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Comparative Studies of the Uptake of Daunorubicin in Sensitive and Resistant P388 Cell Lines by Flow Cytometry and Biochemical Extraction Procedures

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Summary. *The intracellular accumulation of daunorubicin as determined by flow cytometry correlates well with that as determined by extraction of the drug from cell homogenates. Two P388 mouse leukaemia cell lines showing differential sensitivity to the drug have been used to investigate the transport changes associated with resistance. Resistance to daunorubicin in these cell lines occurs through an alteration in the intracellular accumulation of the drug, resulting from the increased efflux of the anthracycline from the resistant cells. The effect of temperature, drug concentration, pH, and metabolic inhibitors on this process have been investigated. Uptake by a carrier-mediated process of the un-ionised form of the drug (pK = 8.25), coupled with an energy-dependent efflux process, is proposed as the mechanism of cellular accumulation in the case of the resistant cell line.*

Introduction

The wide use of the anthracycline antibiotics in the treatment of neoplastic disease has led to many investigations into their modes of action. A major limitation to their clinical effectiveness is the development of resistance to these agents. An understanding of the mechanisms of resistance, together with the prediction of resistance, could be of potential clinical benefit. The detection of resistance in a cellular population which may be heterogeneous in both cell type and response would best be accomplished by examination of individual cells. The technique of flow cytometry allows the rapid examination of large numbers of such cells.

Resistance to the anthracycline antibiotics has been postulated to arise by several different mechanisms. A reduced accumulation of drug in resistant cell lines has been reported in several studies [4, 7, 10, 12, 15, 17, 19, 20]. A differential inhibition of ribosomal RNA together with altered intracellular distribution and metabolism has also been reported [19], although these effects were again accompanied by a decreased uptake.

Resistant cells exclude the drugs by an energy-dependent efflux mechanism [4, 15], which is accompanied in some cases by a decreased influx [15].

The mode of action of the anthracycline antibiotics is probably dependent on the high-affinity binding to DNA [5], resulting in inhibition of the template activity of both DNA and RNA polymerase [2]. Adriamycin, when covalently linked to agarose beads, has also been shown to be cytotoxic by interaction only at the cell surface [18]. However, the effective concentration of drug on the surface of the beads which comes in contact with the cell surface is expected to be several orders of magnitude greater than that experienced by cells when the drug is free in solution. However, many other effects on cellular processes have been reported [1].

The intrinsic fluorescence of daunorubicin (DnR), coupled with speed and sensitivity of the technique of flow cytometry, enables a direct estimate of intracellular drug levels to be obtained in intact cells. Hence subpopulations of cells showing altered drug permeability may be observed.

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Materials and Methods

Cell Lines. A DnR-resistant P388 mouse leukaemia cell line (P388R) was developed from the original (P388S) cell lines by continuous exposure to increasing concentrations of the drug in vitro. Both cell lines were maintained in Fischer's medium containing 10% horse serum (Gibco). The resistant cell line was routinely maintained in the presence of DnR (0.1 μg/ml).

Growth Inhibition Studies. These were carried out by back-extrapolation of growth curves following a 1-h treatment with drug. The doubling times for the cell lines were 16.5 h (P388S) and 28.5 h (P388R). The diploid chromosome number of both cell lines was 38 ± 0.1.

Extraction of DnR. Cells were suspended in 50 ml of serum-free Fischer's medium at a concentration of $2 \times 10^5 \cdot ml^{-1}$ and incubated with DnR (10 μ*M*, 37° C) for times up to 4 h. A 45-ml aliquot was then pelleted by centrifugation (160 *g*, 10 min, 4° C), washed in cold phosphate-buffered saline (PBS), and resuspended in 2 ml distilled water prior to sonication (MSE, 20 μm peak to peak). DnR was extracted with 4 × 3 ml $CHCl_3$: isoamyl alcohol (24 : 1), and estimated fluorimetrically (λ_{ex} = 480 nm, λ_{em} = 570 nm). DNA was estimated by the method of Kissane and Robbins [9], as modified by Setaro and Morley [13] using diaminobenzoic acid (60 mg/ml). Calf thymus DNA was used as a standard. All fluorimetric measurements of DnR and DNA were carried out on an MPF-2A fluorimeter (Perkin-Elmer). Cellular accumulation of DnR was expressed as μg DnR/μg DNA. All estimations were performed in triplicate.

Cytofluorimetry. Cells, harvested in log phase, were resuspended in Fischer's medium at a concentration of between 1

and $2 \times 10^5 \cdot ml^{-1}$. DnR was added with gentle agitation to give the desired final concentration (1–20 μ*M*) and the cell suspensions were maintained at 37° C, unless otherwise stated. This corresponded to the zero time point and the cells used directly for flow cytometric determination of DnR levels. Cytofluorimetry was performed on a laboratory-built flow system [8] interfaced with a Hewlett-Packard 9845A microcomputer. Flow rates of 500–1000 cells/s were used, and each time point was measured by the accumulation of approximately 2×10^4 cells. The excitation source used was the 488-nm line of a Spectra-Physics 5W Argon ion laser. Emission was measured at 560 ± 20 nm.

The coefficient of variation (c.v.) of fluorescently labelled beads (Coulter) was ~ 2%. The c.v. was calculated from the relationship:

$$\text{c.v.} = \frac{\text{FWHM} \times 0.45}{\text{channel max.}}, \quad (1)$$

where FWHM is the full width of the peak at half maximum height, and channel max. is the channel number of the maximum value of the fluorescence distribution.

Chemicals. All chemicals used were of the highest purity available. Daunorubicin was obtained as the commercial preparation Cerubidin (May and Baker Ltd), and was used without further purification. DnR concentrations were determined spectrophotimetrically using a molar absorptivity of 9,650 $dm^3 \cdot mol^{-1} \cdot cm^{-1}$ at 480 nm.

Results

The differential sensitivity of the cell lines to DnR is shown in Fig. 1. The doses at which 50% inhibition of growth occurs are 1.5×10^{-8} *M* and 2.7×10^{-7} *M* for the sensitive and resistant cell lines, respectively.

The accumulation of DnR into both cell lines, as measured by extraction of drug, is shown in Fig. 2. The initial uptake can be seen to be very rapid, as at $t = 0$ h there are significant levels detectable in both cell lines. This time point was obtained by centrifugation and washing immediately after drug addition. Uptake is complete after 1–2 h in both cell lines. The decrease observed in the sensitive cell line at 4 h is due to cellular disruption. The sensitive cell line can be seen to contain approximately three-fold higher levels of DnR than the resistant line.

The correlation between the median fluorescence values (measured from the fluorescence distribution from flow cytometry) and the intracellular levels of DnR obtained by biochemical extraction of drug is shown in Fig. 3. A good linear correlation between fluorescence and drug content can be observed.

The uptake of DnR, as measured directly by flow cytometry, can again be seen to show differential accumulation between the two cell lines (Fig. 4). Here the sensitive line shows an approximately four-fold excess of drug over the P388R cell line when incubated with 10 μ*M* DnR. The effect of drug concentration on rate and extent of uptake is also shown. The decrease in fluorescence at longer times after incubation with 20 μ*M* DnR is again associated with cellular disruption. Membrane damage is clearly visible at this dose level by optical microscopy.

The accumulation is pH-dependent (Fig. 5). Increasing the pH from 7.0 to 8.2 causes an increase in the rate and extent of incorporation. However, the differential uptake between the cell lines is again maintained.

The temperature-dependence of the uptake of drug is shown in Fig. 6. Little accumulation is observed at 2° C. However, if the temperature is raised during the experiment the uptake process is initiated. At 42° C cell lysis took place, resulting in a loss of fluoresence after ~ 20 min in the sensitive cell line. The P388R cell line showed an even greater temperature-sensitivity, with cell loss occurring during the warming period before drug addition.

Suspension of the cells in Hank's balanced salt solution with and without glucose and in the presence and absence of the metabolic inhibitor dinitrophenol (DNP, 1 m*M*) prior to incubation with DnR was used to monitor the effect of energy deprivation on drug uptake. Glucose deprivation in the pres-

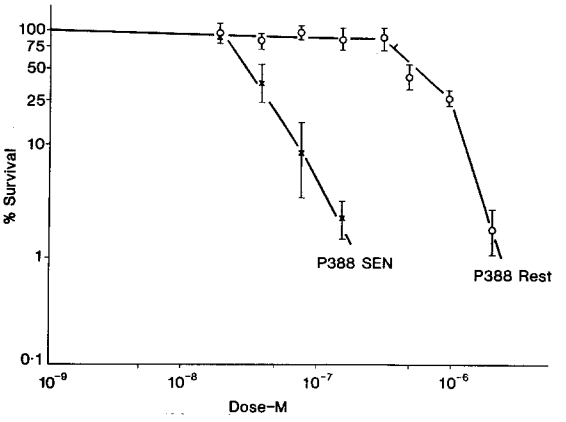

Fig. 1. Survival of sensitive (P388S, X), and resistant (P388R, O) cell lines after treatment with DnR (1 h, 37° C)

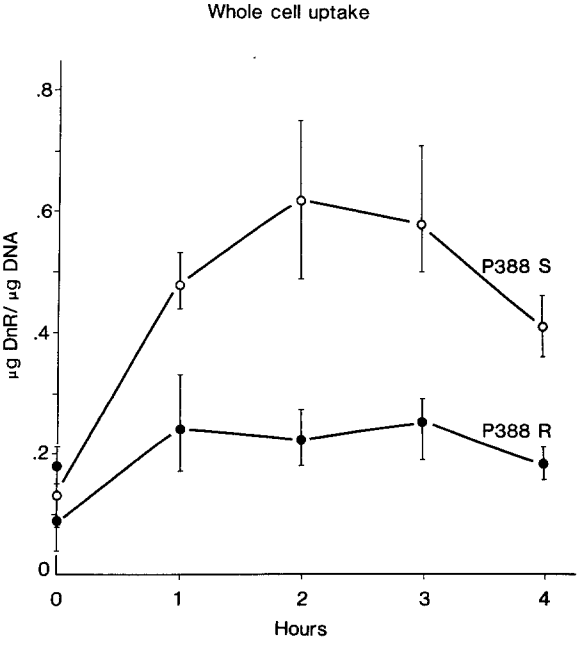

Fig. 2. Cellular accumulation of DnR, measured by drug extraction after incubation (10 μ*M* DnR, 37° C) in the sensitive (P388S, O) and resistant (P388, ●) cell lines

ence of DNP caused the P388R cell line to accumulate DnR to a level similar to that observed in the P388S cells (Fig. 7). Subsequent addition of glucose (1% w/v) caused a rapid decrease in intracellular fluorescence to a level similar to that observed in the resistant cell line incubated with drug in the presence of glucose. No similar effect was observed in the sensitive cell line on glucose addition. DNP in the presence of glucose, or glucose deprivation alone did not cause the resistant cells to show elevated fluorescence levels. This is in agreement with a similar observation made by Inaba et al. [7].

Incubation of the cell lines in medium containing 10% horse serum decreased the extent of incorporation of drug by ~ 60%. This effect could be mimicked by the use of bovine serum albumin. In the presence of serum, the rapid initial

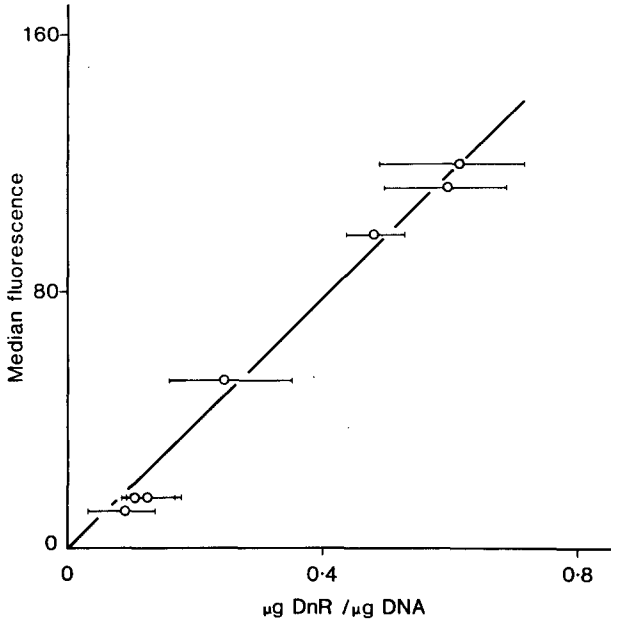

Fig. 3. Correlation of intracellular fluorescence as measured by flow cytometry with the level of DnR estimated by biochemical extraction of drug

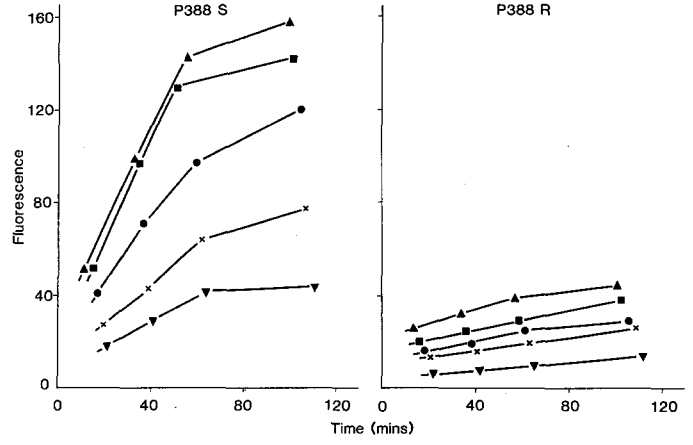

Fig. 4. Cellular accumulation of DnR, measured by flow cytometry, on sensitive (P388S) and resistant (P388R) cell lines. [DnR]: (▲) 20 μ*M*; (■) 15 μ*M*; (●) 10 μ*M*; (×) 5 μ*M*; (▲) 1 μ*M*

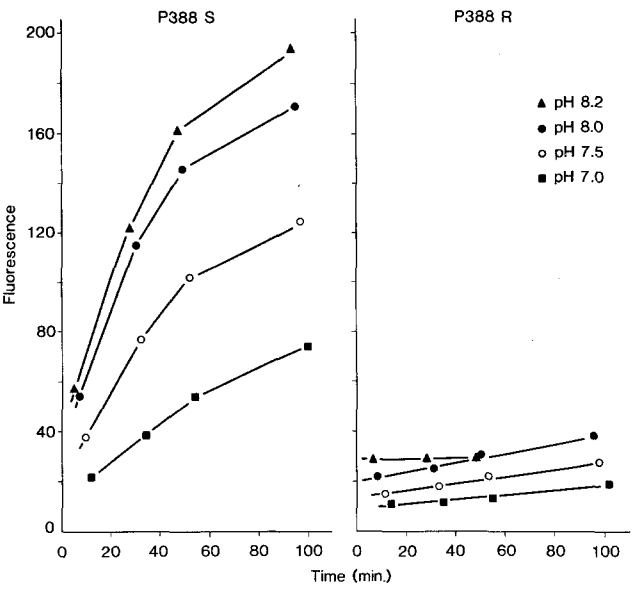

Fig. 5. The effect of pH on the cellular accumulation of DnR in sensitive (P388S) and resistant (P388R) cell lines. ([DnR] = 10 μ*M*)

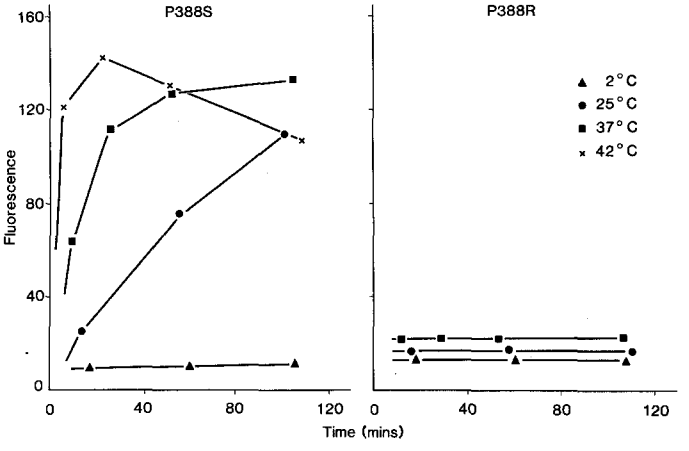

Fig. 6. Effect of temperature on the accumulation of DnR in sensitive (P388S) and resistant (P388R) cell lines. ([DnR] = 10 μ*M*)

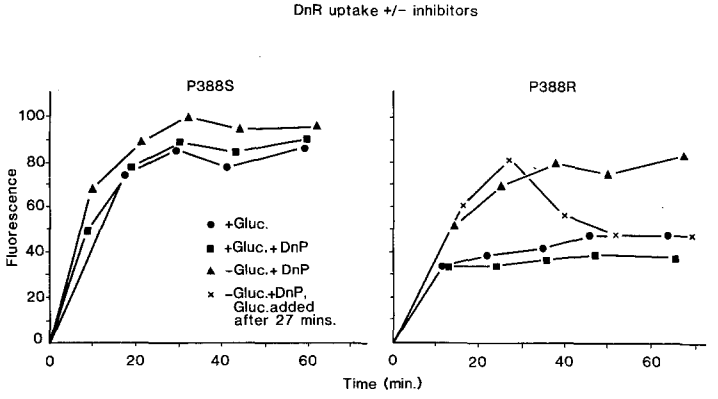

Fig. 7. The effect of glucose deprivation and the addition of DnP on the cellular accumulation of DnR in sensitive (P388S) and resistant (P388R) cell lines

binding of drug is not observed, but no evidence for DnR-albumin interactions could be obtained by optical absorption or fluorimetric methods.

Discussion

Resistance to daunorubicin, and other anthracycline antibiotics, has been attributed to a decreased accumulation of the drug [4, 7, 10, 12, 15, 16, 17, 19, 20], although this may not be the sole mechanism [4]. The ability of the P388R cell line to exclude DnR is maintained even at lethal doses. At an incubation concentration of 10 μM a value of 0.6 μg DnR per μg DNA is obtained after 2 h. This is equivalent to almost 1 drug molecule per DNA base pair (taking 2 μg DNA $\equiv 10^{15}$ base pairs) and is much larger than the known high-affinity site concentration in calf thymus DNA [3]. Hence about 90% of drug must be associated with other sites within the cell. This level, corresponding to 160 arbitrary fluorescence units in Fig. 3, would thus correspond to approx. 8×10^9 daunorubicin molecules/cell. Daunorubicin fluorescence is quenched on intercalation into DNA to a level which is approx. 15% of the free drug [6]. Anthracycline binding to proteins has also been reported [1], although no estimate of fluorescence quenching was given. A good correlation between drug content and fluorescence can be observed (Fig. 3).

The significant level of daunorubicin binding to the cells immediately after addition of drug (observed by both drug extraction and cytometry) could indicate membrane binding, an effect which has been reported by Skovsgaard [15]. The rates of uptake of DNA by the sensitive cell lines at [DnR] = 15 μM and 20 μM are very similar and could reflect saturation of a carrier-mediated uptake process.

The rate and extent of drug incorporation is dependent on the anthracycline concentration in the incubation medium. Considerable cellular damage occurs at the highest dose level (20 μM) in the P388S cell line within the time-scale of the experiment. The rate and extent of drug uptake is dependent on the pH of the incubation medium (Fig. 5). This is in agreement with the work of Skovsgaard [14], who postulated that the drug was accumulated in its non-ionised form (pKa DnR = 8.25).

The resistant cell line can be shown to accumulate levels of drug similar to that observed in the sensitive cell line after glucose deprivation together with the addition of the metabolic inhibitor DNP (Fig. 7). This effect is reversible on addition of glucose. Hence the resistance is due to the ability of cells to exclude the drug by an energy-dependent efflux process. Similar efflux processes have been described in other daunorubicin-resistant cell lines [4, 11, 14]. No similar effect is observed in the sensitive cell line.

The direct measurement of intracellular levels of daunorubicin by flow cytometry offers a very rapid and sensitive test for resistance in this cell system. As each cell is examined individually the test could be applied to heterogeneous systems.

The resistance mechanism in the P388R cell line is ascribed to an energy-dependent efflux mechanism which is absent, or very ineffective, in the original sensitive cell line.

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