~1–15 ATP/s [29–31] with a near stoichiometric substrate transport to ATP hydrolysis ~2ATP/drug, reviewed in [32].

At the molecular level everything sounds fine but what remains unclear however is the low efficacy of Pgp in reconstituted systems. The apparent stoichiometry of the hypothesised ATP coupled active drug transport, i.e. the number of ATP molecules hydrolysed per drug transported, can be enormous (calculated to be up to ~36,000 ATP/drug in reconstituted proteo-liposomes) [33–35]. This suggests that whilst consuming ATP, Pgp does not necessarily lead to drug extrusion. It seems therefore that Pgp-like transporters oscillate between open and close conformations without involving and transporting drugs. Although the history of biology (and evolution in particular) taught us that biological systems do not need to be fully efficient to keep their robustness, it is notable nonetheless that if Pgp was inefficient MDR would not be a problem in clinical oncology. Two paths are now available, either Pgp and relatives are not involved in MDR at all (that is unlikely to be the case) or something else must help Pgp and relatives to gain enough efficacy for MDR to be noticeable by clinicians.

1.3. Paradox three: the role of drug molecular weight (MW) in drug resistance

Today, it is suggested that the ability of many drugs to bind the internal cavity of Pgp is linked to the number of potential binding sites available on the wall of the internal cavity composed of hydrophobic, aromatic, polar and charged amino acid residues [19]. Althoht the later statement is sound from a biochemical point of view, it is important to note that the MW of drugs (namely their size or volume) is known to be a strong predictor of MDR levels in Pgp expressing cells [36–38]. This point was first demonstrated in 1970 [36]. The date is important here as this seminal study on drug resistance comes three years before Dano Keld’s “vacuum cleaner” hypothesis [18]. And six years before the discovery of Pgp [18]. So albeit the notion of drug pumping was inexistant at the time (1970), the drug MW was the main parameter describing MDR then. Why this type work based on drugs MW was not carried forward is not clear but what is remarkable however, is that decades later the pharmaceutical industry discovered that the MW of drugs is indeed paramount for their systemic delivery (bioavailability) and largely responsible for attrition [39]. From the pharmaceutical point of view, the bioavailability of a drug depends also on its ability to cross the multiple membrane layers present in a body (i.e. cells) and, accordingly, it was demonstrated that lipid bilayer membranes do indeed play a fundamental role in drug bioavailability based on their MW [37,40]. The fundamental reason behind this is related to the biomechanical interaction between the drug volume and the surface tension of the cell membrane namely the physical packing of lipids in either leaflet of the cellular membrane (controlled by cells themselves).

So maybe without noticing it, Bielder and Riemi discovered in 1970 [36] a fundamental Law in basic drug delivery [37,40].

1.4. Paradox three: the lack of specificity

As stated by the term used namely “multi drug resistance”, a single transporter should be able to transport many different drugs not related structurally and chemically. Although the molecular model of Pgp has permitted a relatively simple representation of MDR in agreement with the usual concepts issued from the field of biochemistry, a single protein can expel structurally different drugs is still poorly understood. Indeed, “controversy remains over how P-gp recognizes hundreds of different hydrophobic drugs and pump them out of the cell...” [41]. Beyond this last remark, there is something far more significant and important at stake: the Pgp-mediated MDR model does not conform to the fundamental notion of specificity and seems to challenge the roots of biochemistry. This conceptual issue was exposed early and very clearly by Paul Roepe: “…MDR cells are resistant to, and/or exhibit decreased retention of, literally hundreds of different hydrophobic compounds that are structurally divergent... Membrane transporters, like soluble enzymes, are exquisitely substrate-specific... If transporters were not specific, the cell would eventually become a high entropy chaotic mess...[as there are] no structural molecular motifs common to all the many different agents to which MDR cells are resistant... MDR protein is a very unusual enzyme with extraordinarily broad substrate recognition capabilities; that is, it violates the law of enzyme specificity” [35]. Given the paramount importance of the notions of “specificity” or “affinity” in classical biochemistry there was an obvious need to redefine Pgp efficiency.

It is common to define the binding-affinity as the likelihood of drug and transporter interacting upon meeting and in this case the interaction energy becomes a fundamental variable. However there exist chemical reactions that are relatively inefficient and one way to increase the rate of products formed is to raise the temperature. By doing so it is not the interaction energy that is affected but the rate of collisions between chemicals that is increased. By increasing collision rates the chance of a product being formed increase as well.1 Random processes have been studied for more than a century, and it is now well established that the mathematical properties of Brownian diffusions are fully dependent on the dimensions of space. In particular, there is one theorem, known as Polya’s Theorem, that states that portions of space are always left unvisited (whatever the visitation time considered) if the Brownian particle diffuses in dimensions higher than 2 and that, conversely, in dimensions smaller than or equal to 2, all the space will be visited possibly more than one time over a long enough period of time, reviewed in [42]. Recalling that the MW of drugs is important and involved in their residency time in membrane (of course function of the membrane physical properties), the larger the drug the better to improve Pgp-mediated diffusion.

1 Likewise, one has more chance of winning the national lottery if we buy more than one ticket.
drug pumping efficiency, especially if Pgp-like transporters oscillate between open and close conformations (paradox one). In this condition, the diffusion of drugs in the membrane warrants the ability of drugs and transporters to interact without the use of interaction energy (i.e. drug-Pgp affinity).

Altogether the points above highlight the fact that albeit Pgp and relatives are responsible for drug resistance levels they cannot do it alone and that, the membrane is very likely involved in this process. The next point to clarify is to find the parameters allowing the physical changes necessary to impact on the membrane to sustain Pgp function (i.e. drug pumping). For this it is central to review the role of proton dynamic and pH in cancer.

### 2. The role of pH in cancer progression and the related Warburg’s hypothesis

Central to MDR research is being able to understand the physiology of cells and how this differs in cancer. A key event, cause or consequence, in the transformation of normal cells into cancerous cells was discovered by Otto Warburg in 1924. His observations described the switching of cellular respiration to glycolysis, even under aerobic conditions, and this was further discovered to be at least partly as a result of the loss of mitochondrial inhibition of glycolysis [43].

Despite the relative inefficiency of glycolysis (Fig. 1) and the increased metabolic demand for adenosinetriphosphate (ATP), cancerous tumours still undergo this switch to glycolysis.

In 1956, Otto Warburg identified a shift in pH surrounding cancer cells that was found later to be related to an up-regulation of proton exchange mechanisms across the membrane, themselves related to glycolysis. It is now well established that, in cancer cells, the alkalinisation of intracellular pH (pHi) is accompanied by acidicosis. It is now well established that, in cancer cells, the alkalinisation of intracellular pH (pHi) is accompanied by acidicosis. Further studies have concurred that one of the defining characteristics of the tumour microenvironment is the reversal of the pH gradient across cell membranes [46,59].

A low pH, together with hypoxia, results in loss of apoptotic control through activation of intracellular pathways; for example, the extracellular-signal-related kinase (ERK1/2). This pathway is critical to proliferation, transformation, tumourigenicity, invasion, angiogenesis, differentiation and survival [49], and is controlled by the oncoproteins Ras and Raf (Fig. 2). Upon activation through phosphorylation, ERK1/2 translocates to the cell nucleus in order to phosphorylate its gene targets. Acidic conditions have been shown to upregulate the phosphorylation of ERK 1/2 [50], and the expression of ERK1/2 in mucopidermoid carcinoma correlates with both the aggression of tumours and the overall clinical outcome [51]. It is also worth mentioning that additional studies have demonstrated a role for Bcl-2 and GPR65 as mechanisms of resistance to apoptosis mediated by pH [52].

A major consequence of up-regulated glycolysis is the increased production of metabolic acids responsible for the presence of acidic areas within solid tumours. Autophagy is a cellular catabolic pathway leading to lysosomal degradation and recycling of proteins and organelles. Studies have demonstrated that induction of autophagy may represent another adaptation mechanism for cancer cells exposed to an acidic environment [53,54].

In addition to activation of ERK 1/2, the hypoxic and acidic tumoural environment also leads to the increased release and activation of acidic proteases such as cathepsin B [55,56], MMP2 [57] and MMP9 [58], which are overly expressed in tumour ECM, contributing to the degradation of the extracellular matrix, thus promoting invasion and metastasis [59]. Furthermore, acid- and hypoxia-induced up-regulation of ERK 1/2 increases vascular endothelial growth factor (VEGF) expression through hypoxia-inducible factor 1 (HIF-1), thereby promoting angiogenesis [60–63], which is then permissive to further growth and metastasis.

Further studies have concurred that one of the defining characteristics of the tumour microenvironment is the reversal of the pH gradient across cell membranes [46,59]. This aberrant intratumoural pH gradient can also be accredited, in part, to two main events: (i) the intracellular proton overproduction following the dissociation of lactic acid into lactate and hydrogen ions, as end-products of the up-regulated glycolytic pathway and; (ii) the over-expression or over-activity of proton exchange or acid extrusion mechanisms [44,64,65]. This gradient is exaggerated in metastases and increases
with disease progression [66]. Nowadays, the reversed proton gradient of all malignant tumours is emerging as one of the most significant and selective hallmarks of cancer [48]. See Fig. 3 for a schematic overview of these relationships.

3. The role of proton pumps and transporters in maintaining a reversed pH gradient in tumours

Accumulation of intracellular acidity is clearly intolerable to cancer cells and must be counteracted by the use of a detoxification mechanism [67] as there is clear evidence that inhibiting proton pumps leads to an early intracellular acidification and accumulation of toxic chemicals (e.g. ROS) followed by caspase activation in different tumour histologies [68,69]. This is achieved by proton pumps and transporters which are found both in the lipid bilayer of the external cell membrane and in intracellular compartmental membranes. These pumps/transporters, through various mechanisms, have the net effect of externalising protons, ultimately leading to a drop in the pH of the extracellular fluid (ECF) within the tumour (Fig. 3). It is however important to underline that the type of acid extrusion is different between the direct transport of proton from the cytosol across the plasmalemma and the accumulation of proton from the cytosol inside lysosomes to saturating levels followed by the exocytosis of their contents via the endosomal/lysosomal trafficking system. This review focuses on the proton pump V-ATPase and the proton transporters NHE1 and MCTs and the HCO3⁻ transporters.

3.1. V-ATPase

Vacuolar type H⁺-ATPases (V-ATPases) have been identified as mediators of the acidic microenvironment of tumours [70] and as playing a key role in the MDR phenotype [71]. The up-regulation of the pump and its associated increased intracellular alkalinisation has also been implicated in cell transformation and cisplatin resistance [72]. V-ATPases are expressed in the plasma membrane [73] and membranes of intracellular vacuoles [67,74], such as lysosomes and endosomes, resulting in their acidification [75]. The sequestration and inactivation of chemotherapeutic agents in these acidic, endocytotic compartments and their subsequent extrusion from the cell is believed to contribute to resistance, in addition to the drug efflux mechanisms mentioned above. Pérez-Sayáns concluded that induced expression of V-ATPases in MDR is an anti-apoptotic defense: the up-regulation of V-ATPases in most human tumour cells [67,72,76] together with their fundamental role in cellular detoxification [67], suggests that these pumps might present a viable therapeutic target in the overcoming of MDR.

3.2. NHE1

The membrane-bound NHE1 transporter is a sodium/hydrogen exchanger present at the surface of most cells where it has a central role in cellular volume and pH homeostasis [75,77–80]. Hypoxic tumour conditions have been shown to mediate the activity and expression of NHE1 through up-regulation of the transcriptional regulator, hypoxia-inducible factor 1, (HIF-1) [81] and the exchanger has been cited as the primary mediator of acidic tumoural ECF [57,59,75]. Recent studies have also defined an additional role for NHE1, localised at the leading edge of pseudopodia and lamellipodia of cancer cells involved in tissue invasion [59,75]. This process is mediated through activation of phosphoinositide-3-kinase (PI3K) [82] and p38 mitogen-activated protein kinase (p38MAPK) [83] pathways. It is postulated that the extrusion of protons by NHE1 results in proteolysis and degradation of...
the local extracellular matrix (ECM) through activation of acidic proteolytic enzymes, thus facilitating subsequent invasion, [83–85] and metastasis [86]. Indeed, recent work has demonstrated that NHE1 is localised at the invasive structure of aggressive cancer cells, called invadopodia, and is necessary for both their formation and proteolytic activity; [87,88] and that its activity can be stimulated by hypoxia [89]. In support of this idea of the importance of NHE1 in invasion is the recent paper looking at the distribution of proton transporter protein in tissue sections of rat brain C6 gliomas where the NHE1 was most heavily expressed at the invasive edge of the tumour [90].

Direct inhibition of NHE1 or V-ATPases, which are believed to mediate the malignant ΔpH, has been identified as giving rise to cytostasis and/or cytotoxicity. For example, whilst inhibition of NHE1 has been shown to have cytostatic effects in malignant glioma [91], hepatocellular carcinoma cells [57] and breast cancer cells [92]; V-ATPases inhibition gives rise to cytostasis and cytotoxicity in oral squamous cell carcinoma [93], B cell lymphomas [94], doxorubicin-resistant renal cell carcinoma [95], breast carcinoma [96], hepatoblastoma [76] and pancreatic cancer [97,98] cells. Whilst cytostasis is obviously encouraging in cancer therapy, reflecting a lack of disease progression, cytotoxicity is regarded as more desirable as it characterises regression. Based on these findings, NHE1 and V-ATPase represent potentially valuable and specific targets for the mediation of disease progression and metastasis. Further studies, in which resistant cells and tumours have been treated with inhibitors of these pumps, prior to or together with a chemotherapeutic agent, have yielded encouraging results in the reversal of tumour resistance [14,99–102] and NHE1 inhibition has been found to augment paclitaxel [103], imatinib [104], doxorubicin [105] and cisplatin sensitivity in breast cancer cells [106].

3.3. MCTs

There are a total of 14 MCTs; encoded by the solute carrier family (SLC) 16 [107]. MCT1–4 are proton symporters involved in the transmembrane transport of lactate, pyruvate and ketone bodies. The up-regulation of factors mediating hypoxic stress such as HIF-1α is a well documented phenomenon in the cancerous tumour environment [108–113]. It has been demonstrated that HIF-1α increases the expression of MCT4 [114], an important pH regulator. MCT4 not only brings about the expulsion of hydrogen ions, in an attempt to decrease pHe, but is also involved in the removal of lactate from the intracellular cytosol, allowing continuous conversion of glucose to lactate. The role of MCT1 in cancer cell invasiveness could be explained by the extracellular expulsion of lactate and hydrogen ions [115], leading to vascular incursion. The importance of MCTs in cancer cell survival has been indicated in studies showing that inhibition of MCT1, both in vitro [116–118] and in vivo [118], resulted in a decrease in pHi and retarded tumour growth respectively. Whilst much less is known about the role of MCT in tumour cells, compared to NHE1 and V-ATPases, recent research suggests that it has a major role in cell survival in the hyper-glycolytic and acidic conditions brought on by a hypoxic tumoural environment.

3.4. HCO₃⁻ transporters

HCO₃⁻ transporters facilitate the transverse movement of HCO₃⁻ ions that is too hydrophobic to cross lipids membrane otherwise. Doing so, the drop in pHi associated with the metabolism of cancer cells can be buffered into the release of CO₂ and H₂O but also changes in the membrane potential of the cells. Two classes of HCO₃⁻ transporters are fundamental to drive this reaction namely the Cl⁻/HCO₃⁻ exchangers (AEs) and the NBCs family of Na⁺/HCO₃⁻ co-transporters. The function of some AEs involves also the carbonic anhydrase isofoms (CAs) to which they form complex with known as metabolon [119], this has been demonstrated for the AE1-CAZ metabolon [120]. Altogether they allow a net increase in pH. The NBC family of co-transporters is composed of electrogenic (i.e. 3:1 or 2:1 HCO₃⁻:Na⁺ stochiometry) and electroneutral transporters. Their interaction with membrane of the
CAs family is thought to be fundamental to transport efficiently \( \text{HCO}_3^- \) [121]. A role for anion exchangers and related pH metabolism in multidrug resistance was first demonstrated by Paul Roepe in 1997 [122].

4. MDR and pH

As stated above, disease progression and metastases are associated with an exaggerated pH gradient reversal, notably an elevated internal pH (pHi). This gradient reversal has, in turn, been shown to correlate with the development of MDR [15,46,72,123]. This leads to the conclusion that the reversed pHe-pHi gradient somehow interferes with the passage of drugs across the lipid bilayer of cells. Such a supposition seems plausible for the many anti-cancer drugs that are weak bases (such as doxorubicin and mitoxantrone), which are neutralised and inactivated by protonation in the acidic microenvironment surrounding the cells which they are intended to penetrate, or sequestered in intracellular acidic vesicles or endosomes [15,102]. However weak acids and water-soluble molecules (such as methotrexate and 5-FU, respectively) for which the malignant acid-base status ought not to be so problematic, still encounter considerable barriers in poorly-vascularised, solid tumours in the form of polyglutamation and malperfusion [124]. A fundamental question remains: how the pH alteration could also be involved in triggering the MDR state bypassing all the electrochemical properties of drugs and membrane?

All anti-cancer drugs aimed at intracellularly targets will have to cross the cell membrane. Therefore, understanding the biochemical and biomechanical interactions of drugs with the membrane including how they penetrate, accumulate or are extruded represents an important focus in developing future chemotherapy treatments. Up-to-now, we have discussed the role of proton pumps in MDR. For the next part of the review, the biomechanical properties of the cell membrane itself will be examined; it will then become clear that the membrane itself is involved in the MDR state.

5. Fluid mosaic model and MDR

Current MDR theories concerning interactions between drugs and the membrane utilise the ‘fluid mosaic model’ [125] to explain the interaction between drug and cell. It is widely accepted that the physical and chemical properties of the phospholipid bilayer with a hydrophilic outer and hydrophobic inner make a selectively permeable barrier facilitating passive and energy-utilising transport across it [125]. The apical and basolateral membranes contain intrinsic and extrinsic structures which, together with the membrane, are thought to be managed via endocytosis and exocytosis in a process called ‘membrane trafficking’, in which membrane components are regularly renewed and replaced [126]. A number of membrane properties are of interest in MDR including the fluidity and associated lipid density of the membrane. Whilst lipids cooperate with one another via non-covalent interaction of their hydrophobic tails and hydrophilic heads, the overall fluidity of the membrane can be adjusted by the level of saturation of the phospholipid molecules [126]. Where a high density of saturated molecules occurs together with cholesterol and often sphingolipids, distinct micro-domains or ‘lipid rafts’ of highly stiffened regions on the membrane may form [127]. Another important element of the fluid mosaic model in MDR is the concept of lateral mobility of membrane components via interactions between the polar and non-polar regions of the proteins and lipids. This lateral heterogeneity in lipid bilayers has been termed “liquid-ordered micro-domains” and indicates regions with differing chemical and physical properties to other areas of the membrane such as decreased fluidity in lipid raft regions [126]. MDR research utilises the complexity of these systems and the associated intrinsic and extrinsic proteins to interpret the process of drug interaction with the cell.

6. Biomechanics in MDR

The relevance of biomechanics in MDR research relates to the ability of the drug to negotiate the membrane and includes the fluidity, relative lipid density and surface tension of each of the leaflets within the membrane [128,129]. For example, it has been suggested that ‘an excess of packing of lipid in the inner membrane of MDR cells is responsible for blocking drugs mechanically as a function of their sizes at the membrane level’ [37,40,130]. Management of the membrane is facilitated via the processes of endocytosis and exocytosis and it has been shown that rates of endocytosis in MDR cells are higher than in sensitive parent cells [131]. A fundamental concept of membrane biomechanics is the relationship between endocytosis and the differential packing of lipids between membrane leaflets. Conditions of increased endocytosis have been associated with higher levels of endogenously compression of the inner leaflet of the membrane where mechanical packing of lipids in the inner leaflet has been found to drive endocytosis [132]. When vesicles are formed the surface area of the outer leaflet is larger than that of the inner leaflet and therefore the outer leaflet must contain a larger number of lipids than the inner leaflet (Fig. 4A). During the budding stages of endocytosis, the outer leaflet of the vesicle is formed by the inner leaflet of the membrane and, therefore, the inner leaflet of the membrane must contain a proportionately higher density of lipids [130,37] (Fig. 4B).

In this respect, MDR cells have been found to have a two- to ten-fold increase in their kinetic rate of endocytosis which would suggest that they have a much higher lipid density within the inner leaflet of their membrane compared to drug-sensitive cells [40]. There are two types of endocytosis distinguishable in cells including ‘receptor mediated endocytosis’ (RME) and ‘fluid phase endocytosis’ (FPE). RME utilises a form of membrane coating such as clathrin that creates pits or bends in the membrane resulting in vesicles surrounded by a membrane coating [133]. Cytokines and growth factors utilise RME to mediate signalling pathways [134]. FPE is a more passive process with no receptor mediated interactions prior to vesicle formation [135]. FPE vesicles are continuously formed and have been linked with membrane recycling [132]. FPE requires there to be a degree of asymmetry in the phospholipid number between the inner and outer leaflets of the membrane as this asymmetry provides the mechanical moment required to generate membrane curvature and budding [136]. This asymmetry is thought to be mediated by transmembrane proteins called ‘flipases’ [137] which have been also highlighted as being important in potential Pgp mechanisms of action. Rauch and colleagues have hypothesised that the altered endocytosis kinetics seen in drug resistant cells with resultant increases in the difference in surface tensions between membrane leaflets will affect the mechanical interaction between the drug and the membrane. This concept suggests there is a critical cross-sectional area of drug size beyond which the differences in surface tension on the membrane will affect the transit time of the drug through the membrane. Similarly, drugs with different physical properties such as high molecular weight (as it relates to the drug size) will be affected by cellular MDR changes in different ways. Conversely, a low molecular weight will be less affected by lower membrane fluidity and be able to cross the membrane more easily.

There is a double advantage in increasing the mechanical packing of lipids within the inner membrane: not only will this increase the ‘trapping’ of drugs within the inner leaflet of the membrane (as described in Fig. 4C) and therefore increase the residency time of the drug within the membrane but also, it will allow a full explanation of the ‘vacuum cleaner’ model mediated by drug transporters to

\[ \text{The “vacuum cleaner” model was first put forward in 1972 to explain why drugs do not diffuse into cells that are resistant to drugs. It was then suggest that cells “vacuum clean” drugs. It is remarkable that such elusive hypothesis stood up time until recently.} \]
take place (see next paragraph). Indeed, the longer residency time of the drug within the inner leaflet increases the probability that via lateral diffusion the drug will contact one of the multi-drug transporters and be removed from the membrane (Fig. 5). As a result, membrane changes in the specific way described are not only necessary but entangled with the notion of multidrug resistance.

Therefore, inclusion of the biophysical properties of the membrane into MDR theory does have the potential to build on the more classical theories of MDR and make them closer to actual observations.

7. Membrane biomechanics and ‘Pgp’

Biomechanics provides an additional argument as to why cells can become resistant to multiple drugs rather than just the drug to which they are being exposed and in this way can help explain the link of MDR to the non-specificity of Pgp transporters. Fig. 4C shows that all drugs will experience some degree of impairment by the membrane, which is non-specific and thus represents a potential mechanism of action that acts in a totally non-specific manner. In the case where drugs have a very small molecular weight and are not seriously impeded by the membrane, the probability of lateral diffusion of the drug to meet a transport protein decreases due to the decreased residence time of the drug within the inner leaflet [40]. Where this scenario occurs, Rauch [40] suggests that levels of cross-resistance would then become solely a function of the mechanical packing of the lipid and the molecular weight of the drug, lessening any affect of multidrug transporters such as Pgp.
The role of proton pump inhibitors and the cell membrane have so far been discussed independently of one another. The next step is to merge MDR, pH changes as observed and measured in cancer and the physical biology of the cell membrane. This will help to better understand both how the altered pH to pHi gradient in tumours alters the membrane dynamics and how the action of proton transporter/pump inhibitors can result in a blocking of MDR through the re-establishment of ‘normal’ membrane lipid dynamics.

8. Lipid density theory and pH interaction

It has been hypothesised that the resistance attributed to proton-pump associated ΔpH reversal may also be mediated through the biophysical properties and packaging of membrane phospholipids [37]. The individual phospholipids of a lipid bi-layer such as phosphatidylserine, which is found predominantly in the inner membrane leaflet [138–140], carry a negative charge in their polar region (see Fig. 6). As positive charges will therefore be electrochemically attracted to the region, any free protons will accumulate in close vicinity to the membrane. In drug-sensitive cells, this effective neutralising of the membrane nanoenvironment will lead to decreased electrostatic repulsion between the polar groups of the phospholipids, thus optimising the membrane fluidity for successful drug penetration. It therefore follows that in a resistant cell, the up-regulation of proton pumps will lead to depletion of the intracellular supply of protons, such that the inner membrane will continue to carry a negative charge, thus permitting increased repulsion and tighter packing of membrane phospholipids – effectively ‘stiffening’ the membrane – which, in turn, decreases permeability to chemotherapeutic drugs [37]. As a result, a pH difference of about 0.2 units is enough to block classical chemotherapy drugs with a molecular weight around 500 (Fig. 7A) [37,40]. Moreover, the rate of fluid-phase endocytosis will increase as a result of increased membrane lipid packing forcing infolding of the membrane. This will lead to the endocytosis of the previously blocked and/or effluxed drugs, which are sequestered into the aforementioned intracellular vesicles [37,141]. This postulated mechanism is supported by the decreased cytotoxic effects of doxorubicin ([142,143], mitoxantrone [144] and vincristine [145] in the acidic environment of tumours [146].

9. pH and APOPTOSIS with or without drugs

Whereas the precise role of pH in apoptosis has yet to be clarified, it has been discovered that intracellular acidification is key to the cellular process of programmed cell death. Whether intracellular acidification is the trigger or simply an amplifier of apoptosis is still unknown. However, pH changes are paramount to drug resistance. It therefore follows that an alkaline intracellular pH can promote cancer cell survival. As discussed above, alkaline pH can be brought about via the activation of ion carrier (or exchanger) in the cell membrane, for example the Na+/H+ exchanger (NHE1). In cancer cells upregulation of this transporter has been recognised as a mechanism for extruding hydrogen ions and, therefore, creating an alkaline pHi.

Fig. 6. Illustration of the lipid-packing theory. (A) Drug-sensitive cell, showing theoretical ‘neutralisation’ of the intracellular membrane microenvironment, through electrochemical attraction of protons to negatively-charged phospholipids such as phosphatidylserine. This results in decreased electrostatic repulsive forces, maintaining membrane fluidity and potentially altering drug permeability. (B) Drug-resistant cell, in which the externalisation of protons results in increased electrostatic repulsion between polar regions of phospholipids. This effectuates a ‘stiffening’ of the membrane, which renders the passage of drugs more problematic. For a more detailed schematic of the biophysical forces acting on and within the membrane, see Rauch (2009).
The NHE1 has been recognised as a potential therapeutic target in anti-cancer therapies [46,147]. Downregulation of this transporter, or alteration of its activity, results in the accumulation of hydrogen ions within the cells (from the dissociation of lactate), which leads to intracellular acidification and contribute to cell death via apoptosis [46,147,148].

The Warburg effect, as previously discussed, describes a switch in metabolism from oxidative phosphorylation to glycolysis in cancerous cells. Reversal of this characteristic change in cancer cells could understandably be believed to cause apoptosis in these cells. Several mechanisms for the reversal of Warburg’s effect have been proposed; for example, the use of 2-Deoxyglucose which inhibits the action of hexokinase, a rate-limiting enzyme in glycolysis [149]. Inhibition of glycolysis reduces the amount of ATP energy within the cell and, therefore, stimulates AMP-activated protein kinase, which in turn promotes cell death via apoptosis. Inhibition of glycolysis can also be brought about by reducing the pH as this downregulates the activity of hexokinase, pyruvate kinase and phosphofructokinase — all key enzymes which promote glycolysis [149]. A reduction in glycolysis has been suggested to affect the pentose pathway and, therefore, affect nucleic acid production in cancerous cells leading to apoptosis.

Finally, as seen the pH is also expected to be more than a reasonable target for any chemotherapeutic treatment whatever the physical-chemical properties of the drugs.

10. Conclusion: when science and societal needs converge

This review nails the current potential interrelated mechanisms of physiological pH alteration and MDR in cancerous cells (Fig. 7B summarizes some of inter-relationships involved in transformation, and tumourigenesis that plays a critical role in MDR). MDR has been defined, and the indirect role of the proton pumps/transporters NHE1, V-ATPase and MCTs have been examined. In addition, the
biomechanical properties of the cell membrane have been evaluated to provide the theory behind the development of a drug-resistant state.

All together, the works carried out in the field of cancer and MDR over the last 20 years points towards the pH regulatory mechanisms as a unique target to affect tumour development or survival. Provided the lack of effective cancer treatments, or engineered drugs (namely “magic bullet” awaited for too long now and often too expensive), wouldn’t it be more productive as far as human lives are concerned to concentrate on simple, generic and cheap compounds that are used on a daily basis to regulate pH? Let us think about well known and highly efficient molecules blocking stomach acid reflux (e.g. omeprazole) or even the simple use of bicarbonate of soda for example? Why are these compounds not used in large clinical trials right now? Is it because the idea of targeting pH looks too simple and that as a result fundings are scarce from public research councils; or is it because these molecules are not patentable anymore?

As scientists we aim to do outstanding far-reaching science with no ounce of a doubt; but as a simple human being of some empathy towards others it is important to realise that people are dying from cancer and that families are destroyed by the disease. There is a real need to open extra scientific debates to clarify and let patients know our scientific strategy against cancer. As a result, it is important to redefine strategy with patients, and that can be developed easily. Targeting the pH of cancer cells may well be one of these solutions.

In this context the authors would like to acknowledge and present the International Society for Proton Dynamic in Cancer (ISPDC) created few years ago (2009) that is a societe savante regrouping all scientists in the world involved in research on proton exchangers, acidity and cancer, in order to create an efficient and meaningful network aimed at leading new therapeutic strategies in the field of clinical oncology.

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