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Relationship of Chemical Charge of Anticancer Agents to Increased Accumulation and Cytotoxicity in Cardiac and Tumor Cells : Relevance to Multidrug Resistance

Cardiac-muscle, a number of carcinoma cell lines and Friend leukemia cells accumulate relatively high amounts of positively-charged anthracyclines and xanthene dyes. The high intracellular accumulation of these positively-charged compounds correlates with the high electronegative plasma membrane potentials found in these cells and the absence or decreased levels of p-glycoprotein. In contrast, the respective resistant cell counterparts, cardiac fibroblasts, normal epithelial cell lines, and MDR FLC have lower accumulation of positively-charged compounds, increased expression of mRNA MDR_1 (not tested in cardiac fibroblasts), and lower plasma membrane potentials. Thus, chemical charge plays an important role in the selection between intrinsically sensitive cardiac-muscle, some carcinomas, and Friend leukemia cells and their intrinsic or acquired multi-drug resistant cell counterparts. Design of future anti-cancer drugs taking these points into consideration might lead to agents that will be better able to overcome cancer cell drug resistance will relatively lower cardiotoxicity.

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Introduction

Our interests in cancer drug selectivity began 12 years ago when one of us working at the Sidney Farber Cancer Institute became interested in the mechanism(s) responsible for Adriamycin-induced cardiotoxicity. The idea that an anti-cancer agent which supposedly kills cells by affecting their replicating machinery was also toxic to a non-replicating tissue such as the heart was an intriguing problem. With the rather naive belief that Adriamycin was an effective anti-cancer agent because it was selectively toxic to tumor cells, we reasoned that perhaps there was a relationship between some tumor and cardiac cells in their sensitivity to this drug. Twelve years

later we believe that there is compelling evidence to suggest that high negative membrane potentials in some carcinoma and cardiac cells influence their preferential accumulation of, and subsequent increased cytotoxicity to, positively-charged agents of which Adriamycin is one. In conjunction with these findings, we have recently reported that Friend leukemia cells (FLC), with acquired resistance to Adriamycin which have the typical multi-drug resistant phenotype, i.e. accumulate lower levels of positively-charged compounds and express high amounts of MDR-1 mRNA, have lower membrane potentials as compared to their drug-sensitive parental cell counterparts [1].

We will review evidence here which suggests that the chemical charge of MDR drugs is a common factor which influences their differential accumulation and subsequent toxicity in sensitive and MDR cells. Furthermore, it will be illustrated how electronegative membrane potentials, that have been found to be lowered in cells with intrinsic and acquired MDR (which express MDR_1 mRNA), affect the uptake and toxicity of positively-charged drugs. Data will be presented which indicates that Adriamycin does preferentially accumulate in some carcinoma cells shown to have high membrane potentials similar to cardiac-muscle cells. We will also show that cardiac-muscle cells, accumulate high amounts (relative to cardiac derived non-muscle cells) of positively-charged anthracyclines as well as positively-charged xanthene dyes but not their respective zwitterionic, neutral, or negatively-charged analogs. This paper, therefore, seeks to establish the importance of the relationship that exists between high cellular electrical-negative membrane potentials and increased attraction and intracellular accumulation of positively-charged compounds in a variety of sensitive (cardiac-muscle, some carcinoma, and Friend leukemia cells) as compared to multi-drug resistant cell counterparts (cardiac fibroblasts, some normal epithelial cell lines, and Friend leukemia cells with acquired resistance of Adriamycin).

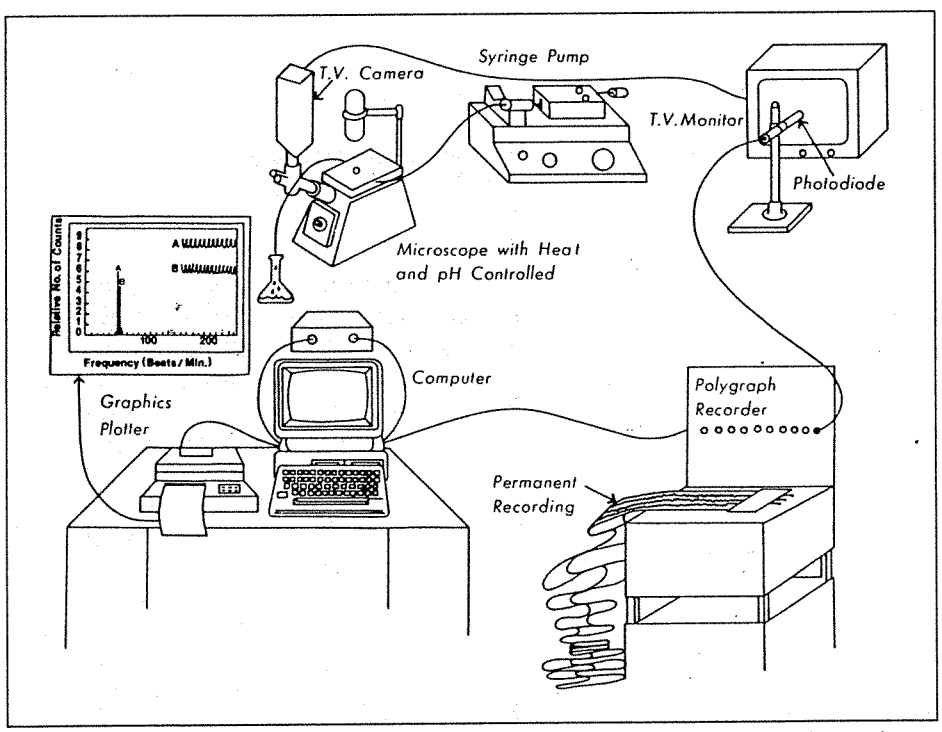

Figure 1. ***Schematic illustration of the microcomputer system for monitoring and recording*** in vitro ***cardiac cell function.*** *A video camera transmits the image of the cardiac cells placed in an environment controlled microscopic stage (pH and temperature) onto a television monitor. A photoresistor detects changes in light intensity resulting from pulsations of the cells. Each beat is permanently documented through interfacing of the photoresistor to a polygraph recorder and to a microcomputer. The construction of a real-time program for the computer allows for the measurement and computation of the interval of time between beats (to the msec level) which is instantaneously converted to beats/min and standard deviations. Thus a precise measurement of chronotropic changes in contraction is made. In addition, a program has been developed which samples the light intensity every 20 msec and a reconstruction of the contraction is produced by the computer and can be stored for later analysis. A peristaltic pump has also been added for continuous perfusion of the cells with medium. In this way, additions of test agents can be made without moving the cells, allowing for uninterrupted monitoring without need for relocating the area of contractility being measured.*

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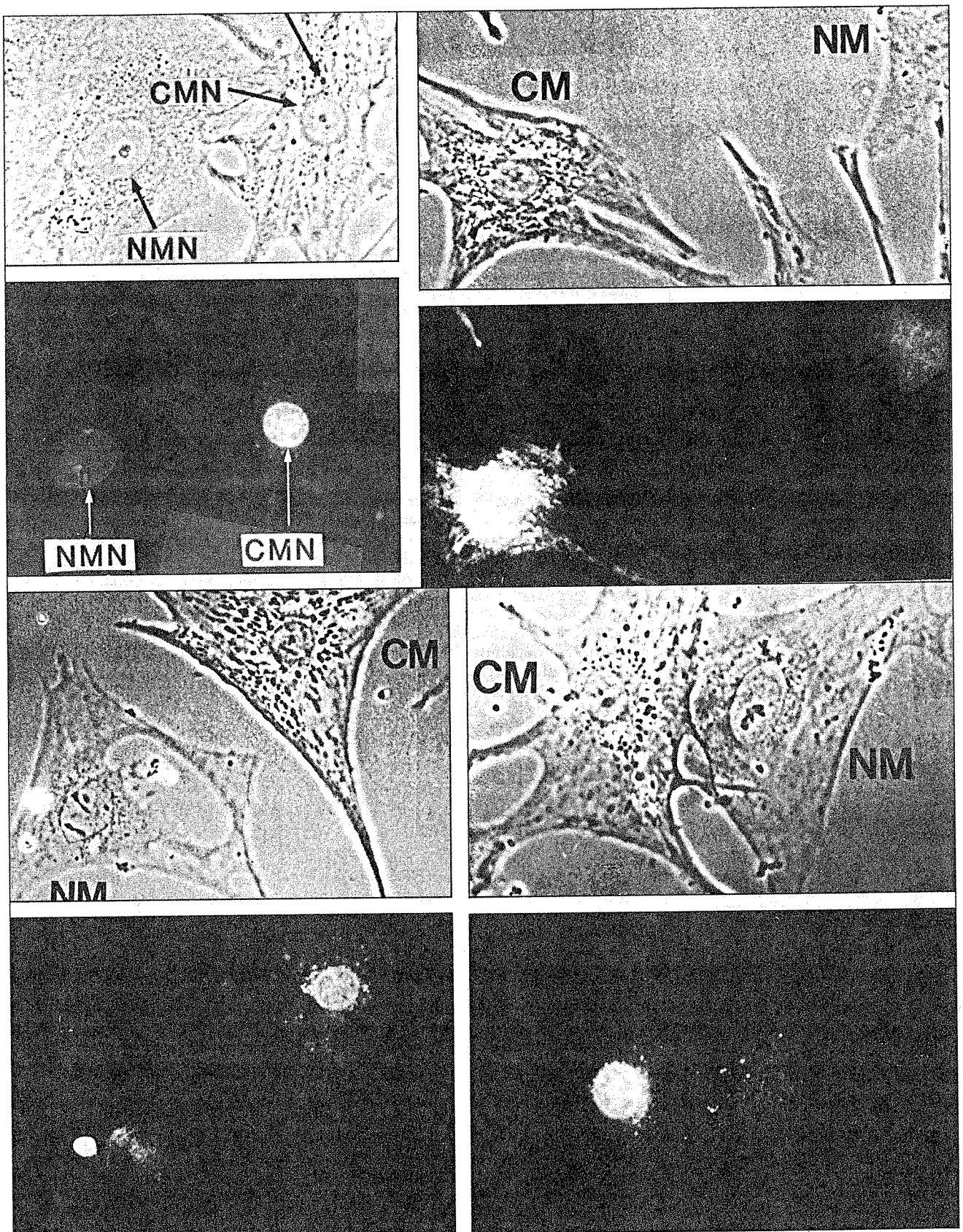

Figure 2. ***Matched phase-contrast and fluorescence micrographs of cardiac cultures treated for 30 min with 10 μg/ml of Adriamycin (upper left). Daunomycin (upper right), Rubidazone (bottom left) and Detorubicin).*** *Note greater drug-specific fluorescence of each positively-charged anthracycline in cardiac-muscle (CM or CMN) vs cardiac non-muscle (NM or NMN) cells.*

Results and discussion

Preferential accumulation of anthracyclines in cardiac-muscle vs non-muscle cells. Using 1-2 day old rat neonates hearts are excised, minced, and serially trypsinized to yield singly isolated myocytes, which upon 36-72 hr in culture, beat spontaneously. By use of this model, we have shown that many of the cardiac effects of Adriamycin reported in animals or from biopsy of patients treated with ADR could be simulated *in vitro* [2]. The system we developed for detecting and measuring chrono and inotropic changes in synchronously beating cardiac cell cultures is illustrated on *figure 1.*
Cardiac fibroblasts, which grow in conjunction with cardiac-muscle cells isolated from the newborn rat, can be used as internal controls when measuring drug uptake in cardiac-muscle cells. Cardiac cultures treated with ADR for 30 min show a striking difference between muscle and non-muscle cells as to the amount of drug taken into the cell and accumulated in the nucleus (*Fig. 2*). Testing a number of anthracycline analogs, it was seen that cardiac-muscle cells preferentially accumulated those analogs which were positively-charged at physiologic pH, Daunorubicin, Detorubicin & Rubidazone (*Fig. 2*). When a neutral analog, AD 32 was used, equal amounts of fluorescence were seen in both cell types (*Fig. 3*). Thus, a trend was detected in that those anthracyclines that are positively-charged are taken up better in cardiac-muscle vs non-muscle cells. However, the significance of the chemical charge to differential cell accumulation was not fully appreciated until working with Rhodamine 123, when we noticed a similarity in the preferential retention of this positively-charged dye in cardiac-muscle vs. non-muscle cells (*Fig. 4*).

Preferential retention of positively-charged xanthene dyes in cardiac-muscle vs non-muscle cells. The importance of the positive charge of xanthene dyes for their specific localization at mitochondria, which are known to have high electronegative membrane potentials, established by Lan Bo Chen and his colleagues [3] suggested to us that perhaps the similarity in charge of these compounds and that of the anthracyclines could account for their preferential uptake in cardiac-muscle cells. Testing a number of other rhodamines and xanthene dyes, we found that those which were positively-charged at physiologic pH, Rhodamine 123, Rhodamine 6G and Saffranin O, were preferentially accumulated in, and cytotoxic to, cardiac-muscle cells [4]. In contrast, zwitterionic analogs Rhodamine 110 and Rhodamine 116 did not select between these two cell types.

Increased accumulation and cytotoxicity of positively-charged xanthene dyes in carcinoma vs normal epithelial cell lines. In conjunction with the observations that cardiac-muscle were retaining more Rhodamine 123 than non-muscle cells, a number of tumor cells (mainly carcinomas) were also observed to retain more of this dye in their mitochondria than normal epithelial cells when treated for short periods (15 min) and monitored hours lated [5]. This selective retention was exploited when a number of carcinoma cells which were able to retain Rhodamine 123 for prolonged periods were killed with continuous exposure to this dye while normal epithelial cell lines were relatively unaffected [6, 7, 8]. The importance of the positive-charge in this selective toxicity became clearer when we found that only those xanthene dyes that were positively-charged at neutral pH were selectively killing carcinoma but not normal epithelial cells [4].

Higher plasma membrane potentials in Adriamycin-sensitive carcinoma vs intrinsically resistant normal epithelial cell lines. Since lipophilic, positively-charged drugs can be attracted across the plasma membrane and retained, by the electronegative plasma membrane potential, we examined this parameter in the high (carcinoma) and low (normal epithelial) drug-accumulating cell lines. Using microelectrodes we found significantly higher plasma membrane potentials in the carcinoma as compared to the normal epithelial cell lines [4] *(Table I)*. Thus, the differences found in membrane potentials between these carcinoma and normal epithelial cell line pairs correlated with their high and low accumulation of positively-charged drugs as well as to their relative levels of resistance to these compounds.

Drug accumulation in sensitive and multidrug resistant Friend leukemia cells. The relevance of the above data to multi-drug resistance is that intracellular levels of a variety of drugs which are positively-charged at physiologic pH have been found to be decreased in multi-drug resistant (MDR) cell as compared to their sensitive cell counterparts which correlates with their selective cytotoxicity in these two cell types *(Table II)*. In contrast, compounds which are zwitterionic or neutral have been shown to be less or non-selective in their intracellular accumulation and cytotoxicity in comparative sensitive and MDR cell types *(Table II)*. Although the intracellular levels of all these compounds does not account for the entire level of resistance to each, the overall correlation of resistance to lowered drug accumulation is strong. Thus, across such different families of compounds as the anthracyclines, xanthene dyes and vinca alkaloids, a com-

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mon feature of these chemicals for reduced accumulation in MDR cells as compared to their sensitive cell counterparts is their positive-charge. In further support of this concept, using a series of 9-anilinoacridines, Baguely and Feguson [9] have found that strongly basic or positively-charged analogs of this group of compounds showed the greatest degree of cross-resistance in P388/ADM resistant cells. Moreover, Burke *et al.* [10] have reported that the higher the pka of the amino sugar of a selected number of anthracyclines they studied, the greater the intracellular accumulation in HL-60 leukemia cells.

The MDR Friend leukemia cell (ADM-FLC$_3$) line used in our studies was developed by stepwise increases to Adriamycin and has the typical MDR phenotype, i.e. resistance is relatively stable in the absence of drug (varies between 1000-8000 fold as compared to parental FLC), expresses very high levels of MDR$_1$ mRNA, is cross-resistant to a large number of drugs (most of which are positively-charged at neutral pH), has increased efflux of positively-charged drugs, has lower total drug accumulation, and its drug efflux is blocked by verapamil [11].

Higher membrane potentials in multi-drug sensitive vs resistant Friend leukemia cells. Using flow cytometry and a computer program system developed by Gunther Valet, we measured a four-fold higher membrane potential value in sensitive as compared to MDR FLC [1]. This finding suggests that positively-charged compounds will be attracted and retained differently by these cell types. Thus, the question arises, is there a relationship between elevated expression of p-glycoprotein, active efflux, lower membrane potential, and lower positively-charged drug accumulation in the MDR cell ? Although we do not have a complete answer to this question, from the above data the following possibilities present themselves : (1) Since the rate of entry of drugs which pass the plasma membrane is partially dependent on their partition coefficients it is likely that those positively-charged agents which are highly lipophilic enter quickest and are governed most by membrane potentials ; (2) On the other hand, if the efflux pump mechanism is distinct from the force of attraction for positively-charged drugs generated by membrane potentials, then those compounds which enter slowly (such as Adriamycin) are more likely to be controlled or affected by the efflux pump. The idea is that regardless of what kind of pumping system there is, this system must have a finite capacity per time period to process these drugs. Therefore, one way this system can be overridden is by use of positively-charged compounds with high partition coefficients ; (3) Non-positively

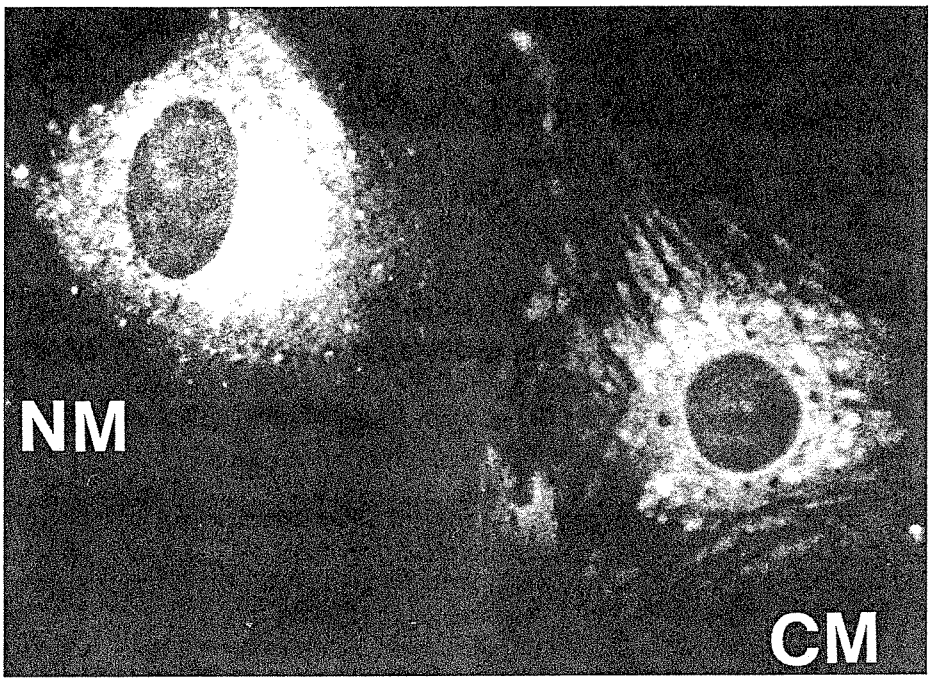

Figure 3. ***Fluorescence micrograph of cardiac culture treated with neutral anthracycline AD32.*** *Note non-selectivity of drug-specific fluorescence between cardiac-muscle (CM) and cardiac non-muscle (NM) cells for this neutral anthracycline.*

Table I. ***Membrane potential differences between cell lines which are high and low retainers of positively-charged drugs***

Cell line	Origin	Plasma Membrane potential (mV)
MCF-7	Human breast carcinoma	-83
CV-1	Normal monkey kidney	-48
HeLa	Human cervix carcinoma	-99
PtK-2	Normal marsupial kidney	-56

Table II. ***Patterns of multi-drug resistance***

Drug	Cytotoxic dose, ID$_{50}$ (ng/ml)		Positively-charged at physiologic pH
	FLC	ADM-RFLC$_3$	
Adriamycin	1.6	5 000	yes
THP-adriamycin	1.7	500	yes
Daunorubicin	11	2 000	yes
Demethoxy-DNR	3.3	130	yes
Mitoxanthrone	2.8	1 000	yes
Aclacinomycin	25	40	no
Vincristine	10	550	yes
Vinblastine	6.5	125	yes
Vindesine	3	450	yes
Rhodamine 6G	30	3 000	yes
Rhodamine 123	300	>30,000	yes
Rhodamine 110	>100,000	>100,000	no
Rhodamine 116	>100,000	>100,000	no
cis-Platinum	170	240	no
Methotrexate	5.5	5	no

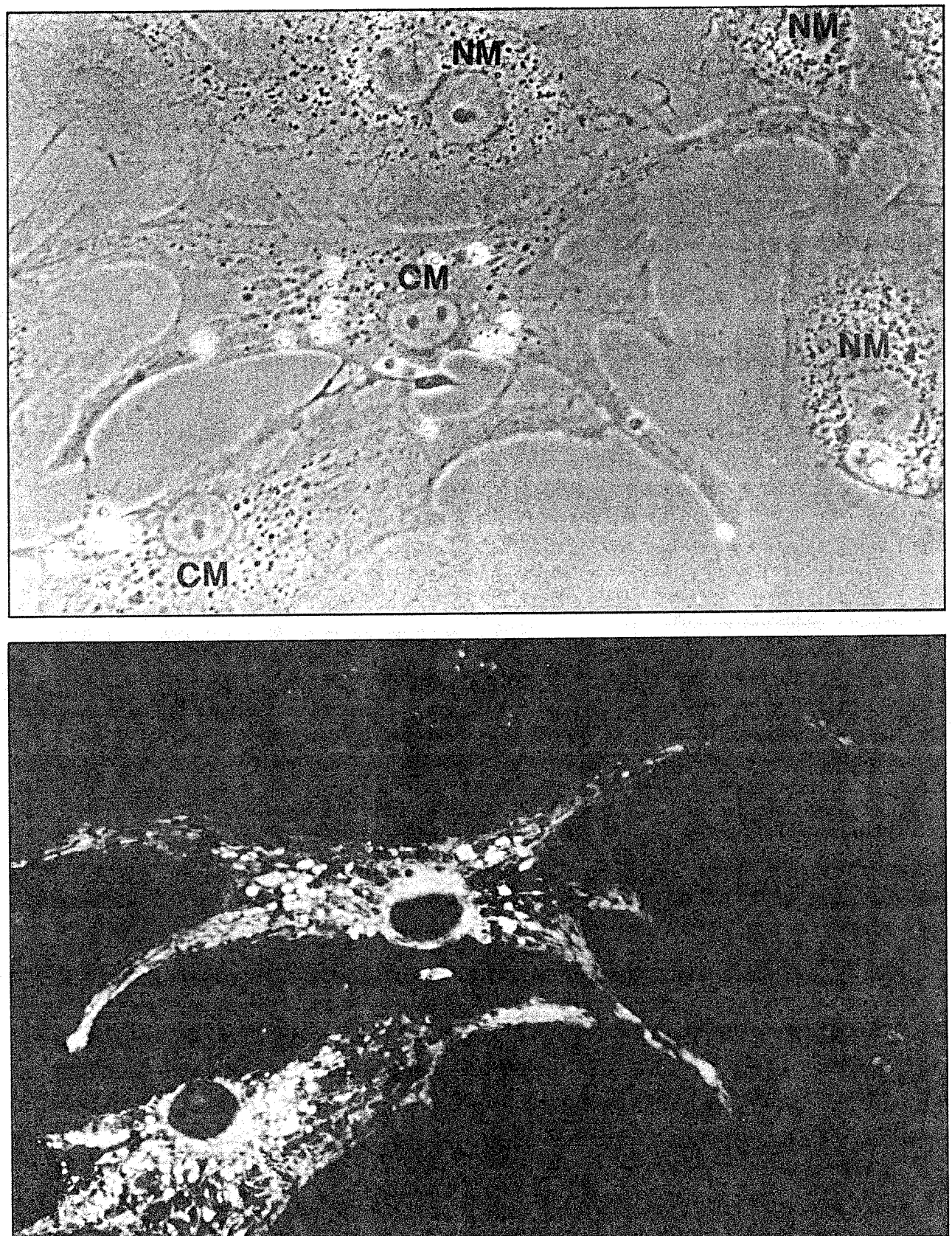

Figure 4. ***Matched fluorescence (upper) and phase-contrast (lower) micrograph of cardiac culture treated with rhodamine 123 (10 μg/ml) for 10 min and observed 24 hr later in drug-free medium.*** *Note preferential mitochondrial rhodamine 123 retention in live cardiac-muscle (CM) vs cardiac non-muscle (NM) cells.*

Table III. *Lower adriamycin levels in normal epithelial vs carcinoma cells are reversed by Verapamil*

Drug	Cell type	Intracellular drug (ng/10^6 cells)
Adriamycin	CV-1	20
ADR + Verapamil	CV-1	54
Adriamycin	MCF-7	149
ADR + Verapamil	MCF-7	110

charged compounds which can be trapped inside of cells for reasons other than membrane potentials, might more equally accumulate in sensitive and resistant cells (cumulative data suggests this to be true for anthracyclines, AD 32, 143, and Aclacynomycin A) and thus overcome one of the major mechanisms of MDR, lowered drug accumulation. In contrast, the zwitterionic or neutral xanthene dyes, Rhodamine 110 and Rhodamine 116 do not appear to bind intracellularly and hence do not get trapped inside of cells. Therefore intracellular concentrations of these non-positively charged compounds do not exceed extracellular levels ; (4) Some high molecular weight compounds could be inhibited in their influx into MDR cells due to permeability problems inherent in cells which contain high amounts of p-glycoprotein. These influx problems could be overcome by compounds which bind or modulate p-glycoprotein such as verapamil. Our data with the uptake of the fluorescent calcium probe, INDO 1AM (see below), indicates a marked effect of verapamil on the influx of this compound in our MDR cell type.

Preferential accumulation of Adriamycin in carcinoma vs normal epithelial cells : Intrinsic MDR in normal epithelial cells. Since we had previously found that positively-charged Rhodamines accumulated less in the normal epithelial cell line, CV-1, which was subsequently shown to have a relatively low membrane potential [4] as well as elevated expression of MDR_1 mRNA [12] as compared to the carcinoma cell line, MCF-7, we wondered whether these cells would show selectivity for the positively-charged anthracyclines. As illustrated in *Table III* the normal epithelial cell line, CV-1, also accumulates less Adriamycin than MCF-7 (carcinoma) and this lowered drug level can be reversed by co-treatment with verapamil. Similarly, in these cell lines differential Adriamycin accumulation correlated with differential cytotoxicity [13]. Thus, lower intracellular levels of positively-charged compounds from two different families of compounds correlated with intrinsic resistance to these agents in cells isolated from normal tissue.

In conclusion, we appear to be closer to answering the original question of whether ADR cardiotoxicity implies a relationship between cardiac and some cancer cells in their common sensitivity to this agent. The absence of an MDR phenotype and the presence of relatively high membrane potentials correlates with increased accumulation of, and sensitivity to, positively-charged compounds in the carcinoma, leukemia and cardiac-muscle cells we have studied which suggests such a relationship.

Verapamil increases uptake of Indo-1/AM with no effect on efflux in MDR FLC. It is generally assumed that active efflux of MDR drugs from resistant cells accounts for the lowered intracellular accumulation of these agents. In uptake studies of MDR drugs it is difficult to separate the effect of efflux on influx since as drug enters the cell it is also able to leave. Thus measurements of the rate of influx are complicated by the rate of efflux. To date most studies done on verapamil's effects on the transport of MDR drugs have demonstrated its inhibition of drug efflux. Our recent finding that uptake of the calcium probe INDO-1/AM in resistant cells is significantly increased by verapamil with no apparent effect on efflux suggests that MDR cells may exclude certain compounds because of an influx problem [1]. More importantly these results demonstrate a new effect of verapamil on drug transport in MDR cells. Since INDO-1/AM enters cells as a neutral species and gets trapped and enriched inside of cells by esterification to a negatively-charged species, efflux of this compound does not appear to be governed by the MDR efflux system nor reversible by verapamil. One interpretation of the above results are that MDR cells have decreased permeability towards high molecular weight compounds such as INDO-AM/1, and verapamil, either by its binding to p-glycoprotein, or by other less specific interactions with the MDR membrane, increases its permability. Further studies on this phenomenon should yield useful insights into MDR drug transport ■