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Review

From delocalized lipophilic cations to hypoxia: Blocking tumor cell mitochondrial function leads to therapeutic gain with glycolytic inhibitors

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An unexpected similarity between cancer and cardiac muscle cells in their sensitivity to anthracyclines and delocalized lipophilic cations (DLC) prompted a series of studies in which it was shown that the positive charge of these compounds is central to their selective accumulation and toxicity in these two distinct cell types. An initial finding to explain this phenomenon was that cancer and cardiac muscle cells exhibit high negative plasma membrane potentials resulting in increased uptake of these agents. However, the *p*-glycoprotein efflux pump was shown to be another factor underlying differential accumulation of these compounds, since it recognizes positively charged drugs and thereby actively reduces their intracellular concentrations. The delocalized positive charge and lipophilicity of DLCs leads to their retention and inhibition of ATP synthesis in mitochondria. Years later it was realized that cancer cells in the hypoxic portions of solid tumors were similar to those treated with DLCs in relying mainly on anaerobic metabolism for survival and could thus be targeted with a glycolytic inhibitor, 2-deoxy-D-glucose (2-DG). This hypothesis has lead to a Phase I clinical trial in which 2-DG is used to selectively kill the hypoxic tumor cell population which are resistant to standard chemotherapy or radiation.

Keywords: Anthracycline / Cancer / Delocalized lipohilic cation / Hypoxia / Mitochondria

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1 Introduction

The impetus for this review dates back to the early 1970s when it became known that the antitumor agent, doxorubicin (Dox) a member of the anthracycline family of compounds, caused serious and sometimes fatal cardiomyopathy in a significant number of treated cancer patients [1, 2]. A fundamental question which arose was, why should a drug that affects actively dividing cancer cells also affect the heart which is comprised mainly of nondividing cells? Thus, it followed that the toxicity of Dox might be reflecting similarities in these very different cell types which could be investigated *in vitro* by measuring the relative accumulation and potency of this drug. In initial studies it was found that Dox (which is naturally fluorescent and thus easy to detect in live cells under microscopy) accumulated preferentially in the nucleus of cardiac muscle cells *versus* cocultured cardiac fibroblasts [3, 4]. Testing a number of other anthracyclines it was observed that those analogs which were positively charged at physiologic pH, daunorubicin, detorubicin, and rubidazone were shown to display similar preferential accumulation in cardiac muscle *versus* nonmuscle cells [5]. In contrast, a neutral anthracycline analog, AD32, accumulated equally in cardiac muscle *versus* nonmuscle cells [5]. Thus, a connection was made between the positive charge of anthracyclines and their preferential accumulation in cardiac-muscle cells (Fig. 1). It is of interest that a charged molecule can permeate the plasma membrane, however the mechanism for the diffusion of anthracyclines remains unclear. A possibility is that the charge is weak enough so that it does not interfere with the lipid solubility of these compounds but further investigation is required to clarify this issue.

This connection was later fortified by the finding that Rhodamine 123 (Rho123), a delocalized lipophilic cation (DLC), also preferentially accumulated and was retained in cardiac muscle *versus* nonmuscle cells [6–8]. Although Dox

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Abbreviations: 2-DG, 2-deoxy-D-glucose; **DLC,** delocalized lipophilic cation; **Dox,** doxorubicin; **HIF,** hypoxia-inducible factor; **MDR,** multidrug resistance; **OxPhos,** oxidative phosphorylation; ***p*-gp,** *p*-glycoprotein; **Rho123,** rhodamine 123

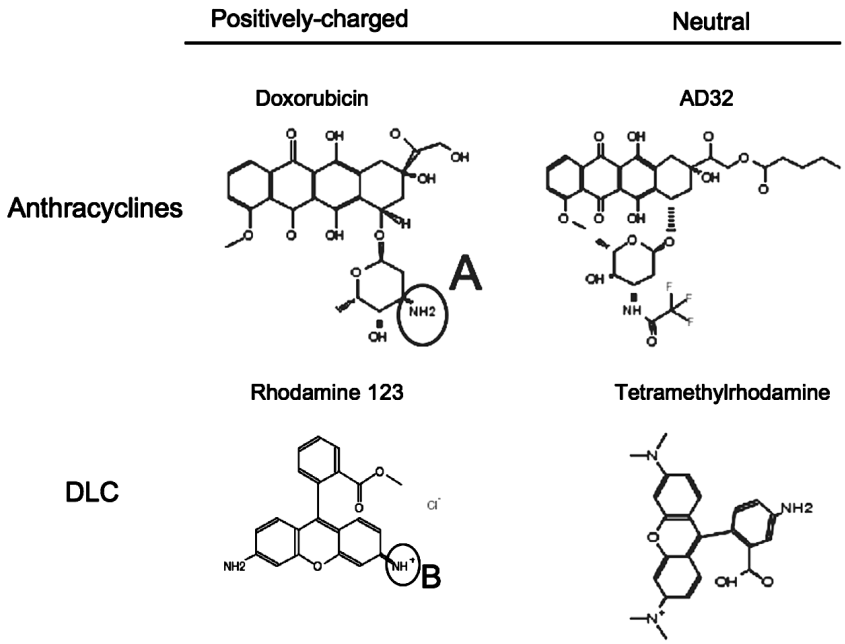

Figure 1. Note that the NH_2 group (A) on the sugar moiety of Dox, as well as daunorubicin, detorubicin, rubidazone is protonated at physiologic pH. On the other hand, Rho123 and other DLCs carry a delocalized positive charge (B) which does not interfere with their membrane permeability while the fixed charge on tetramethylrhodamine does. Overall, positively charged anthracyclines and DLCs selectively accumulate in cells with high plasma membrane potentials.

is an anthracycline and Rho123 is a member of the DLC family, the commonality of their preferential accumulation in cardiac cells led to the realization that the positive charge of these compounds at physiologic pH was a key chemical component responsible for this phenomenon (Fig. 1).

2 Rhodamine 123 and other DLCs preferentially accumulate in mitochondria of living cells

In the late 1970s, while investigating the staining of a surface antigen with a rhodamine-conjugated antibody in living cells, it was observed that so-called "snakelike organelles" were staining which were later shown to be mitochondria [9]. In a following report, it was found that a contaminant in the rhodamine preparation, identified as rhodamine 3B, was responsible for mitochondrial staining of living cells when used at a concentration of 0.5 μg/mL for 15 min [10]. Furthermore, two other rhodamines (123 and 6G) designated by Kodak as laser dyes, were observed to be supravital stains for mitochondria, whereas rhodamine B, 110, 116, and tetramethylrhodamine B were not [11]. Structure function analysis with these rhodamine analogs led to the observation that only those rhodamines that carried a delocalized positive charge were accumulating specifically in mitochondria [10, 11]. These rhodamine analogs are a subset of DLCs which contain an esterified carboxyl group and a positive charge over the molecule that does not interfere with the lipophilic nature of the dye [12]. They differ from the neutral rhodamine analogs which carry a free unesterified carboxyl group and are therefore amphoteric. Thus, the delocalized positive charge of esterified rhodamines allows these compounds to permeate through plasma as well as mitochondrial membranes.

Their intracellular localization is governed by the high negative transmembrane potential of mitochondria which attracts positively charged agents such as DLC, in accordance with the Nernst equation [13]. Staining of mitochondria by Rho123 was shown to be inhibited by dissipation of mitochondrial membrane potential further indicating that the negative charge in the mitochondrial inner membrane acts as an attractive force for positively charged agents [10, 11, 14]. In contrast, pretreatment of cells with nigericin which increases mitochondrial transmembrane potential resulted in increased Rho123 uptake and staining of this organelle [10, 15]. These data suggested that the uptake of lipophilic cations such as Rho123, could be used in living cells as a relative measure of their mitochondrial transmembrane potentials ($\Delta\psi m$) [10].

The fact that one group of compounds (the DLCs) localizes intracellularly in mitochondria while the anthracyclines accumulate in nuclei is most likely due to the overall size, lipophilicity, and nature of the DNA binding properties of each group, respectively. The free amino group in the glycosidic portion of the anthracyclines appears to be a key determinant for actual covalent bonding to nuclear DNA while the delocalized positive charge of the DLCs does not favor this kind of bonding to DNA. Thus, it appears that the nature of the positive charge (localized *vs.* delocalized) plays an important role in determining nuclear *versus* mitochondrial localization although the degree of lipophilicity has also been shown to affect the intracellular localization of analogs within each family of compounds [12].

3 Positively charged anthracyclines and delocalized lipophilic cations accumulate preferentially in carcinoma *versus* normal epithelial cells

The finding that preferential accumulation and toxicity of anthracyclines and DLCs in cardiac cells was related to the positive charge of these compounds, prompted experiments in which it was shown that these drugs selectively accumulate in, and are toxic to, a number of different carcinoma cell lines as compared to normal epithelial cell types [16]. These early studies led to a series of publications in which it was shown that Rho123 and other DLCs, exhibited anticancer activity both *in vitro* [17, 18] and *in vivo* [19]. Although the mechanism of toxicity induced by anthracyclines was different from that by Rho123 [18, 20], the data in these reports established that positive charge for both anthracyclines as well as DLCs was an important chemical component in the preferential accumulation of these different families of compounds in cardiac muscle and carcinoma cells as compared to cardiac nonmuscle and normal epithelial cells.

3.1 Increased plasma and mitochondrial membrane potentials are mechanisms for selective accumulation of anthracyclines and DLCs

A biophysical parameter that could account for increased uptake of positively charged compounds is increased transmembrane potentials at both plasma ($\Delta\psi p$) and mitochondrial ($\Delta\psi m$) membranes. Since all cells generate an electrical gradient which is negative on the inside of the cell, $\Delta\psi p$ could act as an attractive force for intracellular accumulation of agents of this nature. The evidence obtained suggested that cardiac muscle as well as some carcinoma and leukemic cells exhibit higher $\Delta\psi p$ as compared to cardiac nonmuscle, normal epithelial, and other cell types which influences their preferential accumulation of positively charged compounds [21]. In contrast to positively charged anthracyclines which are mainly affected by $\Delta\psi p$, intracellular accumulation of DLCs are influenced by the magnitude of both $\Delta\psi p$ and $\Delta\psi m$. Using valinomycin to depolarize $\Delta\psi m$ and high K to lower $\Delta\psi p$, it was shown that $\Delta\psi m$ had a more profound effect than $\Delta\psi p$ in determining the overall accumulation and retention of DLCs [22]. Thus, the difference in accumulation of Rho123 between carcinoma and normal cell types has been related to a more negative transmembrane potential of mitochondria extracted from carcinoma cells which results in the higher retention of DLCs in these cells [22].

Moreover, it has been suggested that increased $\Delta\psi m$ appears to correlate with aggressive features of carcinoma cells in a model of gastric cancer [23]. In a recent study using several isogenic carcinoma cell lines which have differences in their $\Delta\psi m$, it was found that those cell lines with higher intrinsic $\Delta\psi m$ exhibited enhanced tumorogenic properties, *i.e.*, survival in low oxygen conditions by inhibiting hypoxia-induced apoptotic pathways, increased anchorage-independent growth ability as well as invasion of the basement membrane [23]. This study provides a rationale for the possible clinical application of DLCs since cancer cells with a more aggressive character may have higher $\Delta\psi m$ and thereby preferentially accumulate and be more sensitive to these positively charged agents.

The mechanisms underlying the differences in $\Delta\psi m$ between carcinoma and normal cells, however, remain unclear. It was suggested that the deficiencies in ATP synthase observed in cancer, as compared to normal cells, correlate with their higher $\Delta\psi m$ [24–31]. However, in reports where defects in mitochondrial ATP synthase was detected, electron transport deficiencies were also observed and furthermore $\Delta\psi m$ was not directly measured [25–31]. It is reasonable to presume, a priori, that reduction in electron transport chain (ETC) should lead to decreased $\Delta\psi m$. Thus, the effects of reduced ATP synthase activity in increasing $\Delta\psi m$ would be negated by ETC interruption. This contention is supported by the findings that mutations in cancer mitochondria cause electrons to be retained in complexes I and III. This, in turn, facilitates the direct transfer of electrons onto molecular oxygen and thereby higher production of reactive oxygen species (ROS) which are shown to be involved with oncogenic transformation [32, 33]. Thus, it appears that mitochondrial deficiencies in cancer cells correlate with high ROS levels, but not necessarily with high proton gradient or increased $\Delta\psi m$. Moreover, the studies, in which $\Delta\psi m$ was found to be higher in cancer cells, used DLC accumulation as a measure of $\Delta\psi m$ rather than direct measurement of this parameter [25–31]. Taken together, to date there is direct evidence supporting a link between the retention of positively charged compounds in tumor cells and increased $\Delta\psi p$ [21] while the role of $\Delta\psi m$ in this phenomenon remains to be further investigated.

3.2 *p*-Glycoprotein (*p*-gp) mediated multidrug resistance is an underlying mechanism for selective accumulation of DLCs

Another explanation for increased DLC retention in carcinoma mitochondria comes from the observation that most of the compounds recognized by cancer cells expressing the clinically important mechanism of *p*-gp mediated multidrug resistance (MDR), are also positively charged. Juliano and Ling have shown that tumor cells which develop the MDR phenotype, earlier described by Riehm and Biedler [34], and are typified by lower drug accumulation, have increased levels of a 170-kDa plasma membrane glycoprotein [35]. Extensive work after this initial finding revealed specific MDR genes encoding this glycoprotein which conferred cellular drug resistance [36]. It is known that the

p-gp acts as an efflux pump for a variety of structurally unrelated compounds, which accounts for their lower accumulation in MDR+ cells [34, 37]. To reiterate, our observations and results [21, 38] confirmed by others [39–46] indicate that most compounds effluxed by MDR+ cells are positively charged.

Further evidence to support this idea comes from studies in which a single negatively charged amino acid in the MDR transporter, Mdfa, of *Escherichia coli* was found to account for recognition of positively charged compounds [47]. Additionally, it was demonstrated that when this amino acid was substituted with a positively charged one, recognition of positively charged drugs was abolished but the transporter still functioned for other uncharged compounds, *i.e*., chloramphenicol [47]. Although this result was reported in prokaryotic cells, the authors speculated that similar recognition for *p*-gp mediated transporters in eukaryotic cells may exist due to single amino acid residues. This work is particularly pertinent for supporting the hypothesis that the positive charge of a number of different families of compounds, including DLCs, is an important chemical characterestic for determining whether a drug will be recognized by *p*-gp mediated MDR in a variety of human and mouse cells [47].

Moreover, using a series of nine anilinoacridines, Baguley and Ferguson [48] have found that strongly basic or positively charged analogs of this group of compounds showed the greatest degree of crossresistance in P388/ADR resistant cells, which further supports this concept. It was also reported that the higher the pK_a of the amino sugar of a selected number of anthracyclines studied, the greater intracellular accumulation in drug sensitive HL-60 leukemia cells [49]. Beck and coworkers, in a series of papers have shown the necessity of cationic charge for compounds to act as MDR modulators [40, 41]. Lampidis *et al*. using a selected series of anthracycline analogs in which lipophilicity and charge were altered found the following: (i) positively charged anthracyclines as compared to their neutral counterparts are better recognized by MDR+ cells; (ii) with increasing lipophilicity charge becomes less important for MDR recognition; (iii) resistance to anthracylines can be reduced >1000-fold with an analog that does not contain a protonatable nitrogen and is highly lipophilic; and (iv) highly lipophilic analogs regardless of charge can act as modulators of *p*-gp [50]. This latter finding suggests that at a high enough lipophilicity either drug transport overcomes the speed of the efflux pump and or that highly lipophilic compounds nonspecifically bind to or interfere with the effluxing function of *p*-gp and thereby act as self-modulators.

To define more precisely the influence that charge and lipophilicity have on MDR recognition, a series of simple aromatic (pyridinium) and nonaromatic (guanidinium) cations were designed and synthesized which differ in lipophilicity by stepwise addition of single alkyl groups [51]. Using these simple compounds it was found that an aromatic ring and a minimal degree of lipophilicity (log-$P > 1$ = at least five alkyl groups) in addition to positive charge, were required for *p*-gp mediated MDR recognition [51]. With increasing lipophilicity (above five alkyl groups) the resistance ratio between MDR- and MDR+ cells increased as a function of increasing chain length [51]. This study, which is further supported by other labs, emphasizes the importance of positive charge but also defines a minimal degree of lipophilicity necessary for MDR recognition [51–55].

It is clear that the chemical structure of Rho123 and other DLCs is suitable for recognition by the *p*-gp efflux pump [56] which suggested that the selectivity we had originally observed between carcinoma *versus* normal epithelial cells with Rho123 [6, 16] could, at least in part, be due to the presence of the MDR1 gene product. Indeed, the normal epithelial cell line, CV-1, which was originally used as a model for cationic drug selectivity between normal and tumor cell lines, was later found to intrinsically express MDR1 [57] and display reduced uptake of a number of "MDR recognizable drugs," *i.e*., Dox, vinblastine, taxol, as well as Rho 123 [57]. In this report, it was also shown that the classic MDR modulator, verapamil, reverses the resistance to each of these agents in CV-1 cells [57]. However, not all normal cells are known to express MDR1 and therefore, may not be resistant to DLC treatment. In fact, when one of the DLCs, MKT-077, was tested in phase 1 clinical trial, it was found to be nephrotoxic [58]. It appears that the use of DLCs for cancer treatment is limited due to the absence of *p*-gp in various normal tissues [59, 60].

In summary, the significance of these findings is that cancer cells that do not express MDR and contain high $\Delta\psi p$, are most likely to retain and be sensitive to Dox as well as DLCs. The same can be said of cardiac muscle cells, *i.e*., their unusual sensitivity to Dox may, at least in part, be explained by their high negative $\Delta\psi p$, whose electrical force would contribute to the intracellular attraction of positively charged compounds, and the absence of MDR to rid itself of these types of agents.

4 Targeting hypoxic tumor cells with glycolytic inhibitors: Lessons from DLCs

Most anticancer agents, which are currently used clinically, target the aerobic rapidly dividing cells of a tumor. Therefore, slow-growing anaerobic (hypoxic) cells found in necrotic centers and at the inner core of most solid tumors, will be resistant to these chemotherapeutic drugs. Thus, slow-growth may be considered another form of MDR. The fact that the most common toxicities of currently used chemotherapeutic agents in normal cells are found in the fastest dividing tissues, *i.e*., bone marrow, gut, and hair, provides further evidence that the selectivity of anticancer drugs in

general lies not as much between tumor and normal cells as it does between rapidly dividing and slow, or nondividing cells.

The hypothesis on how to overcome these slow-growing drug-resistant tumor cells found in the hypoxic areas of tumors derives from work in which Rho123 was shown to inhibit mitochondrial oxidative phosphorylation (OxPhos) [61, 62]. Consequently, tumor cells treated with this drug have to rely solely on glycolysis for ATP production and thus become hypersensitized to inhibitors of glycolysis, such as 2-deoxy-D-glucose (2-DG). In fact it was shown that cotreating human breast carcinoma cells, MCF-7, with Rho 123 and 2-DG, 100% of the colony forming units was inhibited whereas similar treatment in normal epithelial cells showed little or no toxicity [17]. This concept was carried over to *in vivo* studies in which it was found that tumor bearing animals treated in combination with 2-DG and Rho123 were cured whereas when treated with either drug alone, only partial or no responses were obtained [18]. This latter result provides evidence that manipulation of OxPhos and glycolysis simultaneously can cure tumors in animals. Furthermore, these *in vivo* data also demonstrate that 2-DG can be administered safely to animals, at doses which are effective for antitumor activity in combination with an OxPhos inhibitor. In this regard, several reports have shown that low levels of 2-DG can be safely administered to animals for various reasons including hypersensitization of tumors to irradiation [63].

Since hypoxia, similar to Rho123 treatment, forces cells to rely mainly on anaerobic metabolism of glucose for survival, hypoxic tumor cells could be selectively targeted with inhibitors of glycolysis such as 2-DG [64]. The switch from aerobic to anaerobic metabolism in cells that lie in hypoxic regions of tumors creates two windows of selectivity that we believe will ultimately prove beneficial to cancer patients when 2-DG is used in combination with cytotoxic agents. The first is that tumor cells under hypoxia upregulate both glucose transporters and glycolytic enzymes [64], which favors increased uptake of 2-DG in these cells as compared to normal aerobic cells. The second is based on the principle that even if enough 2-DG is accumulated in normal cells to block glycolysis, they can survive by using oxygen to burn fats and proteins through their mitochondria to produce ATP. In contrast, when glycolysis is blocked in hypoxic tumor cells they die, since their mitochondria at these oxygen levels (8–57 μM) [64, 65] are less efficient in converting these alternative energy sources to ATP. These two windows of selectivity are based on fundamental principles of biochemistry and as such provide the basis for using glycolytic inhibitors to raise the efficacy of current chemotherapy by targeting the slow-growing hypoxic cell population found in most, if not all, solid tumors.

In order to investigate the mechanisms involved with hypersensitivity to glycolytic inhibitors, we developed three distinct models of simulated hypoxia which are referred to as chemical model A, genetic model B, and environmental model C [66–68]. Model A approximates hypoxia by using chemicals such as Rho123, rotenone, antimycin A, and oligomycin to interfere with mitochondrial function thereby rendering the cell unable to produce ATP *via* OxPhos [66]. Model B uses tumor cells that have been permanently genetically altered by depletion of their mitochondrial DNA and cannot undergo OxPhos [66]. Since models A and B are growing under oxygen, but are unable to produce ATP *via* their mitochondria, they simulate hypoxic cells by relying exclusively on glycolysis for this function, whereas, model C are actually tumor cells grown under decreased atmospheric oxygen [68]. In all three models, glycolysis is increased (as measured by lactic acid) and most importantly, they are all found to be hypersensitive to glycolytic inhibitors as compared to their aerobic counterparts [66, 68]. However, model C is found to be less sensitive to glycolytic inhibitors than models A and B, which suggests that certain variables influencing the cellular response to glycolytic inhibitors differ between these models. One such variable is hypoxia-inducible factor-1 (HIF-1), which is expressed only in model C. HIF-1 is known to be a key regulator of a wide range of cellular responses to lowered oxygen tension [69]. Among the numerous genes activated by HIF-1, are glucose transporters and glycolytic enzymes [70–73]. Since HIF-1 is activated in model C [69], but not in other models, it seems likely that HIF-1 induction is contributing to the increased cellular resistance to glycolytic inhibition by 2-DG found in this model. In this regard, a recent publication demonstrated that HIF-1 induced hexokinase 2 expression confers resistance to glycolytic inhibition by 2-DG, suggesting that combining inhibitors of HIF with 2-DG may be a more effective strategy than either agent alone, particularly for targeting the slow-growing hypoxic cell populations found in most solid tumors.

5 Conclusions

The studies which began by investigating the reasons why Dox seemed to preferentially affect cardiac and cancer cells has lead to a number of findings which have resulted in clinical applications. Notably, nonpositively charged analogs of Dox such as annamycin and AD 32 are shown to cause little or no cardiotoxicity and are able to overcome MDR in tumor cells expressing this mechanism of drug resistance [74, 75]. Similarly, a member of the DLC family, MKT-077, has been used in clinical trials as an antitumor drug based on its selectivity for accumulating in cancer mitochondria [58]. However, this approach has not yielded successful clinical results which is most likely due to a variety of factors including, the presence of MDR in many tumor types, the ineffectiveness of mitochondrial inhibition as a sole means of killing a cell and the toxicity in various normal tissues due to lack of MDR expression. On the other hand,

hypoxic regions in solid tumors are shown to be hypersensitive to glycolytic inhibitors such as 2-DG [76]. Thus, combined with chemotherapeutic agents, 2-DG is currently in a Phase I clinical trial to selectively target the hypoxic cell population found in most solid tumors [77]. The biochemical differences in glucose metabolism between slow-growing hypoxic tumor cells and slow-growing aerobic normal cells provides a natural window of selectivity that can be exploited for therapeutic gain using glycolytic inhibitors.

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6 References

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