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COMMENTARY

CELLULAR TRANSPORT OF ANTHRACYCLINES BY PASSIVE DIFFUSION

IMPLICATIONS FOR DRUG RESISTANCE*

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Adriamycin (ADR; doxorubicin) is one of the primary chemotherapeutic agents for the treatment of solid tumors and leukemias. Resistance to this agent developed by target tumor cells, even in cancer patients who were initially responsive, may be a critical factor in the therapeutic efficacy of the drug. This is especially important because cross-resistance may develop to other chemotherapeutic agents concurrently with the emergence of ADR resistance [1, 2]. Cell lines isolated from leukemias, lymphomas, and solid tumors with decreased drug sensitivity have been developed for studying resistance to anthracyclines in culture [2–13]. Studies concerning the mechanism of acquired resistance have focused mainly on transport of ADR and its analog daunomycin (DNR; daunorubicin). Alternative mechanisms, unrelated to transport, have been explored in only a few studies [12, 13]. Some degree of reduction in steady-state intracellular drug levels is usually found when resistant populations are assayed. A model proposed by Danø [3], Skovsgaard [4, 5], and others [6, 8, 9] suggested that an active efflux pump exists which selectively removes ADR from the cells in an energy-requiring process; by this explanation, resistant cells are presumed to have a more active pump than sensitive parent lines. This proposal, which will be discussed in more detail below, was based on a few simple experiments which were carried out in a variety of resistant and sensitive cell types. The existence of the postulated transport protein has never been proven, nor has it been isolated or studied in any detail. Although this transport model for ADR resistance is attractive in its ability to provide a straightforward explanation of reduced drug sensitivity, several lines of evidence suggest that it may not be entirely correct. Harder *et al.* [14] showed that cells resistant to ADR accumulated *more* drug than sensitive cells. This finding poses considerable difficulty for a model which proposes that the level of drug accumulation directly controls the cellular response. In addition, work by Dalmark and associates [15–17] demonstrating the effect of self-association of ADR on its uptake points to passive diffusion as the mechanism of transport and calls into question earlier interpretations of uptake studies. Lastly, studies from our laboratory comparing a series of ADR-resistant cell lines indicate that decreased drug content is only one component of resistance [12]. The purpose of this article is to review the subject of anthracycline transport as it relates to drug resistance. Three models have been suggested for ADR transport: (1) a "leak and pump" mechanism involving diffusion of ADR into the cell and selective removal through an "efflux pump"; (2) facilitated diffusion via a carrier molecule; and (3) passive diffusion. Data supporting each of these mechanisms will be discussed. Our view is that, although definitive evidence is still lacking, passive diffusion can explain the observations for anthracycline transport. For an alternative viewpoint, the interested reader is referred to a recent review by Skovsgaard and Nissen [18].

THE "EFFLUX PUMP" MODEL

Evidence for an efflux pump comes from experiments in which cultured cells have been deprived of glucose in the presence of various uncouplers of oxidative phosphorylation. The steady-state level of ADR and DNR increased under these conditions. When glucose was added back, drug was observed to leave the cell. Such effects were observed to a greater extent in resistant than in sensitive cells [3–6, 8, 9]. The interpretation of these experiments was that energy is required to transport ADR out of the cell, against a concentration gradient. This model presumes that there is a driving force for movement of anthracyclines into the cell, against which the proposed pump must work. The resulting equilibrium would be determined primarily by the action of the pump. Such a driving force might exist because ADR has an enormous capacity to bind phospholipids [19, 20], nucleic acids [21], ATP [17], and other cellular constituents, so that the majority of intracellular drug is in a bound form. Only free drug would participate in the concentration gradient, so

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that even though ADR accumulates in the cell up to 20-fold [22], the free drug concentration remains low. If an efflux pump exists, however, it must serve some purpose for the cell. The usual function of a transport protein is to aid in bringing nutrients into the cell. Xenobiotics could be transported if the carrier had some affinity for the particular compound. A carrier might be postulated whose function is to remove toxins from the cell, but in this case other toxins should compete with ADR for transport. *N*-Acetyl-ADR is the only compound for which competition has been demonstrated at relevant concentrations.

Because all chemical processes are the net result of forward and reverse reactions, a transport protein would be capable of establishing a net outward movement of anthracycline if the equilibrium constant for the outward direction were favorable or if the equilibrium extracellular concentration were lowered relative to the internal concentration. The latter process would not require energy. It is reasonable to propose that resistance could arise if the equilibrium constant of a transport protein were altered in the varient cell line. No direct documentation of this possibility exists, however.

It is also important to point out that, although the efflux pump model has been proposed by many investigators, no detailed experiments have ever been presented in support of the "pump". The kinetics, stoichiometry, ion requirements, and other biochemical properties of the measured efflux have never been comprehensively investigated. The fact that these observations have been made in many cell systems and with other drugs, such as vinblastine and actinomycin D [1, 10], indicates that the data are of importance in understanding the pharmacology of these agents, but the existence of the "pump" is far from proven. We believe the only useful experimental approach to this problem would be to utilize membrane vesicles of an appropriate cell type. This is necessary because the superposition of intracellular binding, sequestration and metabolism of the two proposed equilibria (active transport and simple diffusion) makes the system too complex for accurate analysis. To date no work of this type has been carried out.

FACILITATED DIFFUSION MODEL

Evidence for carrier-mediated diffusion of anthracyclines further complicates the issue of whether an efflux pump exists. Saturation kinetics have been demonstrated for DNR and ADR in L1210 cells [23, 24] and erythrocytes [25]. Skovsgaard [22] has suggested a carrier-mediated mechanism based on the following observations in Ehrlich ascites cells: (1) steady-state levels of anthracyclines vary among different derivatives; (2) some competition between ADR and DNR was noted for uptake (although less than that predicted based on the apparent K_m); (3) saturation kinetics were demonstrable; and (4) a strong temperature dependence was found for uptake. We have also observed saturation kinetics and a similar temperature dependence in Sarcoma 180 cells [26]. The apparent K_m for DNR was 125 μM, as opposed to 70 μM in Ehrlich ascites cells [22]. It is striking that the apparent K_m observed for DNR is extremely high in comparison to cytotoxic concentrations of this drug. In Sarcoma 180 cells, the IC_{50} for DNR is 0.05 μM, while the apparent K_m is over three orders of magnitude higher. This indicates that transport of DNR by the carrier is never rate-limiting at pharmacologically relevant concentrations. Yet differences in steady-state levels have been noted between sensitive and resistant cells in many cell types, including Sarcoma 180 [2–12, 26]. Resistant Sarcoma 180 cells did not show a significantly different K_m for DNR compared to the parent sensitive line [26]. This raises the distinct possibility that differences in steady-state levels are unrelated to carrier-mediated transport.

If a carrier protein for uptake exists, it raises many questions about the "efflux pump" theory. Do two carriers exist, both of which are essentially undirectional by virtue of their respective equilibrium constants? Does one carrier exist, with an equilibrium far favoring the outward direction? With such a low affinity for anthracyclines, how could such a carrier operate as an effective pump? A model could be proposed in which the coupling of transport with the hydrolysis of ATP changes the affinity of the protein for anthracyclines, but it would have to selectively occur for the outward movement only, since the inward transport clearly does not require energy [4–6, 22]. Such models would seem to be thermodynamically unlikely. Thus, it appears that neither theory can adequately explain all the reported observations. Recent data which describe formation of anthracycline dimers [14, 18, 27, 28] point to an explanation of why carrier-like properties have been observed for ADR transport. In addition, the affinity which protonated ADR has for many cellular constituents may explain why uncoupling of oxidative phosphorylation by metabolic inhibitors has an effect on steady-state drug levels.

PASSIVE DIFFUSION MODEL

The chemical properties of ADR and DNR make it likely that they would move across the cell membrane by passive diffusion in both a forward and reverse direction, and that the relative intra- and extracellular concentrations would be determined by several factors: (1) pH of the cell and pK_a of the compound, (2) extent of binding to cellular constituents, (3) extent of dimerization and higher complex formation, and (4) temperature. These factors have been explored by several investigators, the most recent of which has been Dalmark and coworkers [15–17]. The anthracyclines can exist in neutral and ionized forms; at physiological pH, the main ionization occurs at the amino group on the sugar moiety, daunosamine, which has a pK_a of 7.6 to 8.2, depending on the ionic strength [15]. ADR will be increasingly protonated at this site as the pH decreases. Because charged molecules do not move as readily across the cell membrane as neutral compounds, the uncharged species is preferentially taken up by the cell. An intracellular equilibrium will be established between charged and uncharged species which will be dependent on both cellular pH and on

binding of the charged species to phospholipids, DNA, and other cellular sites [19–21].

As was noted earlier, free drug accounts for very little of the intracellular anthracycline content. Therefore, differences in drug levels must be determined primarily by the extent of binding to cellular constituents. A decrease in pH would shift the intracellular equilibrium in favor of the ionized form, and since the ionized form binds cellular constituents with greater affinity, the overall equilibrium would be shifted in favor of intracellular drug. An increase in intracellular drug content would result because the equilibrium between intra- and extracellular free drug would be shifted. 2,4-Dinitrophenol was found to decrease intracellular pH by one full unit in Ehrlich ascites cells [27]. Skovsgaard [5] has also noted this possibility.

One of the sites of cellular binding of ADR is to negatively charged phospholipids [19, 20]. In light of experiments on glucose deprivation, it is interesting to point out that glucose also has considerable ability to bind phospholipids. Up to 35% of cellular monosaccharide content is extractable from phospholipids [29]; this may represent a depot form of glucose in the cell. ADR may compete with glucose for binding sites on phospholipids. When glucose is added back to cells deprived of ATP, two factors may result in a decreased cellular ADR content: (1) a shift in pH as noted above, or (2) displacement of ADR by glucose. It is important to note that extremely high glucose concentrations (10 mM) have been used, in comparison to micromolar levels of ADR. It is also important to point out that changes in drug levels have never been measured in control experiments in which glucose concentration alone was manipulated, without uncoupling phosphorylation. Thus, glucose alone may alter the equilibrium of free ADR. Such a model could explain why efflux is still somewhat inhibited when preloaded cells are placed in drug-free medium in the presence of uncoupling agents, because the pH decrease and lack of glucose would be factors in determining the equilibrium, not just the concentration gradient.

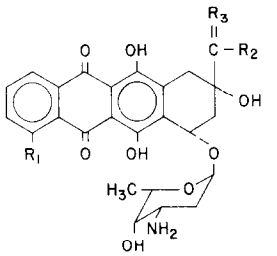
But what of data which have been presented in support of facilitated diffusion? We believe the predominant evidence favors the idea that self-association of anthracyclines is responsible for the observations that have been made. Barthelemy-Clavey *et al.* [28] described complexes of DNR in solution by measuring absorption spectra, circular dichroism, and nuclear magnetic resonance. Dimerization and higher order complex formation were detected, the extent of which was temperature and concentration dependent. Complex formation is reduced by raising the temperature; it is maximum at 0° [29]. Chaires *et al.* [30] found that daunomycin self-associated in aqueous solution at concentrations greater than 10 μM. Eksborg [31] described similar results with ADR.

Dalmark and coworkers [15–17] have demonstrated that the extent of complex formation determines the transport characteristics of anthracyclines and accounts for the carrier-like properties that have been observed. Because the monomeric form of ADR or DNR is much more permeable than aggregates, the apparent permeability coefficient decreases with concentration and increases with temperature [15, 16]. Saturation kinetics and a high temperature coefficient will result [15]. The permeability coefficient into red blood cells and the partition coefficient between 1-octanol and Tris buffer had the same concentration dependence: unchanged below 10 μM, with an inflection point between 70 and 100 μM. This is exactly the concentration range where saturation kinetics begin to occur in Ehrlich ascites cells [22] and Sarcoma 180 cells [26], and explains why the apparent K_m is so much higher than pharmacologically relevant concentrations. This interpretation is supported by the observation of Chaires *et al.* [30] that the apparent extinction coefficient of DNR began to decrease at 10 μM and dropped steadily up to 1 mM, in the same concentration range where saturation kinetics and aggregation are observed.

Further evidence that transport occurs through passive diffusion has also been presented by Dalmark [17]. The rate of ADR transport in red blood cells was pH dependent, efflux occurring twenty-five times faster at pH 8.3 than at pH 6.1. In addition, 1-alcohols increased the rate of transport in proportion to their chain length, charged and uncharged local anesthetics enhanced transport, and phloretin, an inhibitor of facilitated diffusion, did not affect ADR efflux [17]. Heavy metals and anions which inhibit various facilitated diffusion systems also had no effect on anthracycline transport [17].

The findings of Skovsgaard [22] that steady-state levels of anthracyclines varied among different derivatives could be explained by different pK_a values, permeability constants, and binding affinities for cellular constituents. Limited competition between ADR and DNR could also be accounted for by complex formation. Competition between ADR and DNR was observed at total drug concentrations above 6 μM [22], at which point effects of complex formation begin to be seen [15, 16]. Likewise, self-competition of [^{3}H]DNR by unlabled DNR was only seen at concentrations about 100 μM [22].

Comparison of the physiochemical properties of several anthracyclines with their cellular uptake provides further support for the passive diffusion model. The most straightforward comparisons are between drugs which differ by a simple substitution in the aglycone. Adriamycin and carminomycin (Fig. 1) are structural analogs of daunomycin which meet this criterion. The polarity of the aglycone portion of the drug molecule is one of the two major factors to consider when comparing anthracycline structure with transport properties for a given cell line. (The other major consideration, pK_a, is discussed below). Octanol:buffer partition coefficients have been routinely used in the past to serve as a first approximation of the polarity of a drug molecule. The relative octanol:buffer partition coefficients for adriamycin, daunomycin and carminomycin have been calculated to be 1, 2.2, and 14.4 respectively [32]. Predictably the uptake of these drugs in L1210 [32] and Ehrlich ascites [33] cells directly correlates with polarity. Reduction of the C-13 carbonyl of adriamycin and daunomycin results in formation of the polar analogs adriamycinol and daunomycinol respectively. Bachur *et al.* [34] showed that the



COMPOUND	R_1	R_2	R_3
DAUNOMYCIN	OCH_3	CH_3	O
ADRIAMYCIN	OCH_3	CH_2OH	O
CARMINOMYCIN	OH	CH_3	O
4-DEMETHOXYDAUNOMYCIN	H	CH_3	O
RUBIDAZONE	OCH_3	CH_3	$NNHCOC_6H_5$

Fig. 1. Structural modifications in the aglycone portion of the anthracyclines.

uptake by L1210 cells for adriamycinol and daunomycinol was reduced significantly relative to their parent compounds. Likewise, 4-demethoxydaunomycin is more hydrophobic (less polar) than daunomycin [35], and its uptake by HeLa cells has been shown to exceed that of daunomycin [36]. Thus, the available data show a strong correlation between drug hydrophobicity and uptake, suggesting the involvement of general membrane association and permeability properties in uptake; this is to be expected for the passive diffusion model but not necessarily for a carrier-mediated process.

Skovsgaard [22] based the carrier-mediated transport theory for the anthracyclines partly on the observation that rubidazone, despite having a higher octanol:buffer partition coefficient than daunomycin, exhibited reduced influx relative to daunomycin in Ehrlich ascites cells. This observation was used as evidence against the passive diffusion model. Rubidazone differs from daunomycin by replacement of the C-13 carbonyl with a bulky benzoylhydrazone substituent. The other daunomycin analogs presented in Fig. 1 have structural modifications which introduce only small steric changes in the drug molecule (the most radical change being the replacement of a methoxyl with a proton) but which result in significant changes in aglycone polarity, especially when the alteration occurs at position R_1 which is directly conjugated to an aromatic ring. Rubidazone contains a structural change which not only alters the polarity of the drug but introduces a significant steric consideration. This complicating steric factor makes comparison of rubidazone and daunomycin uptake data with drug hydrophobicity as determined by simple octanol:buffer partition coefficients somewhat difficult to interpret.

The transport properties of an anthracycline have also been shown to change due to structural modifications in the aminosugar portion of the molecule [37, 38]. The presence of an ionizable amino group whose pK_a is sensitive to structural changes makes it difficult to separate the effects of alterations in aminosugar structure from effects on basicity and the consequent role of these properties in cellular transport. Because of this problem, the role of aminosugar structure in governing transport is poorly understood; thus, it is difficult to reconcile all of the uptake data available in the literature concerning the aminosugar analogs with just one of the proposed transport models, although certain conclusions can be tentatively made. Skovsgaard and Nissen [18] have reviewed how the degree of ionization of a drug molecule affects its membrane transport. Assuming free permeability of the unionized molecule and impermeability of the ionized molecule, weak bases such as the anthracyclines are concentrated in cells or cellular compartments where the internal pH is less than the extracellular pH. Jacobs [39] derived an equation which predicts that the ratio of free intracellular drug to free extracellular drug increases with increasing pK_a of the ionizable group, when the internal pH is less than the external pH. Consistent with this prediction, Egorin *et al.* [37] have shown that N,N-dimethylation of the daunosamine residue of both adriamycin and daunomycin resulted in a 3- to 5-fold enhancement in the uptake by L1210 cells relative to those of the parent compounds. Acton [40] synthesized the N,N-dimethylamino derivative in order to enhance the basicity of the amino group. Although the pK_a values of the N,N-dimethylamino derivatives have not been reported, it is likely that the placement of two electron releasing methyl groups on the nitrogen of the aminosugar would increase the pK_a of the derivative. If the N,N-dimethylamino derivatives are protonated (i.e. charged) to a greater extent that their parents, then why do their rates of cellular influx and final steady-state levels exceed those of their parents if transport occurs by simple diffusion of the neutral drug molecule? The explanation, based upon the theory described above, is that an increase in pK_a shifts the final equilibrium in favor of a greater intracellular free drug concentration. Once within the cell, the enhanced ionized nature of the N,N-dimethylamino derivatives may lead to greater binding to cellular components, consequently driving the intracellular/extracellular free drug equilibria to high ratios. By this explanation the ultimate uptake is driven both by the relative ability of the uncharged species to passively diffuse into the cell and by the tendency of the charged species to be bound at intracellular sites.

A similar argument may be made to explain why the rate of influx and steady-state levels of the 4-deoxydaunosamine derivatives of adriamycin and daunomycin exceed those of their parent anthracyclines in L1210 cells [38]. It was observed that the pK_a values of the 4-deoxydaunosamine derivatives of adriamycin and daunomycin increased relative to the parent by values of 0.32 and 0.22 respectively. Thus, the increased uptake is as expected for simple diffusion. However, it was also observed in the same study that the 4′-epidaunosamine derivatives of adriamycin and daunomycin, despite having pK_a values 0.26 units less than their parents, had uptake levels which exceeded those of their respective parents. Thus, a strict correlation between the pK_a of the aminosugar and the rate and extent of membrane

transport is violated in this case. No doubt this is due to the fact that several structural properties of the molecule govern uptake, and consideration of any one of these by itself may lead to erroneous conclusions.

In summary, structural modification in the aglycone and aminosugar portions of the anthracycline molecule have been shown to alter cellular uptake. It appears than octanol:buffer partition coefficient measurements may be useful in predicting how an alteration in the aglycone will affect uptake, provided that steric considerations remain relatively unchanged. Unfortunately the literature is lacking in data which lead to a complete understanding of how stereochemical and substituent changes in the aminosugar affect uptake. We believe that information essential to the further characterization of the pharmacologically relevant structure–transport relationships for the anthracyclines will come from a better understanding of how structural changes alter the binding of the drugs to model and biological membranes. If the passive diffusion model is accurate, it seems likely that the initial anthracycline influx into cells is determined by the extent of drug binding to the cell membrane, the location and dynamics of bound drug in the surface membrane, and the pK_a of the aminosugar. Systematic studies of these variables should deepen our understanding of the uptake process.

IMPLICATIONS FOR ANTHRACYCLINE RESISTANCE

If the transport of ADR occurs through passive diffusion, and no "active efflux pump" exists, how can decreased drug levels be explained in cells resistant to anthracyclines? There are several possibilities: (1) A general change in permeability of the cell membrane, for instance through a change in lipid composition. This is, in fact, observed in S180 cell lines selected for ADR resistance (M. Adler and T. R. Tritton, unpublished observations). (2) A change in the capacity of resistant cells to bind anthracyclines, such as a decrease in negatively charged phospholipids or a change in the extent of phosphorylation of proteins. Kessel [41] has shown that the surface charge of drug-resistant cell lines is altered from controls. (3) A change in metabolic production, for instance through increased production of the aglycone, which would be uncharged at physiological pH and presumably does not interact with cellular components. Data against this idea exist in S180 cells [12] but metabolism differences may be a factor in resistant cells of other types.

General permeability of the cell membrane may be less important in determining levels of anthracyclines in resistant cells than drug sequestering. Resistant Ehrlich ascites cells preloaded with ADR in the absence of metabolic inhibitors extrude drug at a faster initial rate than sensitive cells, so it does not appear that the membrane permeability is actually lower in the variant line. Since most intracellular drug is bound, it is more likely that changes in binding capacity account for lower steady-state levels than changes in permeability. Changes in binding capacity might also explain cross-resistance to other chemotherapeutic antibiotics. Other drugs which often show cross-resistance to ADR are Vinca alkaloids and actinomycin D. Like ADR, these are relatively high molecular weight compounds which are capable of forming dimers and higher complexes [42] and exist as partially protonated, positively charged molecules at physiologic pH. Intracellularly, these drugs are also primarily found in bound form. Active efflux has been proposed as a mechanism of resistance to these agents but, like ADR, these drugs may also enter and exit the cell by passive diffusion of the neutral species. Extent of binding and pools of free drug are most likely pH-dependent. Differences in binding of vinblastine were noted in resistant versus sensitive cells by Beck *et al.* [43]; although such differences were proposed to be due to energy-dependent binding, pH differences could also make a contribution. Beck *et al.* [43] also found that anthracyclines do not compete with transport of Vinca alkaloids.

Changes in drug levels are not the only alterations which determine resistance to anthracyclines. In a series of Sarcoma 180 cells of increasing resistance to ADR and DNR, we found that drug levels did not progressively decline as the degree of resistance increased [12]. Although cells which displayed a 5-fold increase in IC_{50} showed a 30–40% decrease in steady-state of [^{3}H]DNR, no further change in steady-state was found in cells of up to 125-fold resistance. Cross-resistance to actinomycin D and vincristine was observed in cells of 5-fold ADR resistance; as the degree of insensitivity toward ADR increased, however, the level of cross-resistance remained constant [12]. Membrane fluidity of these resistant cells progressively increased, however [44]. Likewise, Ramu *et al.* [45, 46] have shown that resistance to anthracyclines is positively correlated with membrane fluidity and that alterations in lipid metabolism can alter the relative sensitivity of the cells to anthracyclines.

Since ADR and DNR have many effects on cell membranes [47], and since ADR does not need to enter cells in order to be cytotoxic [48], membrane changes in resistant cells may reflect a change in how ADR interacts with the plasma membrane, rather than a change in transport of anthracyclines. In this way a general change in drug uptake or binding may be responsible for a low level of resistance towards many xenobiotics, although without specificity for one class of agents. Higher levels of resistance, however, may be accounted for by specific alterations which modify cellular response to a particular agent.

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