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INTRACELLULAR DRUG SEQUESTRATION EVENTS ASSOCIATED WITH THE EMERGENCE OF MULTIDRUG RESISTANCE: A MECHANISTIC REVIEW

Muralikrishna Duvvuri and Jeffrey P. Krise

The University of Kansas, Department of Pharmaceutical Chemistry, 2095 Constant Ave., Lawrence, KS 66047

TABLE OF CONTENTS

1. ABSTRACT

The acquisition of multi-drug resistance (MDR) in cancer cells subjected to anticancer agents remains a formidable obstacle to successful therapeutic outcomes in cancer patients. As the name implies, the resistance phenotype (MDR) is not typically limited to the drug initially used to eradicate cancer but is often transferred to structurally unrelated chemotherapeutic agents. The mechanisms underlying the development of MDR have been extensively studied and are considered multifactorial. Interestingly, recent observations have shown that altered intracellular distribution of drugs may play an important role in the establishment of the MDR phenotype. Such intracellular redistribution events may reduce the opportunity for a drug molecule to permeate into a drug target-containing compartment and thus limit its therapeutic effect. This review summarizes cases in which intracellular redistribution of drugs has been associated with the emergence of MDR in cancer cells. The review also provides a general overview regarding intracellular compartmentalization mechanisms of drugs in cells, which will include some of the known factors/conditions that influence the accumulation of drugs into specific cellular compartments. Finally, potential strategies for overcoming this resistance phenotype are discussed.

2. INTRODUCTION

The resistance of tumor cells to anticancer agents continues to be a major cause of treatment failure in patients diagnosed with cancer. Multi-drug resistance (MDR) is a term used to describe a situation in which cancer cells become simultaneously resistant to different drugs with no obvious structural similarities or mechanisms of action (1). Over the past two decades research has revealed that MDR is multifactorial whereby multiple resistance mechanisms are typically operative in a single cancer cell line (2). Decreased drug accumulation and/or increased efflux, increased detoxification capacity, increased DNA repair, alterations in drug target susceptibility and apoptosis defects have all been implicated. Each of these topics has been reviewed elsewhere (3-9). Recently, a new type of drug resistance phenotype has been observed in some MDR cancer cell lines that can reduce the effectiveness of drugs by limiting their access to intracellular compartments housing drug targets. Considering the fact that most drug targets are indeed contained within specific intracellular compartments, the ability of a drug to accumulate into these sites is a critical determinant in the observed therapeutic response or lack thereof. Using cultured cancer cells, several research groups have demonstrated an altered intracellular distribution pattern of drugs in MDR cell lines relative to drug sensitive lines (10-12). Specific cancer cell lines in which this redistribution occurs will be reviewed. This review also outlines some of the known driving forces responsible for promoting drug accumulation into specific cellular compartments of cells and will subsequently address, mechanistically, how the development of MDR can result in changes in intracellular drug distribution. Finally, potential strategies that may be useful in combating this particular resistance phenotype will be discussed.

3. INTRACELLULAR LOCALIZATION OF DRUG MOLECULES – A MECHANISTIC OVERVIEW

Research in the past few decades has provided us with intimate knowledge regarding the nature of targets that drugs are designed to interact with. Moreover, much is known about the precise intracellular localization of such targets; the majority of which are contained within specific organelle populations. Unfortunately, little is known regarding the intracellular distribution of small molecule type drugs. As anticipated, the complexity of the intracellular environment limits our ability to unequivocally rationalize exact mechanisms for drug distribution within cells. However, many cellular conditions exist that can be

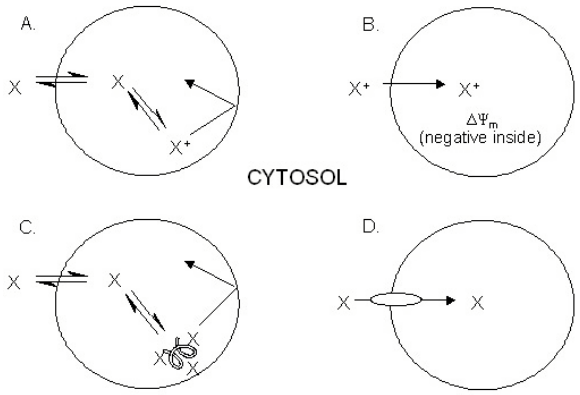

Figure 1. Schematic representations of mechanisms for intracellular drug sequestration. A.) pH partitioning, B.) Electrochemical gradient, C.) Macromolecular binding and D.) Active transport. Refer to text for detailed explanation.

expected to predictably influence the intracellular localization of drugs.

In the most general view of the process, the ability of a drug molecule to permeate lipid bilayers will play a defining role in its intracellular localization. Very polar and/or large molecular weight molecules with poor membrane permeability are typically limited to endocytic uptake and end up in the lysosomes of cells, which are the terminal endocytic compartments. Alternatively, membrane impermeable molecules may be substrates for endogenous transporter proteins (i.e., nutrient transporters) located on the plasma membrane and may be directly translocated into the cell cytosol. Recent interest in gene delivery to cells has initiated an area of drug delivery research, which attempts to devise mechanisms, which promote either the transfer of such polar molecules either directly across the plasma membrane or through bilayers of endocytic compartments (13-14). This review will not address these drug delivery challenges but will instead focus on the intracellular fate of membrane permeable small molecules that can freely diffuse across biological membranes, including the plasma membrane of cells. Considering the fact that most commercially available drugs are membrane permeable, understanding the intracellular distribution of these drugs is a highly important area of research. As will be discussed, a number of factors can influence the intracellular distribution of drug molecules thus directly affecting a drug's activity.

Following passive diffusion across the plasma membrane into the cell cytosol, the subsequent distribution within the cell is an intricate process governed by many factors. Firstly, one must realize the extensive compartmentalization of mammalian cells; over half the total cell volume is comprised of membrane bound compartments/organelles into which drug molecules may partition (15). These compartments are designed to carry out specialized cellular functions and hence have unique properties, such as intralumenal pH, electrochemical potential, lipid bilayer composition and resident proteins. Such properties/conditions can provide driving forces for the accumulation of drugs into distinct organelles to levels greater than the concentration contained within the cell cytosol.

As early as the nineteenth century investigators, through empirical observations, have noted that selected compounds had the tendency to accumulate into specific cellular compartments. von Gerlach, in the year 1858, was among the first to make such an observation when he noted that the biological stain carmine appeared to concentrate in the nuclei of animal cells. He speculated that such accumulation was a result of attraction between the dye and intra-nuclear components (16). Since this time extensive research has allowed us to rationalize the molecular basis for the nuclear accumulation of carmine, noted by von Gerlach, as well as the accumulation of additional compounds in the array of compartments contained within mammalian cells.

The phenomenon known as pH partitioning is one of the better-understood mechanisms for the selective accumulation of weakly acidic/basic drugs within cellular organelles. The theoretical basis for pH partitioning has been described in detail elsewhere (17). Briefly, this mechanism is applicable for the distribution of drug molecules between two compartments having different pH values separated by a biological membrane. In the context of a cell, this is referring to the pH values of the cell cytosol and the intralumenal environment of a membrane bound organelle. The magnitude of the pH differential and the ionization constant (pKa) of a drug are two principal determinants of the degree of drug accumulation in intracellular compartments. For a pH partitioning type event to occur the ionized form of the molecule must have no intrinsic membrane permeability across the organelle lipid bilayer; however, even if the ionized form of the compound has reduced membrane permeability relative to the unionized form one can observe a pH partition type accumulation in a time dependent manner. A simplistic illustration of this phenomenon is shown in Figure 1A. In this figure, "x" represents a weakly basic molecule permeating into a relatively acidic compartment from the cytosol. The schematic is also applicable for pH partition mediated accumulation of a weak acid in an organelle that has a slightly alkaline pH compared to the cytosol (in this case "x" would be a weak acid). It is obvious yet important to note that such pH partitioning type mechanisms cannot occur for neutral molecules or for molecules whose pKa is far removed from the pH values associated with the cellular environments in question. For the latter scenario, the degree of ionization is virtually unchanged regardless of the cellular compartment in which the drug is located and thus a pH partitioning type mechanism is not realized.

The specific pH values associated with intracellular compartments have been evaluated in numerous cell types and these measurements vary to some degree depending on the cell type. Typically, cytosolic pH is near neutrality (pH ~7.2); alternatively, compartments of the endocytic pathway are relatively acidic (pH ~ 4.5 - 6) with the lysosomes being the most acidic (18-19).

Mitochondria can have slightly alkaline pH values (~8.0) that are greater than cytosolic pH (19).

There are numerous examples where pH partitioning mechanisms have been shown to be responsible for influencing the intracellular distribution of various molecules. This phenomenon has been exploited in the creation of weakly basic vital stains for labeling acidic organelles of cells and is widely used in cell research; an array of such molecules, including so called Lysotrackers and Lysosensors are commercially available from Molecular Probes (20-21). These are all weakly basic compounds that would exist to a greater extent in the ionized form within the lysosomes than in the cytosol. Theoretically, weak bases with pKa near cytosolic pH are the best substrates for pH partition mediated sequestration into acidic intracellular compartments such as lysosomes (22). The overall extent of accumulation of such compounds into acidic compartments is dictated by the pH differential that exists between the cytosol and the organelle. Considering the fact that this pH differential can approach 3 pH units in some cell lines, the expected accumulation can be quite dramatic (i.e., 1000 fold difference if the pH differential is 3 pH units). Through a similar mechanism, weakly acidic compounds could be expected to preferentially accumulate in the mitochondria relative to cytosol, since mitochondria typically have a higher pH than cytosol. Rashid *et al.* (1991) have qualitatively assessed this scenario, although more work needs to be done to conclusively establish if this is truly a prominent mechanism for significant mitochondrial accumulation of weakly acidic compounds (23). However, it may be unlikely that such compounds could achieve high mitochondrial accumulation, purely based on the fact that the pH differential between cell cytosol and the mitochondrial lumen is not that pronounced.

Another important parameter that can influence the intracellular distribution of certain drugs is differences in electrochemical gradients across intracellular compartments. Mitochondria are unique organelles in that they posses a net negative inner membrane potential of about 160 mV (24). This can provide a driving force for the accumulation of lipophilic cations into these compartments. The Nernst equation relates the magnitude of membrane potential across a barrier to the ratio of concentration of highly permeable ions on either side of the barrier. According to this equation, every 61.5 mV increase in membrane potential can result in a 10-fold higher mitochondrial concentration of cations as compared to the cytosol (25). Accordingly, delocalized lipophilic cations (DLCs) that are membrane permeable in their charged form are expected to achieve mitochondrial concentrations 1000 fold higher than cytosol under equilibrium conditions. DLCs such as rhodamine123 and tetraphenyl phosphonium (MPP^+) have been shown to preferentially distribute into mitochondria by this mechanism (26-27). A simplistic illustration of this phenomenon is illustrated in Figure 1B where the DLC is depicted as "x^+"

The binding of drugs to endogenous molecules within the cell can also be expected to alter the intracellular localization of drugs. The number of binding sites, affinity and intracellular localization of these binding partners can impact the distribution of drugs in a cell. An example of this scenario where the drug-binding molecule is contained within an organelle is depicted in Figure 1C. If the drug bound molecule has dramatically reduced membrane permeability relative to the free drug, selective intracellular accumulations can be expected. The best example of this type of influence on intracellular localization of drugs is preferential nuclear accumulation of DNA binding drugs such as the anthracyclines (e.g. doxorubicin) (28). Lansiaux *et al.* (2002), in their evaluation of derivatives of the antiparasitic drug furamidine, established that the dicationic compound DB75 accumulated predominantly in the nuclei driven by a strong propensity to bind to DNA, while a monocationic analogue DB607 that lacked affinity to DNA failed to accumulate in the nuclei (29). Utilizing a series of furan-based compounds the authors were able to correlate apparent nuclear accumulation with DNA binding capacity, which was assessed by monitoring the melting temperature of isolated DNA in the presence of the compound evaluated.

Another molecule that has been reported to influence intracellular localization of cationic compounds is cardiolipin, an anionic phospholipid that is found enriched exclusively in the inner mitochondrial membrane of mammalian cells. The cationic dye 10N-nonyl acridine orange is known to bind strongly to cardiolipin, mediated by both electrostatic and hydrophobic interactions and hence the molecule accumulates in mitochondrial membranes (30). Several polycations such as spermine, neomycin and gentamycin are also thought to accumulate in mitochondrial membranes due to their affinity for cardiolipin (31). Although limited research in this area has been performed to date, it is expected that future research will dramatically improve our understanding of the distribution of drugs within cells that result from similar types of interactions with endogenous molecules.

It is noteworthy to mention that apparent enhancement of drug accumulation in target containing organelles due to interactions with macromolecules (discussed above) might not necessarily improve the therapeutic efficacy of a drug if the interacting molecule is not the target. It is the concentration of free (unbound) drug that is important since it is what typically interacts with the target to elicit the pharmacological response.

Finally, a large number of cellular proteins have been implicated in the transport of drugs across biological membranes. The human cell is thought to contain about 50 proteins that through hydrolysis of ATP could function in translocating drugs across biological membranes (32). Much of the original work on these proteins has suggested that they function in transporting endogenous and drug molecules across the plasma membrane. For example, uptake transporters such as organic cation transporters and organic anion transporting polypeptides have been responsible for the uptake of several drug molecules into cells, while efflux transporters such as P-glycoprotein and the multi-drug resistance associated protein (MRP1) are

Table 1. Drug sequestering organelles hypothesized to operate in different resistant cancer cell lines

Cell line	Sequestering organelles	References
NIH/3T3	lysosomes, Golgi, ER	41-42
DKLP	cytoplasmic vesicles	43
CHO	lysosomes	44
MCF-7	Golgi, lysosomes, recycling endosomes	45-49
V-79	cytoplasmic vesicles	50
HL-60	Golgi, mitochondria, lysosomes	51-53
P388	Golgi, secretory vesicles	52, 54
KB3-1	Golgi, lysosomes	52, 55-56
U 937	lysosomes	12, 57
EPG85	secretory vesicles	58
SW-1573	cytoplasmic vesicles	59-61
AUXB1	cytoplasmic vesicles	60, 62
K562	lysosomes, mitochondria, Golgi	10, 63-68
LoVo	golgi	69
CEM	lysosomes, Golgi	67, 70
A2780	Golgi	71
PKSV-PR	lysosomes	72
MES-SA	lysosomes	73
Bone Marrow	cytoplasmic organelles	74
AML cells	Golgi	68, 75

involved primarily in extruding drugs from cells (33-34). Such transporters have received a great deal of attention in the literature and they presumably have very wide substrate specificity. Apart from being expressed at the plasma membrane there is sufficient evidence that many of these transporters may be expressed on the membranes of intracellular compartments. It can be reasonably postulated that these transporters could be involved in intracellular transport of drugs in normal cells; however, currently there is little evidence to support this claim. The involvement of such transporters in the intracellular distribution of drugs in MDR cancer cells has received much more attention and is discussed in detail in the following section. An illustration of this phenomenon is shown in Figure 1D.

4. ALTERATIONS IN DRUG DISTRIBUTION IN MDR CANCER CELLS

For any drug to elicit a pharmacological response it must achieve sufficiently high solution concentrations in the microenvironment surrounding the drug target. Most commercially available anticancer drugs have nuclear targets including DNA and topoisomerase (35-36). Likewise, many newer drugs are currently being investigated that have targets in other cellular organelles including mitochondria, endosomes, Golgi apparatus and endoplasmic reticulum (37-40). Considering the fact that many of these targets are localized into discreet subcellular compartments the success of a drug lies in its ability to concentrate in the desired organelle. If a drug were specifically concentrated into a target containing organelle one would anticipate a high therapeutic efficacy; otherwise therapeutic failure would be predicted.

It is obvious that the intracellular distribution of a drug can have a profound impact on its activity; however, evaluating the intracellular distribution of most anticancer drugs is not easily accomplished. One exception is when the drug is fluorescent and the intracellular distribution can be visualized using a fluorescence microscope. Such investigations have been conducted using the fluorescent anthracycline anticancer agents (i.e., daunorubicin and doxorubicin). Intriguingly, marked differences have been observed with regard to the intracellular distribution of these agents in drug sensitive and MDR cancer cell lines. MDR cell lines often show an increased capacity to sequester drugs into cytoplasmic compartments, which in the case of daunorubicin, would result in decreased interactions of the drug with its nuclear targets. Using either daunorubicin or doxorubicin this phenomenon has been documented in numerous pairs of drug sensitive and resistant cell lines. A summary of these observations is illustrated in Table 1. As is shown in Table 1, much confusion exists regarding the identity of the drug sequestering organelle (10, 12, 41-75). These results may suggest that different MDR cancer cell lines sequester drugs into different organelles; however, it must be noted that the majority of these studies only speculated on the identity of sequestering organelles and did not perform rigorous evaluations to positively identify them. Representative micrographs revealing the differences in drug distribution in three human leukemic cells from work conducted in our laboratory are shown in Figure 2. The weakly basic anticancer drug daunorubicin is shown to have predominant nuclear accumulation in drug sensitive cell lines, while in the MDR lines the drug is sequestered away from the nucleus into cytoplasmic organelles. We have also quantitatively shown that the extent of nuclear accumulation directly correlates with sensitivity of these cells to the cytotoxic effects of daunorubicin (unpublished results), which is consistent with other similar studies (76).

What are the differences between drug sensitive and MDR cancer cell lines that cause alterations in intracellular drug sequestration? This is a central question that must be addressed in order to rationalize these

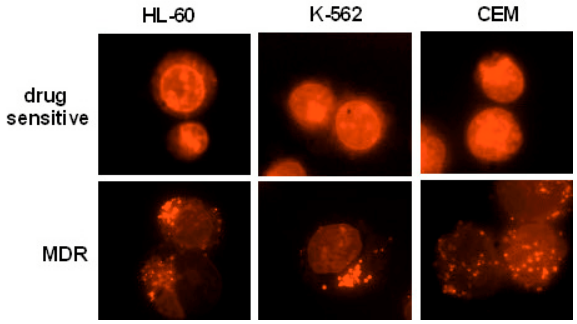

Figure 2. Distribution of the chemotherapeutic agent daunorubicin in three human leukemic cell lines (HL-60, K-562 and CEM). Top row are drug sensitive cells and bottom row are the corresponding MDR variants.

sequestration events and subsequently develop strategies to overcome redistribution and resistance. For the examples illustrated in Figure 2, one could speculate that the lowered nuclear accumulation could be due to changes in the nucleus itself. For example, the nuclear envelope of MDR cells might have reduced permeability to drugs or express efflux transporters that extrude drugs out of the nuclear compartment. Hurwitz *et al.* investigated this possibility in a human leukemic cell line U 937 (12). The authors isolated nuclei from both resistant and sensitive cell lines and compared the uptake of [^{3}H] daunorubicin in these isolated nuclei and found no difference in drug accumulation behavior. This finding hence suggests that cytoplasmic organelles from MDR cells possess an enhanced capacity to sequester daunorubicin and this in turn limits the access of drug to the nucleus.

We, along with others, have compared intracellular pH values between drug sensitive and resistant cell lines in an effort to rationalize the altered sequestration capacity (47, 77). These studies have shown that drug sensitive cancer cell lines have defective acidification of lysosomes (i.e., pH values near cytosolic pH). Considering the fact that daunorubicin and doxorubicin have pKa values slightly above neutrality it is predicted that these agents are candidates for a pH partitioning mediated sequestration into acidic compartments of lysosomes. The fact that the lysosome to cytosol pH differential is disrupted in the drug sensitive cell lines may explain why these agents are not sequestered into lysosomes and thus have a greater opportunity to enter the nucleus. As is shown in Table 2 the MDR cell lines appear to have re-established normal lysosomal pH, which in turn provides a driving force for the sequestration of these drugs into the lysosomes, away from the nucleus. The mechanistic basis for differences in pH gradients between sensitive and MDR cell lines has not been clearly elucidated to date; however, there is evidence that the gene encoding subunit C of V-ATPase is overexpressed in MDR cell lines (78). Interestingly, the defective acidification of lysosomes in drug sensitive cancer cells may provide a significant benefit to improve the overall therapeutic utility of weakly basic anticancer agents such as daunorubicin. Since normal, non-transformed cells have acidic lysosomes (Table 2, 79) they are expected to sequester such agents away from the nucleus to protect themselves from the cytotoxic effects. It is anticipated that normal non-transformed cell lines may have different degrees of lysosome-to-cytosol pH gradients depending on the cell type and thus may have different degrees of susceptibility to weakly basic anticancer agents such as daunorubicin. Unfortunately, such quantitative evaluations of these pH values have not been reported in the literature to our knowledge. Nevertheless, the defective acidification associated with drug sensitive cancer cell lines appears to play a significant role in the selective toxicity to these cells and the emergence of MDR through re-establishment of 'normal' lysosomal acidification may result in a loss of this selectivity.

Proteins involved in drug transport such as those belonging to the ABC superfamily of drug transporters have long been implicated in the cellular transport of drugs and have been evaluated for a potential role in altered intracellular distribution in MDR cancer cell lines. Studies have shown that drug transporters such as P-gp, MRP, breast cancer resistance protein (BCRP) and others are overexpressed in MDR cancer cells relative to drug sensitive lines. As previously mentioned, much of the earlier work on these proteins suggests that they are localized to the plasma membrane and function in extruding drugs from cells. This is certainly the case for many, but not all MDR cancer cell lines. Recent investigations have shown that many of these drug transporter proteins are localized to intracellular compartments (77, 80-81); however, some debate exists as to whether or not they are functional at these intracellular sites or alternatively, may simply be in transit to the plasma membrane as part of their normal biosynthetic pathway.

Although proteins that belong to the ABC family are the most studied, it is quite possible that proteins outside of this group may also play a role in MDR. The major vault protein or the lung resistance-associated protein (LRP) was originally thought to be an excellent predictor of MDR as increased resistance of certain cancer cells correlated with LRP expression without any obvious functional relevance (82). Recently, Meschini *et al.* (2002) have established that LRP expression on cytoplasmic organelles of A549 non-small lung cancer cell line correlates with cytoplasmic distribution of doxorubicin (83). The authors suggest that the lower sensitivity of A549 cells to doxorubicin, as compared to sensitive MCF-7 cells that do not express LRP in the cytoplasm, may be due to LRP mediated cytoplasmic sequestration. However, in a recent study by van Zon *et al.,* it was suggested that although LRP expression is enhanced in MDR SW1573 cells compared to the sensitive counterparts, this protein might not participate in the extrusion of daunorubicin from the nuclei of resistant cells (84). The exact role of LRP in conferring resistance to anticancer agents is not well understood yet. Nevertheless, it is reasonable to speculate that alterations in intracellular LRP expression may be an important factor to be considered in the context of MDR due to altered drug distribution.

There have been studies showing that the electrochemical gradient across the mitochondrial membrane is expanded in cancer cells as compared to non-

Table 2. Intracellular pH gradients in drug sensitive and MDR cancer cells

	Lysosomes	Cytosol	Lysosome to cytosol pH differential	Reference
Non-transformed cells				
3T3 fibroblasts	<5.0	7.40	> 2.4	79
Transformed cells (drug sensitive[a] and MDR[b])				
HL-60[a]	6.44 ± 0.17	6.96 ± 0.17	0.52	77
HL-60/ADR[b]	5.17 ± 0.14	7.12 ± 0.04	1.95	77
MCF-7[a]	>5.8	6.75 ± 0.3	<0.85	47
MCF-7/ADR[b]	5.1 ± 0.1	7.15 ± 0.1	2.05	47

transformed cells, which results in enhanced mitochondrial accumulation of lipophilic cations targeted to the mitochondria and hence their selective cytotoxicity to cancer cells (85). Although there is no direct experimental evidence, it is possible that resistance to such drugs may be due to a diminished membrane potential in corresponding MDR variants, affecting the extent of mitochondrial drug accumulation. It is also possible that other biophysical factors may be altered in MDR cancer cells affecting intracellular drug distribution that have not been discussed in this review due to lack of supporting evidence.

Thus far, this review has demonstrated that a multitude of resistance mechanisms could be responsible for altered intracellular distribution in MDR cancer cell lines; however, a possibility that has not received much attention is that multiple drug sequestration mechanisms may be operative in a single MDR cell line and that the drug sequestration phenomena could be more complex than originally thought. Recent work in our laboratory, in the MDR HL-60 human leukemic cell line, has indeed revealed this possibility. Specifically, we have shown that MDR HL-60 cells have increased expression of MRP1 on the Golgi apparatus as well as expanded intracellular pH gradients (lysosome to cytosol) as compared to their drug sensitive counterparts. An increased pH gradient across lysosomes results in sequestration of the weak base anticancer agent daunorubicin in these compartments, while MRP1 is involved in the active sequestration of a zwitterionic fluorescent dye sulforhodamine 101 in the Golgi apparatus (77). Although SR101 is not an anticancer drug, these findings may have implications for other anticancer agents with similar structural/physicochemical attributes.

5. STRATEGIES TO OPTIMIZE INTRACELLULAR DISTRIBUTION OF DRUGS

From previous discussions, it is clear that cells in general have multiple factors/conditions that can profoundly impact the intracellular distribution of drugs. When cancer cells acquire a MDR phenotype, a variety of changes can occur that have been shown to alter the intracellular distribution of drugs in such a way as to decrease the interactions between a drug and its target, thus decreasing therapeutic efficacy. Currently, it is not clear if these alterations were favored through the drug selection process as a means to promote the survival of these cells or if they occurred serendipitously. Nevertheless, it is clear that there are driving forces, both protein mediated and biophysical alterations, that can influence the intracellular distribution of drugs. As illustrated in the previous section, evidence for altered intracellular drug distribution associated with MDR is abundant; however, only limited work has been done to elucidate the underlying mechanisms. Much more work is required to completely understand these mechanisms as well as how they differ between different cancer cell types.

One of the most essential steps to be taken is to further characterize cellular differences between drug sensitive and resistant cell lines. DNA microarray studies have shown that hundreds of genes are differentially expressed between sensitive and resistant cancer cells (86); however, such studies do not provide relevant information regarding protein expression or function on drug sequestering organelles. A more viable approach would be to assess differences in protein expression/function and other biophysical factors (e.g. intracellular pH and electrochemical gradients) between drug sequestering organelles in sensitive and MDR cancer cells. Such evaluations will provide the necessary knowledge to rationalize the mechanistic basis for the specific sequestration event(s).

To ultimately combat this unique resistance phenotype it is not just sufficient to understand the mechanistic basis for sequestration events, as mentioned above, but it is equally important to be able to design drugs that are not susceptible to undesirable sequestration and can in turn reach the desired intracellular site of action despite emergence of MDR. The most practical approach to this is to evaluate organelle structure transport relationships in MDR cancer cell lines. In other words, it is essential to establish key structural/physical properties that make compounds less susceptible to inappropriate compartmentalization. Although conceptually appealing, such studies are experimentally challenging and have been traditionally limited to viewing the intracellular distribution of fluorescent compounds in cultured cancer cells. Fluorescence microscopy is a simple technique to assess gross changes in subcellular distribution of drugs; however, it is limited to fluorescent anticancer drugs such as the anthracyclines. Moreover, this approach is not quantitative and hence not amenable to establish accurate structure transport relationships for drug accumulation in intracellular organelles. Successful evaluation of substrate specificity for a sequestration mechanism warrants assays sensitive to differences in intracellular distribution resulting from minor structural modifications. Also, such assays need to be adaptable to drugs regardless of their fluorescent properties since most drugs are not fluorescent. One such approach was published by Andrew *et al.* (1997) who evaluated lysosomal accumulation of weak bases based on their ability to offer osmotic protection to these organelles

(87). Our laboratory has developed and utilized various cell fractionation approaches to evaluate intracellular distribution of drugs that are independent of fluorescent properties of drugs (88). This approach involves isolation of intact organelles from cells pre-incubated with a given drug. Several techniques including density-based separation, immunoisolation, magnetic chromatography etc., have been described in the literature for the isolation of subcellular compartments. Drug contained within the isolated fractions can be extracted and quantitated using high performance liquid chromatography coupled with a suitable detector. The obvious advantage of this approach is that it is not limited to fluorescent compounds. A mass spectrometric detection system would enable quantitation of compounds irrespective of their fluorescent properties. A significant concern with the cell fractionation approach is the potential diffusion of accumulated compounds from isolated cellular fractions, which may result in an underestimation of actual concentrations within intracellular compartments prior to isolation. Using model compounds and appropriate experimental conditions we have shown that diffusion had little effect on our quantitative estimations (88). This would be true as long as the sequestration mechanisms responsible for the accumulation of compounds within specific intracellular compartments (i.e., pH gradients, DNA binding etc.) are not disrupted during isolation. Recently, we have made progress in quantitative structure transport evaluations using the cell fractionation approach and have shown that permeability characteristics of weak bases can predictably influence the extent of their lysosomal versus mitochondrial accumulation in MDR HL-60 cells (89). Regardless of the sequestration mechanism, similar structure transport relationships will provide us with vital structural information required to design anticancer agents with desired intracellular distribution behavior. One of the drawbacks of structurally modifying compounds to manipulate drug disposition is the potential loss of therapeutic activity, in which case permanent structural modifications may be undesirable. Novel prodrug strategies may be required to overcome this concern.

A mechanistic understanding of sequestration processes with appropriate assays to evaluate structure transport relationships may facilitate incorporation of structural attributes into drug molecules that could not only avoid sequestration into unwanted sites, but perhaps promote specific delivery to target containing organelles. Such efforts will provide us with the necessary knowledge to overcome this unique and poorly understood drug resistance mechanism.

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Send correspondence to: Jeff Krise, Ph.D, 2095 Constant Ave., Simons Labs 236B, Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, KS 66047-372, Tel:785-864 2626, Fax: 785-864 5736, E-mail: krise@ku.edu

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