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FAST KINETIC ANALYSIS OF DRUG TRANSPORT IN MULTIDRUG RESISTANT CELLS USING A PULSED QUENCH-FLOW APPARATUS

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One major feature of multidrug resistance is the reduced cellular level of drugs maintained by MDR cells. Although there is now strong evidence that drugs are actively pumped out of MDR cells, transport experiments have indicated decreased initial rates of influx at the earliest times at which measurements could be made. We have used a pulsed quench-flow apparatus to study transport characteristics of colchicine resistant MDR cells on a very fast time scale. A rapid association of daunomycin with drug sensitive cells occurred within 0.11 sec. This association is virtually absent in MDR cells. In efflux experiments performed on the same rapid time scale, greater than 50% of daunomycin efflux occurred within 0.1 sec. No substantial efflux from B1, drug sensitive cells was observed. On the other hand, vinblastine accumulation by both cell types was similar for approx. 10 seconds. Thus, kinetically, not all drugs are handled in a similar fashion by MDR cells. The pulsed quench-flow apparatus was useful in making fast time measurements of drug influx and efflux and in demonstrating the differences between drug recognition patterns by MDR cells. © 1990 Academic Press, Inc.

Multidrug resistance (MDR) is characterized by resistance to several structurally and functionally distinct compounds, reduced cellular accumulation of these drugs and the over-expression of P-glycoprotein, a 170 KDa membrane protein [1-3]. The homology of P-glycoprotein to bacterial transport permeases [4-5] together with its ability to bind ATP [4-6] and ATPase activity [7,8] indicates that this protein may function as an energy dependent efflux pump in order to maintain reduced intracellular drug levels. However, it has not been clearly established kinetically that this active efflux can entirely account for reduced steady state drug levels. Accurate measurement of both influx and efflux at short times is difficult and can be complicated by drug binding to membranes. For example, vincristine was shown to bind to Chinese hamster cells and their vincristine resistant mutants within three seconds [9]. Similarly, daunomycin-resistant Ehrlich ascites tumour cells bind daunomycin within 15 seconds [10]. Certain experimental

ABBREVIATIONS:
Multidrug resistance, MDR; pulsed quench-flow apparatus, PQF; phosphate buffered saline, PBS.

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techniques allow for fast separation of cells from drug-containing transport buffer or medium. One of these is the rapid centrifugation of samples through silicone oil which had a time resolution of 5 seconds when used to study vinblastine uptake by MDR human leukemic lymphoblasts [11]. In this study, we used a Pulsed Quench-Flow apparatus (PQF) [12] to study drug transport in parental and colchicine-resistant Chinese hamster ovary cells. The data show that a rapid association of daunomycin with drug sensitive cells occurs while a rapid efflux of approximately 50% of drug from daunomycin-loaded resistant cells was observed within the same time period.

MATERIALS AND METHODS

^{3}H-vinblastine (specific activity 14 Ci/mmol) was obtained from Amersham and ^{3}H-daunomycin (s.a. 4.8 Ci/mmol) was from New England Nuclear. Non-radioactive vinblastine was from Eli Lilly and Co.; daunomycin and Lubrol PX were from Sigma. Aqualuma, 1,2,4-trimethyl benzene based scintillation cocktail was obtained from J.T. Baker. Trypsin and powdered medium was from Gibco. The PQF apparatus has been described [12]; a schematic diagram is shown in Fig 1.

The culture of wild type (Aux B1) Chinese hamster ovary cells and a multidrug resistant variant (B30) was carried out as described previously [13]. Experiments were performed on cells during the late log phase of growth. Following trypsinization and suspension in Hepes-buffered growth medium, cells were gently spun in a roller wheel to maintain them in suspension. After 1 hr, cells were pelleted. The pellet was resuspended to a concentration of approx. 2 x 10^6 cells/ml. At least 98% of the cells excluded trypan blue.

For uptake experiments, this cell suspension was loaded directly into one of the reactant syringes. The other reactant syringe was loaded with medium containing drug at the concentrations indicated in the figure legends. For efflux experiments, the cell suspension was incubated in the presence of the appropriate concentration of tritiated drug. After 1 hr these cells were loaded into one reactant syringe; the other was loaded with drug-free medium. The use of PQF is described in the legend to Fig 1.

Samples were collected in 1 ml ice cold phosphate buffered saline (PBS). To process samples for scintillation counting, cells were pelleted in an eppendorf centrifuge and the pellet was washed with 2 x 1.2 ml ice cold PBS. The pellet was solubilized in 100 µl 10% Lubrol PX for 1 hr at room temperature. Following the addition of 1 ml Aqualuma, samples were counted in a Packard Tricarb Scintillation counter. Zero time values for uptake experiments were determined by mixing ice cold cells with ice cold drug-containing medium and processing as above. Zero time values for efflux experiments were determined by counting an aliquot of pre-loaded cells.

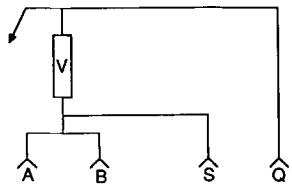

Fig 1 Schematic representation of the pulsed quench-flow apparatus. Reactants are loaded in syringes A and B; PBS in S and Q. Syringes A and B and syringes S and Q are driven by two separate motors. At time zero 22.3 µl of each reactant is driven into reaction vessel, V. To terminate the reaction 100 µl PBS is driven from both S and Q in order to quench the reaction and drive the reaction mixture from the reaction vessel to the point of collection indicated by the arrowhead.

RESULTS

Use of the PQF requires dissociated cells. It was therefore necessary to determine if the drug uptake characteristics of cell suspensions are similar to cell monolayers. Fig 2A shows that this is in fact the case: ^{3}H-daunomycin was taken up at a substantial rate by wild type cells during the course of a 15 min incubation whereas there was very little uptake by the MDR variant.

Measurements made using the PQF revealed a marked initial association of daunomycin with drug sensitive cells (Fig 2B); at approximately 0.5 sec an uptake of nearly 0.4 pmol/10^{6} cells was observed. A second, slower phase of drug accumulation which begins at 2.5 sec is in agreement with the shape of the curve seen in Fig 2A. However, no drug uptake by resistant cells could be detected over the time range of either the rapid or slow drug accumulation phases observed in drug sensitive cells.

As an attempt to determine what might be responsible for the absence of the initial rapid association of drug with resistant, as compared to parental cells, efflux experiments were performed. Fig 3 shows that after preloading MDR cells with daunomycin, approximately 50% of the total drug was released within 0.11 sec. There was very little detectable efflux of daunomycin from drug sensitive cells over the 10 seconds studied. It is important to note that reactants can only be diluted into an equal volume in the PQF. Therefore, we did not use metabolic inhibitors to load either drug sensitive or resistant cells since we felt the inhibitor would not be sufficiently diluted to measure energy dependent drug efflux. This accounts for the relatively high level of daunomycin in drug sensitive cells compared to drug resistant cells (34 pmol/10^{6} cells compared to 6 at time zero).

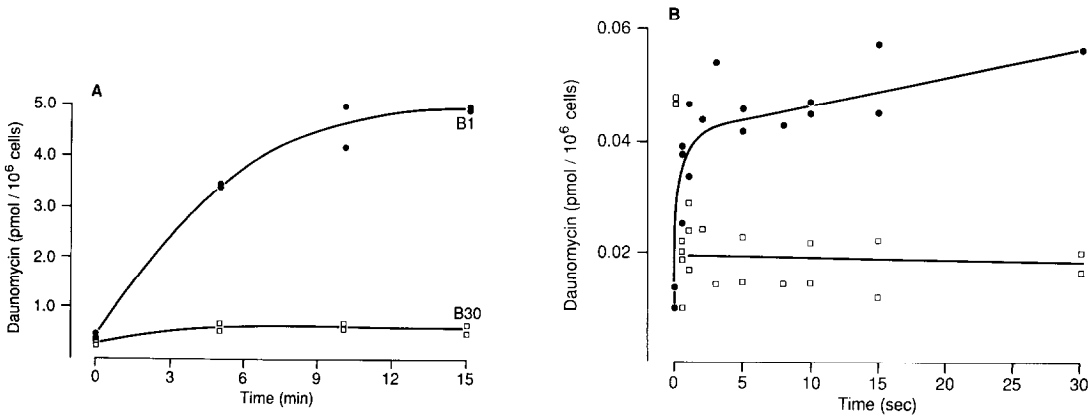

Fig 2 Time course of accumulation of daunomycin over the long term (A) and as determined by PQF apparatus (B). Suspensions of drug sensitive (B1, ●) and drug resistant (B30, □) cells were incubated for the times indicated at 24° in the presence of 1 μM ^{3}H-daunomycin.

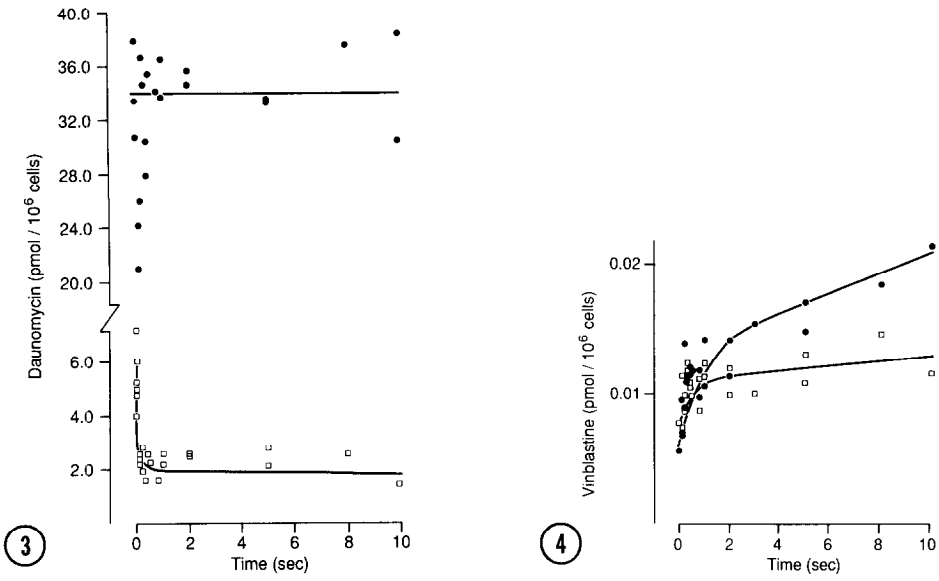

Fig 3 Efflux of daunomycin from drug sensitive (B1, ●) and drug resistant (B30, □) cells. Cells were preloaded by incubation for one hour in the presence of 2 μM ^{3}H-daunomycin. Efflux was determined at the times indicated by mixing drug-loaded cells with an equal volume of Hepes buffered growth medium.

Fig 4 Accumulation of 1 μM vinblastine by drug sensitive (B1, ●) and drug resistant (B30, □) cells as determined in the PQF apparatus.

In order to compare the uptake of daunomycin with the uptake of a representative of another major class of compounds to which MDR cells are resistant, the uptake of vinblastine was examined (Figure 4). In contrast to the finding with daunomycin, during the first second the cell-associated vinblastine was not significantly different for sensitive (B1) and resistant (B30) cells. Between 1 and 10 seconds however, sensitive cells exhibited a much greater rate of uptake than resistant cells which showed only a very slight increase in rate of vinblastine uptake. This is consistent with previously reported longer term uptake data [14].

DISCUSSION

We present here kinetic data which compares the accumulation and efflux of certain drugs by MDR Chinese hamster ovary cells at very short times (i.e.,110 ms). A PQF originally designed for measuring enzyme kinetics, was modified to measure cellular uptake or efflux of drug without compromising cell viability. We detected marked accumulation of daunomycin by drug sensitive (B1) cells within 0.11 seconds. This rapid cell association of daunomycin is virtually absent in drug resistant cells. Conversely, a rapid loss of approximately 50% of daunomycin within 0.11

seconds was observed in resistant (B30) cells but not sensitive (B1) cells. The rapid efflux of daunomycin may account for its lack of accumulation in resistant (B30) cells.

Previously published reports using various fast measurement techniques have provided conflicting data as to whether enhanced drug efflux can totally account for reduced intracellular drug levels or if impaired permeability to drug also contributes. Beck et al [11] reported that binding of vinblastine was greater to CCRF/CEM cells than their MDR variants within 5 seconds but that both these and vinblastine resistant mutants show vinblastine exodus with sensitive and resistant cells retaining approximately 10 and 40-50% of drug, respectively. Sirotnak et al [9] found that cell associated vincristine or daunomycin was about the same for both sensitive and resistant Chinese hamster lung cells at 10 seconds. Using the PQF apparatus we have been able to reduce the shortest measurement time to 0.11 seconds. Our data indicate that over this time period, there is very little, if any, accumulation of daunomycin by MDR cells, whereas its efflux is rapid and substantial from B30, drug resistant cells. Although our data are consistent with the conclusion that enhanced efflux is occurring at this short time, we cannot state unequivocally that enhanced efflux can account for the lack of accumulation of daunomycin over the same time period by these cells. Differences between our study and those cited here may reflect different measurement techniques, different cell types studied or as discussed below, alternative methods of handling different drugs by P-glycoprotein.

Another result of the present study is that MDR resistant cells handle daunomycin and vinblastine differently. That is, while daunomycin is rapidly accumulated by sensitive (B1) cells before 2.5 seconds, vinblastine accumulation by both cell types is approximately the same until one second after which the rate of accumulation by sensitive cells increases, while that for resistant cells decreases. This is consistent with the finding that Chinese hamster lung cells showed the same amount of surface bound vincristine at early times [9]. Furthermore, that MDR cells transport different drugs in a distinct manner has been documented by the fact that verapamil was unable to increase the accumulation of colchicine by resistant (B30) cells at concentrations which did enhance both vinblastine and daunomycin accumulation [14]. In addition, the fact that different anticancer agents have different abilities to inhibit photobinding of azido-vinblastine analogues to P-glycoprotein [15,16] suggests that although the drugs are all recognized by P-glycoprotein, the nature of their interactions with MDR cells varies. Our data using the PQF apparatus show that while vinblastine accumulation does not differ greatly for sensitive (B1) and resistant (B30) cells

until one second, a greatly reduced daunomycin accumulation by B30 cells is detectable even at 0.11 sec. This may be due to an enhanced efflux of daunomycin which is occurring even at this short time.

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REFERENCES

1. Riordan, J.R. and Ling, V. (1985) Pharmac. Ther. 28, 51-76.
2. Gerlach, J.H., Kartner, N., Bell, D.R. and Ling, V. (1986) Cancer Surveys 5, 25-46.
3. Kartner, N., Riordan, J.R. and Ling, V. (1983) Science 221, 1285-1288.
4. Gros, P., Croop, J. and Housman, D. (1987) Cell 47, 371-380.
5. Chen, C-J, Chin, J.E., Ueda, K., Clark, D.P., Pastan, I., Gottesman, M.M., Roninson, I.B. (1987) Cell 47, 381-389.
6. Cornwell, M.M., Tsuruo, T., Gottesman, M.M., Pastan, I. (1987) FASEB J. 1, 51-54.
7. Hamada, H., Tsuruo, T. (1988) J. Biol. Chem. 263, 1454-1458.
8. Hamada, H. and Tsuruo, T. (1988) Cancer Res. 48, 4926-4932.
9. Sirotnak, F.M., Yang, C.H., Mines, I.S., Onibe, E. and Biedler, J.L. (1986) J. Cell Physiol. 126, 266-274.
10. Danø, K. (1973) Biochim. Biophys. Acta (1973) 32, 466-483.
11. Beck, W.T., Cirtain, M.C., Efko, J.L. (1983) Mol. Pharmacol. 24, 485-492.
12. Langowski, J., Urbanke, C. and Schuppe, E. (1984) Analyt. Biochem. 142, 91-97.
13. Stanners, C.F., Eliceiri, G.L., Green, H. (1971) Nature, New Biol. 230, 52-54.
14. Cano-Gauci, D.F. and Riordan, J.R. (1987) Biochem. Pharm. 34, 2115-2123.
15. Safa, A.R., Glover, C.J., Meyers, M.B., Beidler, J.L. and Felsted, R.L. (1986) J. Biol. Chem. 261, 6137-6140.
16. Cornwell, M.M., Safa, A.R., Felsted, R.L., Gottesman, M.M. and Pastan, I. (1986) Proc. Natl. Acad. Sci. 83, 3847-3850.