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RELEVANCE OF THE CHEMICAL CHARGE OF RHODAMINE DYES TO MULTIPLE DRUG RESISTANCE

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Abstract—Previously, we have shown that multiple drug resistant (MDR) Friend leukemia cells (FLC) are cross-resistant to the positively-charged dye, Rhodamine 123 (Rho 123), and that this resistance can be reversed by verapamil (VER). In the present study we used two zwitterionic rhodamine analogs, Rhodamine 116 and Rhodamine 110, and another positively-charged analog, Rhodamine 6G, to determine whether drug accumulation, resistance and modulation were affected by changes in the charge of these compounds. While there was no differential sensitivity between sensitive and resistant FLC to zwitterionic rhodamines, there was marked differential toxicity between these cell types for the positively-charged analogs. The IC_{50} values were 1000- and 100-fold greater in resistant than in sensitive cells for Rho 123 and Rho6G respectively. Intracellular drug accumulation was significantly higher in sensitive as compared to resistant cells for both Rho 123 and Rho 6G, but little difference in drug uptake between these two cell types was observed for Rho 110 and Rho 116. It was also found that the intracellular to extracellular ratio of the positively-charged compounds was greater than unity in both sensitive and resistant cells whereas for the zwitterionic analogs this ratio was less than 1. Furthermore, this ratio of drug uptake was found to be significantly higher for Rho 6G than for Rho 123, which correlated with the high oil:water partition coefficient of Rho 6G (115.6).

In MDR cells, verapamil increased Rho 123 and Rho 6G accumulation by 9.4- and 8.6-fold respectively. In addition, IC_{50} values in resistant cells were reduced >100-fold for Rho 6G and >1000-fold for Rho 123 in the presence of 10 μg/ml of verapamil. In contrast, <2-fold reduction of IC_{50} values for both of the zwitterionic analogs could be obtained under the same conditions. These results indicate that the chemical charge of rhodamines plays an important role in their differential accumulation, cytotoxicity and sensitivity to modulators such as verapamil, in sensitive and multi-drug resistant cells. The data also suggest that increased lipophilicity of the positively-charged rhodamines may increase their ability to accumulate in, and subsequently kill, MDR cells.

Manipulation of plasma and or mitochondrial membrane potentials has been shown to influence the cellular uptake and localization of positively-charged rhodamines [1] and other lipophilic cations [2]. Recently, we have shown that Friend leukemia cells (FLC) with acquired multiple drug resistance (MDR) have reduced membrane potentials when compared to their sensitive FLC counterparts [3]. These results correlate with previous data in which we found that multiple drug resistant FLC are cross-resistant to the positively-charged rhodamine analog, Rhodamine 123 (Rho 123), and accumulate significantly less of this drug than the sensitive FLC line [4]. Thus, lowered membrane potentials could be a contributing factor in decreased uptake of, and consequent resistance to, Rho 123 in FLC.

In this paper we have used several rhodamines differing in their lipophilicity and charge to test the possibility that differential Rhodamine 123 uptake and toxicity are related to these parameters.

MATERIALS AND METHODS

Cells and cytotoxicity assay. The FLC line was derived from a clone of Friend virus-transformed 745A cells. Cells were seeded and grown as described previously [4]. The cell variant resistant to Adriamycin® (ADM) (ARN 15) was selectively derived from the above clone by continuous exposure to ADM. Resistant cells, grown for more than 150 passages in drug-free medium, maintained their resistance to ADM. To assay for cytotoxicity, exponentially growing cells were seeded at 1×10^5 cells/ml (7 ml total) and treated with various concentrations of either of the positively-charged rhodamines (Rho 123 and Rho 6G) or their zwitterionic analogs (Rho 116 and Rho 110) (Eastman Kodak, Rochester, NY) in the presence or absence of 10 μg/ml verapamil (Sigma Chemical Co., St Louis, MO). At 72 hr, cells excluding trypan blue were counted and survival curves were generated as previously described [4].

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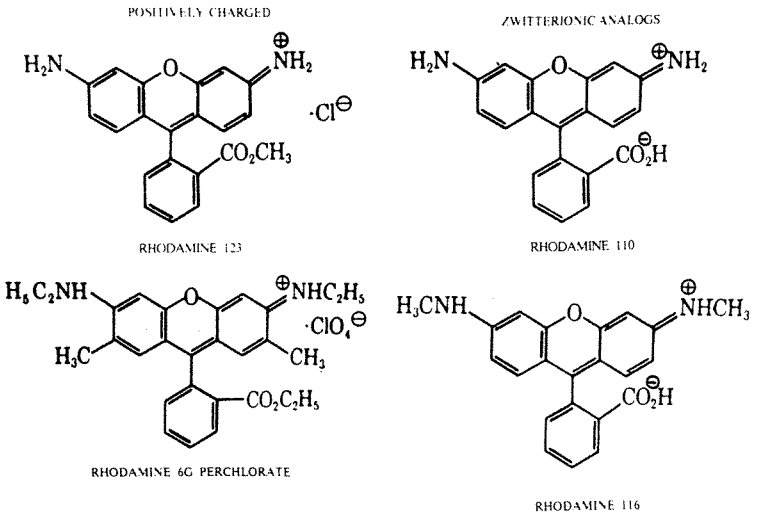

Fig. 1. Chemical structures of rhodamines at neutral pH.

Intracellular drug determinations. To measure drug uptake, 5 ml of exponentially growing cells at 10^6 cells/ml were treated in 35 mm tissue culture dishes for 3 hr at the various specified concentrations and immediately centrifuged and resuspended in ice-cold drug-free Hanks' solution. The wash medium was decanted, and the test rhodamine extracted from the pellets with 0.1 ml of butanol and subsequently analyzed by HPLC (Waters Associates, Milford, MA).

Rho 123, 110 and 116 were chromatographed isocratically with a Bondapak phenyl column (30 cm × 39 mm) using 0.035 M ammonium formate (pH 4.0):acetonitrile (75:25) as eluent at a flow rate of 2 ml/min. All rhodamines were assayed fluorometrically by emission at 530 nm after excitation at 450 nm. The retention times for Rho 123, 110 and 116 were 5.5, 4.0 and 3.0 min respectively. The limit of detection was 1 ng for Rho 123 and Rho 110 and 5 ng for Rho 116. The separation for Rho 6G was achieved by using a Bondapak phenyl column with the eluent consisting of citrate buffer (pH 4.4):methanol:acetonitrile (2:1:2) with an elution time of 6 min. The limit of detection was 10 ng.

pK and partition coefficients. Measurements of pK were performed by pH titrations with a conventional pH meter. Though all the studied rhodamines exhibit a reversible deprotonation at pH 10, Rho 110 and Rho 116 exhibit another pK value at 4.2. This value correlates with the pK of carboxylic acids, so that at physiologic pH, Rho 110 and 116 exist only as the zwitterionic molecules, as shown in Fig. 1. The existence of such dipolar zwitterions is a result of the charge of the aromatic carboxylate not interacting electromerically with the delocalized cationic xanthene ring.

Partition coefficients for each compound were evaluated using *n*-octanol and 10 mM Tris–HCl buffer, pH 7.4. The concentration of each rhodamine compound in each phase was determined spectrometrically.

RESULTS

Relative selective toxicity of positively-charged and zwitterionic rhodamines. Upon treatment of sensitive (FLC) and resistant (ARN 15) cells with rhodamines, we found that for the positively-charged analogs (pK_a values >10, see below) there were large differences in IC_{50} values between the two cell variants (Figs. 2A and 3A). Thus, the difference in IC_{50} values between sensitive and resistant FLC was 1000-fold for Rho 123 and 100-fold for Rho 6G. In contrast, IC_{50} differences in these same types were negligible for the zwitterionic rhodamine analogs (Rho 110 and 116) (Figs 2C and 3C).

Since verapamil has been shown to reverse MDR and increase drug accumulation in these cell types [5] and others [6], we tested its effects on enhancing the cytotoxicity of the four rhodamine analogs used in this study (Figs 2B, 2D, 3B and 3D). Verapamil reversed a large proportion of resistance to both of the positively-charged rhodamines (Figs 2B and 3B), while having comparatively little effect on resistance to the zwitterionic analogs (Figs 2D and 3D). In sensitive cells, the cytotoxicity of both the positively-charged and zwitterionic analogs was not appreciably enhanced by co-treatment with verapamil (Figs 2 and 3).

Differential accumulation of positively-charged versus zwitterionic rhodamines. To determine whether differences in drug accumulation between sensitive and resistant FLC were related to chemical charge, cells were exposed for 3 hr to each of the four rhodamines and assayed for intracellular drug accumulation by HPLC. To attain maximal intracellular levels for each drug with 3-hr exposure (time at which sensitive cells are still viable), extracellular drug concentrations were determined for each analog and cell type. For the positively-charged rhodamines, Rho 123 and 6G, drug uptake was 9.4- and 8.6-fold higher, respectively, in sensitive versus resistant cells (Fig. 4, panels A and B). This factor was reduced markedly to less than 2 when cells were exposed to the zwitterionic rhodamine analogs (Fig. 4, panels C and D).

Table 1 demonstrates that, when the amount of dye each cell type was exposed to is compared to the total amount taken up by the cells, the uptake ratios

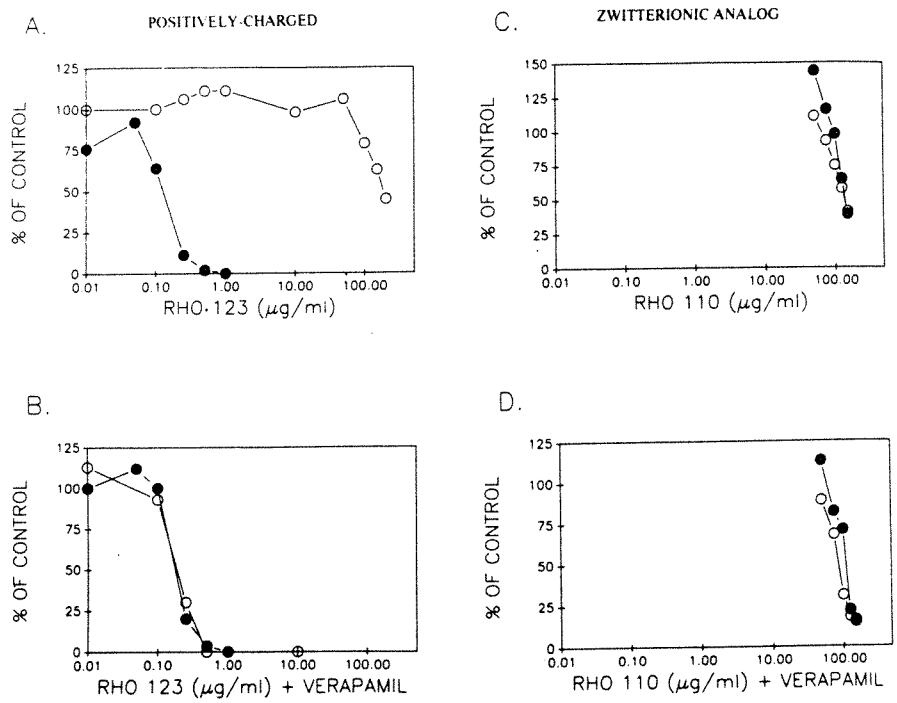

Fig. 2. Cytotoxic effects of Rho 123 and its zwitterionic analog Rho 110 on FLC (●) and ARN 15 (○) cell lines. Cells (1×10^5/ml, 7 ml total) were treated continuously with increasing concentrations of: (A) Rho 123; (B) 123 + 10 μg/ml verapamil; (C) Rho 110; and (D) Rho 110 + 10 μg/ml verapamil, and assayed for survival after 72 hr.

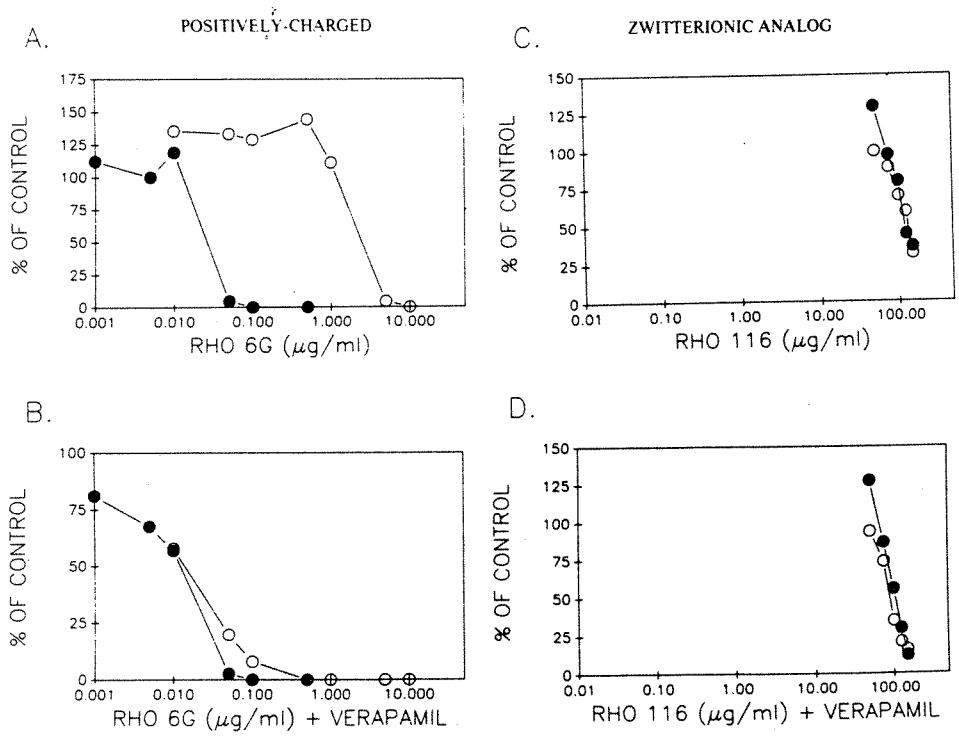

Fig. 3. Cytotoxic effects of Rho 6G and its zwitterionic analog Rho 116 on FLC (●) and ARN 15 (○) cell lines. Cells (1×10^5/ml, 7 ml total) were treated continuously with increasing concentrations of: (A) Rho 6G; (B) Rho 6G + 10 μg/ml verapamil; (C) Rho 116; and (D) Rho 116 + 10 μg/ml verapamil, and assayed for survival after 72 hr.

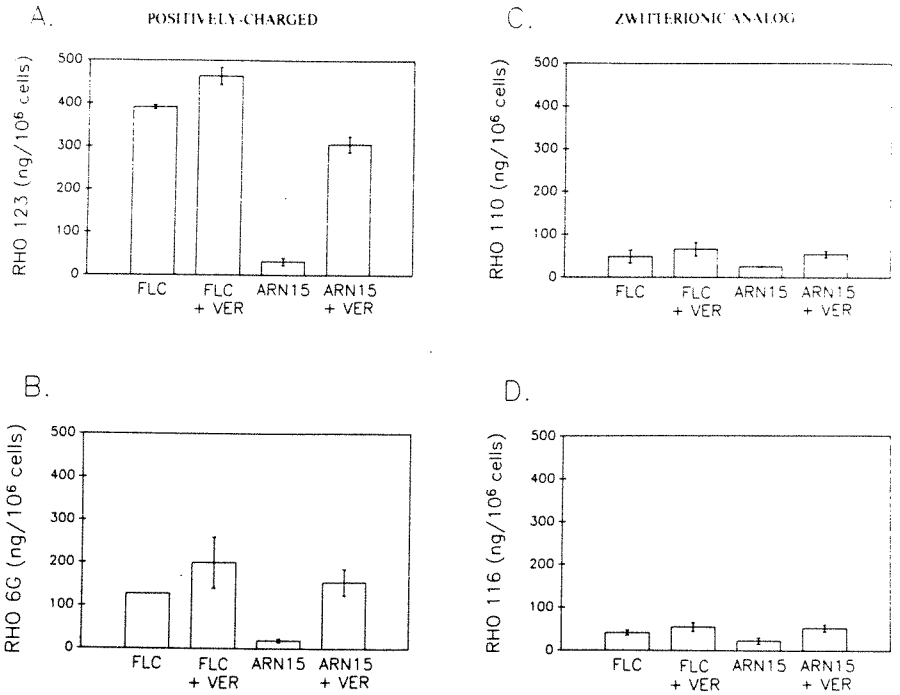

Fig. 4. Comparative intracellular drug accumulation. FLC and ARN 15 (1×10^6 cells) were exposed for 3 hr to (A) 30 μg/ml Rho 123, (B) 0.1 μg/ml Rho 6G (C) 100 μg/ml Rho 110 and (D) 100 μg/ml Rho 116 in the presence or absence of 50 μg/ml verapamil (VER). The cells were then washed, the drug was extracted with butanol, and drug concentrations were measured by HPLC.

Table 1. Intracellular versus extracellular concentrations of rhodamines

Cell type*	Drug	Ratio (intracellular/extracellular)†
FLC	Rho 6G	1270
ARN 15	Rho 6G	200
FLC	Rho 123	13.1
ARN 15	Rho 123	1.17
FLC	Rho 110	0.49
ARN 15	Rho 110	0.26
FLC	Rho 116	0.39
ARN 15	Rho 116	0.21

* FLC = Friend leukemia cells; ARN 15 = cell variant resistant to Adriamycin®.

† Intracellular = ng/10^6 cells; extracellular = μg/ml.

are in the following order: Rho 6G > Rho 123 > Rho 110 > Rho 116 for both sensitive and resistant cells. The difference in uptake between Rho 6G and Rho 123, which have net positive charge at physiologic pH, correlated with differences in their partition coefficients, i.e. the octanol: H_2O partition coefficient of Rho 6G (115.6) was much greater than that of Rho 123 (3.4). On the other hand, the two zwitterionic rhodamines, Rho 110 and 116, had smaller differences in their partition coefficients (0.8 and 5.7 respectively) yet their abilities to accumulate intracellularly were similar (Table 1).

When resistant cells were co-treated with verapamil, the accumulation of the positively-charged rhodamines was enhanced markedly (~12-fold for Rho 123 and ~9-fold for Rho 6G), whereas for the zwitterionic analogs accumulation was increased only slightly in both sensitive and resistant cells (Fig. 4).

DISCUSSION

Our findings that the cationic rhodamines (Rho 123 and Rho 6G) were selectively cytotoxic between multi-drug resistant and sensitive FLC, whereas their zwitterionic analogs (Rho 110 and 116) were not, suggest an important role for the chemical charge of these compounds in their differential accumulation and toxicity. Recently, we found that membrane potentials in MDR FLC are lower than in sensitive FLC [3]. These data agree with a recent preliminary report by Gupta *et al.* [7] in which two different MDR tumor cell lines were found to have lower membrane potentials than their sensitive cell counterparts. Since cellular membrane potentials are negative in an inward direction, differential attraction of positively-charged compounds, which are able to diffuse into the cell, will be (at least partly) dependent on the magnitude of the membrane potential. The delocalization of the positive charge of rhodamines by the xanthine ring suggests a chemistry which would allow for a charged compound to traverse a biological membrane without being hindered by a high charge density. When considering the overall charge of Rho 110 and 116, however, the position of the negatively-charged carboxylic group on the isolated benzene rings indicates that these compounds may not be able to enter the cell because of their localized high density negative charges at physiologic pH. The zwitterionic rhodamines carry

no net charge, which is consistent with their inability to accumulate to any substantial degree in either cell type. That is, the driving force of the electrically negative membrane potentials (plasma and mitochondrial) to attract and retain positively-charged lipophilic compounds obviously will not affect neutral analogs.

It has been reported previously by a number of investigators that certain resistant tumor cells have the ability to efflux a number of seemingly unrelated compounds which correlates with their ability to resist these agents [8–12]. It remains unclear, however, how this is accomplished. Based on studies done with metabolic inhibitors and manipulation of glucose levels, some authors have proposed an active efflux pump system to explain, at least in part, lowered drug accumulation in these types of resistant cells [8, 9, 13]. In view of our findings here, it is not surprising that most of the compounds found to be actively effluxed from MDR cells are positively-charged. Recently, others have attempted to link this pumping system to one found in bacteria [14]. Homology in the amino acid sequence of a protein in bacteria and one found in resistant cells (P-180 glycoprotein) lends support to the idea that these pumping systems are similar [15, 16]. The question remains of whether the appearance of the glycoprotein in the plasma membrane (acting as a pump for positively-charged compounds) is related to a lowering of membrane potential. A possible explanation could derive from alterations in ion flux as a result of glycoprotein expression in MDR cells.

Regardless of the mechanisms responsible for enhanced efflux of drugs from tumor cells which display the MDR phenotype, there should be a finite number of molecules that can be accommodated by this system in a given period of time. Thus, Rho 6G with its higher partition coefficient (115.6) relative to that of Rho 123 (3.4) would be expected to be transported more rapidly through the cell membrane and, therefore, be more able to overcome the efficiency of the MDR efflux "pump" system. In further support of this concept, the positively-charged fluorescent membrane potential probe DiOC6(3), which has a partition coefficient of 644, kills both sensitive and resistant cells at nearly the same dose (data not shown). If our hypothesis is correct, then it would appear that, in general, as we increase the lipophilicity of positively-charged (delocalized) compounds we increase their abilities to accumulate in, and subsequently kill, MDR cells. Thus, even though membrane potential differences will dictate differences in overall intracellular accumulation of these compounds, their lipophilicity will also be a factor in determining their ability to override the efflux pump. Although our experiments do not distinguish definitively between the effects of the efflux (glycoprotein) pump and those of membrane potential on the compounds studied here, they suggest that efflux pump will be most effective on positively-charged compounds with low partition coefficients. However, since drugs can enter and accumulate in cells via several different mechanisms, if a given compound were to be transported intracellularly independent of membrane potential, then not being positively-charged at physiologic pH could be an advantage in overcoming the MDR system.

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