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COMPARATIVE INTRACELLULAR UPTAKE OF ADRIAMYCIN AND 4′-DEOXYDOXORUBICIN BY NON-SMALL CELL LUNG TUMOR CELLS IN CULTURE AND ITS RELATIONSHIP TO CELL SURVIVAL

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Abstract—4′-Deoxydoxorubicin (4′-deoxy) is a new adriamycin analogue with a similar spectrum of antitumour activity but is significantly more lipophilic than the parent compound. We report the kinetics and uptake of the two drugs by human non-small cell lung tumour cells in monolayer culture and the relationship between intracellular drug levels and cytotoxicity. The rate and degree of cell uptake of 4′-deoxy (V_{max} = 30 ng/10^5 cells/min) was greater than that of adriamycin (V_{max} = 0.15 ng/10^5 cells/min). Although for a given intracellular drug concentration adriamycin was more lethal, on the basis of extracellular drug concentration, cell kill was virtually identical. The log cell survival vs intracellular drug concentration plot was linear for adriamycin but biphasic for 4′-deoxy. Intracellular distribution of the two drugs was followed by fluorescent microscopy and it was apparent that adriamycin was localized mainly within the nucleus whereas 4′-deoxy accumulated within the cytoplasm. Our results suggest that the relationship between intracellular distribution of the two drugs could reflect different modes of action for the drugs with respect to binding sites or could be a non-specific phenomenon, unrelated to lethal effects.

4′-Deoxydoxorubicin (4′-deoxy) is a new adriamycin analogue synthesized by removal of the hydroxyl group on the 4′ position of the daunosamine sugar (Fig. 1). The compound is less cardiotoxic than the parent drug and in a series of experimental tumours in the mouse, it showed antitumoral activity similar to and potency generally superior to that of adriamycin [1–3]. Although the derivatized drug differs only in substitution of a single hydroxyl group, this confers notably different physico-chemical properties on the compound. 4′-Deoxy is considerably more lipophilic than adriamycin and would therefore be expected to cross the lipid domain of the tumour cell membrane with greater facility. In this study, we report the relationship between intracellular levels of the two drugs and the corresponding cytotoxicity to human non-small cell lung tumour cells in the exponential phase of monolayer growth.

MATERIALS AND METHODS

Cell culture. The L-DAN cell line was derived from our own patient with squamous cell lung cancer. The cells were maintained as a monolayer in exponential growth on Ham's F-10/DMEM medium (50:50) with 8 mM sodium bicarbonate supplemented with foetal calf serum. All experiments were carried out in the exponential phase of growth and the cloning efficiency of these cells ranged between 20 and 25%.

Conditions of drug exposure and determination of cell survival. L-DAN monolayers were exposed to a range of drug concentrations for differing times. The drugs were kindly supplied by Farmitalia Carlo Erba, and were administered to the cells in culture medium after dissolution in normal saline. After drug exposure, the cells were washed twice with ice-cold phosphate buffered saline in order to remove loosely bound or surface adsorbed drug. The cells were then harvested with 0.25% trypsin in PBS, centrifuged, washed once in ice-cold medium and counted (Coulter Counter Ltd, Poole, Dorset, U.K.). The cells were then resuspended in distilled water and the resulting cell lysate was frozen and stored at −20° until drug extraction and analysis was performed.

During the clonogenic assay for cell survival, the monolayers were either exposed to the cytotoxic

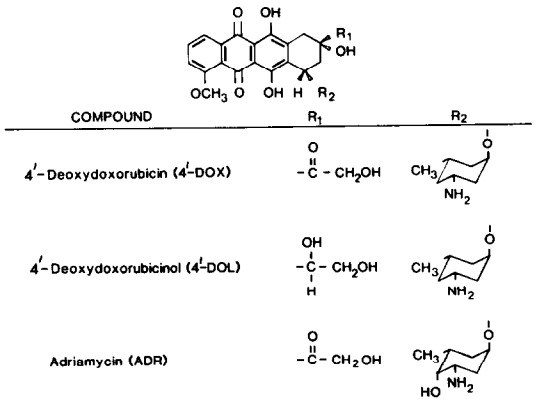

Fig. 1. The structural formulae of adriamycin, 4′-deoxydoxorubicin and 4′-deoxydoxorubicinol.

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agents for one hour over a wide concentration range (0.1–10 μg/ml) in growth medium or were exposed at a constant concentration of 5 μg/ml for differing times (15–60 min). After drug exposure the cells were trypsinized, centrifuged and washed with ice-cold medium. The cells were then diluted in medium and plated at 200 cells/ml into 5 cm diameter Petri dishes. The plates were incubated for 12 days in a humid 2% CO_2 atmosphere. The colonies were then fixed and stained with a solution of methylene blue (0.1%) in 70% ethanol and colonies of ≥40 cells were counted. Following the usual convention the cloning efficiency of the untreated cells was normalized to 100% and the cloning efficiency of the treated cells was expressed as a percentage of control survival.

Drug measurement. Intracellular levels of drug were measured by extraction from the cell lysate with a mixture of chloroform and isopropanol and subsequent analysis by high pressure liquid chromatography utilizing a fluorescent detector. This method has been described in our laboratory [4] and allows detection of both parent drug and metabolites. All solvents were of HPLC grade and the limit of detection of the assay was 1 ng. Results were expressed as ng/10^5 cells.

Determination of oil–water partition coefficient. Lipid solubility was determined by measuring the partition of the drug between *N*-butanol and Tris–HCl buffer and expressing the result as the solvent/aqueous concentration ratio [5].

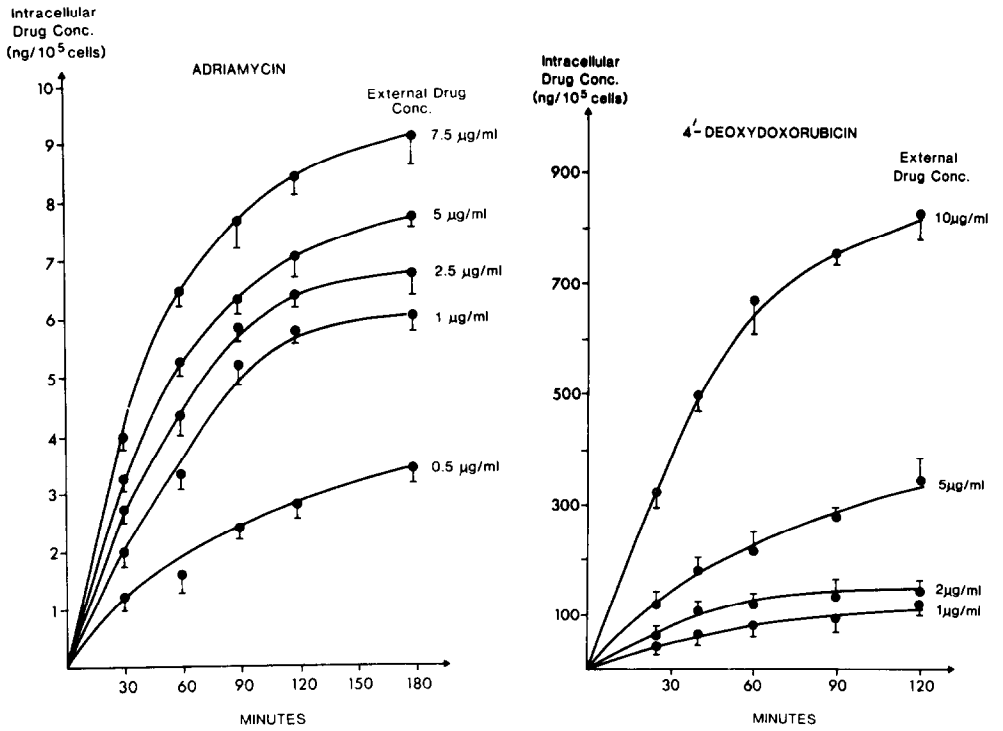
Intracellular localization of the drug. The cells were exposed in monolayer to the drugs at a concentration of 5 μg/ml in medium for up to 6 hr. When the incubation had been completed the drug containing medium was removed and the cells washed with ice cold medium. A coverslip was mounted in medium and the cells examined under a Polyvar fluorescent microscope (λ excitation = 468 nm; λ emission = 550 nm) using oil immersion (×400 magnification). In a separate experiment the cells were fixed with formol saline and stained by Altmann's technique in order to demonstrate the cytoplasmic distribution of mitochondria. In addition, after exposure to both drugs for 2 hr, the cells were prepared routinely for electron microscopy.

Statistical analysis. Drug uptake curves were fitted using a non-linear regressive technique by the University main frame computer.

RESULTS

The effect of intracellular drug concentration and duration of exposure on uptake by cells

The time course of uptake of adriamycin and 4′-deoxy (Figs 2 and 3) showed that the intracellular drug concentration was dependent on the drug concentration in the medium and the duration of drug–cell contact. Clearly 4′-deoxy (oil–water partition coefficient, 16.2) was taken up more rapidly and to a greater extent than the less lipophilic parent compound adriamycin (oil–water partition coefficient, 6.0). Using computer-fitted data it was possible to calculate the initial rate of drug uptake. The respective maximal rates of drug influx were V_{max}, adriamycin = 0.156 ng/10^5 cells/min and V_{max}, 4′-deoxy = 30 ng/10^5 cells/min. Figure 4 shows the



Figs 2 and 3. Intracellular levels of adriamycin and 4′-deoxy after exposure to a range of external drug concentrations for up to 3 hours. Each point represents the mean of 4 experiments (±S.D.)

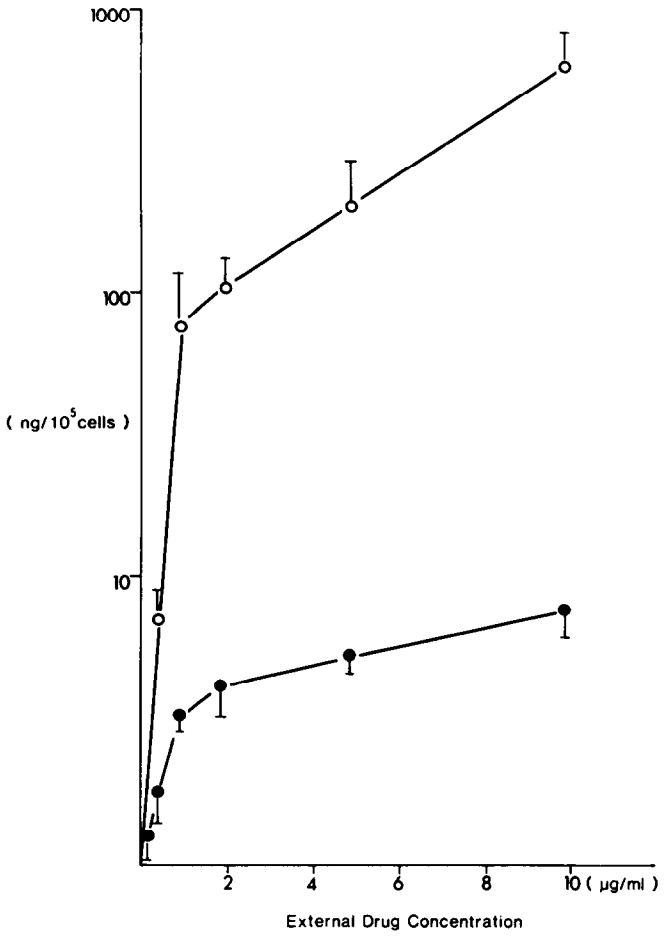


Fig. 4. Intracellular concentrations of adriamycin (●) and 4′-deoxy (○) after incubation for 1 hour over a range of external drug concentrations. Each point represents the mean of 4 experiments.

relationship between intracellular and extracellular drug levels after exposure of one hour (the duration of cell–drug contact during the clonogenic assay).

Correlation between external and internal concentrations and cell survival

Based on extracellular drug concentrations, there is no significant difference in cell survival comparing adriamycin and 4′-deoxy (Fig. 5). The ID_{90} for adriamycin (1.3 μg/ml) and 4′-deoxy (1.2 μg/ml) are virtually identical, on the basis of medium concentration. However, if intracellular levels of drug are plotted against cell survival (Figs 6 and 7), adriamycin achieves greater cell kill at relatively lower internal drug concentrations (LD_{90} adriamycin = 3.5 ng/10^5 cells; intracellular LD_{90} 4′-deoxy = 72 ng/10^5 cells).

Relationship between cell survival and the duration of drug exposure

The greater the duration of drug exposure at constant concentration, the greater the degree of cell kill (Fig. 8). The relationship between cell survival and time was biexponential for 4′-deoxy, with relatively greater cell kill after short drug exposures than adriamycin.

Intracellular drug metabolism

There was no evidence of drug metabolism occurring in the medium. 4′-Deoxy was metabolized within the cells to its alcohol, 4′-deoxyrubicinol (Fig. 9). The enzyme responsible for this [4] is a ubiquitous cytoplasmic enzyme, NADPH-dependent aldo-keto reductase. The rate of alcohol formation was relatively constant over a wide range of external drug concentrations which may imply that the enzyme was not saturated despite the relatively high intracellular drug concentrations achieved. We were unable to detect adriamycinol in the adriamycin treated cells. It is possible that the alcohol is being formed but is below the limit of detection in our HPLC assay as the intracellular levels of the parent drugs are low.

Intracellular localization of drug

The monolayers were examined sequentially for 6 hr after exposure to both drugs at a fixed concentration (5 μg/ml). Intracellular adriamycin was confined to the nucleus as far as could be determined

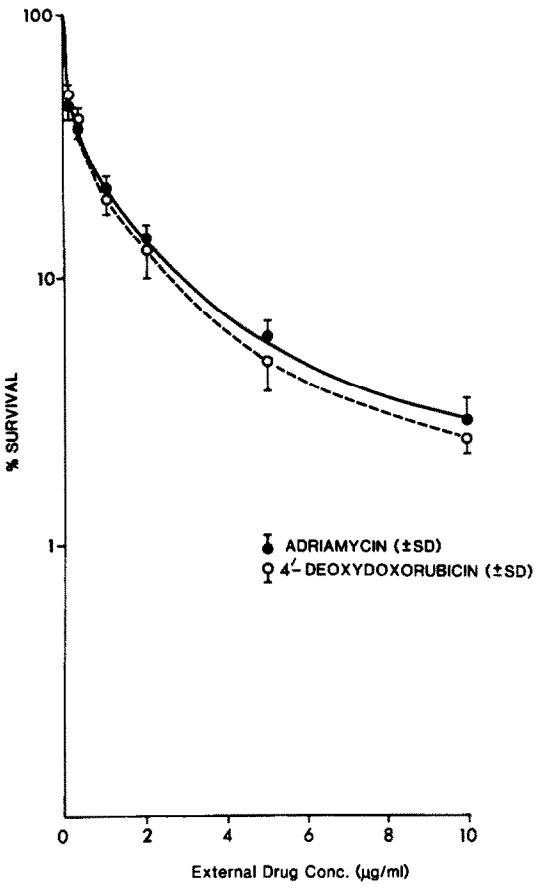


Fig. 5. % Clonogenic survival relative to external drug concentration (adriamycin ●, 4′-deoxy ○). Each point represents the mean of 4 experiments (±S.D.).

by light microscopy. 4′-Deoxy was localized solely within the nucleus with incubations for 10 min but granular cytoplasmic fluorescence had appeared by 20 min and thereafter increased in degree until it predominated. We considered the possibility that 4′-deoxy was binding to mitochondria within the cytoplasm, however, Altman's mitochondrial stain demonstrated a different size and distribution of granules from those "stained" by 4′-deoxy. Ultrastructural studies (Fig. 10) showed marked nuclear disruption and chromatolysis after treatment with both drugs. However, large lysosomal vesicles were seen in the cells treated with 4′-deoxy.

DISCUSSION

We have demonstrated that deletion of a hydroxyl group on the daunosamine moiety profoundly alters the physico-chemical properties of adriamycin, particularly lipid solubility. The increased lipophilicity of 4′-deoxy as manifested by the elevated oil–water partition coefficient, is probably the main factor contributing to the increased rate and cellular uptake seen with this drug in our monolayer systems. Cassaza [6] has reviewed the relationship between cell uptake *in vitro* and the partition coefficient of anthracycline analogues, and for groups of homogeneous derivatives it would seem that lipid solubility is an important determinant of cell uptake. Di Marco *et al.* [3] have compared cell uptake of the two drugs over a limited concentration–time range in a suspension of L1210 cells. They expressed intracellular drug levels as the ratio of moles drug bound/mole DNA and found a 14-times greater accumulation of 4′-deoxy in that cell system.

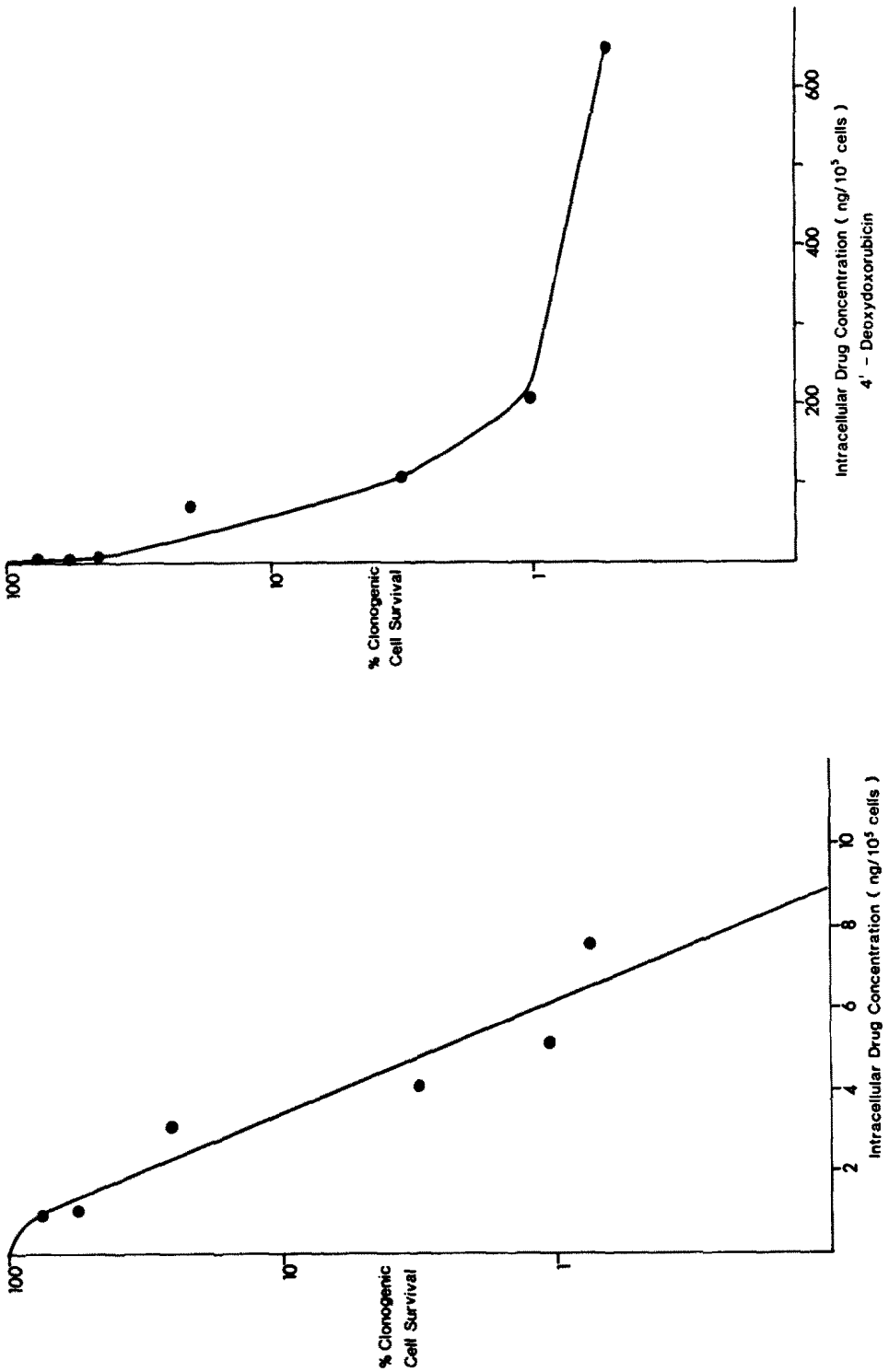
Biological membranes are lipoid in nature and the ease with which a drug molecule will cross a membrane will depend, in part, on its ability to dissolve in the lipid domain of the cell membrane. Skovsgaard [7] demonstrated that membrane transport is rate limiting for adriamycin uptake in Ehrlich ascites tumour cells and he proposed a carrier mediated drug transport mechanism. However, more recently, Dalmark [8–10] has presented transport data from human erythrocytes and Ehrlich ascites tumour cells, from which it appears that adriamycin transport takes place by free diffusion of the electroneutral molecule through the lipid domain of the cell membrane. Adriamycin can self-associate through hydrophobic interaction in aqueous solution and form dimers to which the cell is relatively impermeant. This situation would mimic a carrier mediated transport process although transport of the permeant takes place by simple Fickian diffusion. This may be the explanation for the earlier findings of Skovsgaard. Our results would tend to support Dalmark's hypothesis in that the more lipid soluble drug is accumulated to a greater degree.

On the basis of extracellular drug concentrations, cell kill was virtually identical for both adriamycin and 4′-deoxy. However, the correlation between intracellular drug concentrations and cytotoxicity differed for adriamycin and 4′-deoxy. For a given intracellular concentration, adriamycin is more cytotoxic than 4′-deoxy. The log cell survival plot (Fig. 8) for adriamycin is linear over the intracellular concentration range whereas the curve for 4′-deoxy is biphasic with a relative plateau after 1% cell survival.

The relationship between cell survival and the duration of drug exposure is mono-exponential for adriamycin and biexponential for 4′-deoxy. There is relatively greater cell kill during short drug exposures for 4′-deoxy, which is probably related to the rapid rate of cell uptake. It is interesting to note that the curves for cell survival vs intracellular drug concentration, and cell survival vs duration of drug exposure are both biphasic. It is possible that the initial rapid phase of drug uptake is the major determinant of cytotoxicity.

These results are similar to those reported by Bhuyan *et al.* [11] who studied intracellular uptake of another lipophilic anthracycline, 7-con-*O*-methylnogarol (7-OMEN) in chinese hamster ovary, B16 and L1210 cells in culture. 7-OMEN was taken up more rapidly in B16 cells than adriamycin and had a biphasic cell survival curve. The biphasic curve may implicate a resistant subpopulation or, perhaps more likely as Bhuyan commented, may be a characteristic of a drug which shows predominant accumulation in the cytoplasm.

The time course of drug distribution within the cell is conspicuously different, as demonstrated by fluorescent microscopy. Adriamycin was located



Figs 6 and 7. The relationship between clonogenic cell survival and intracellular drug concentration. Each point is the mean of 4 experiments.

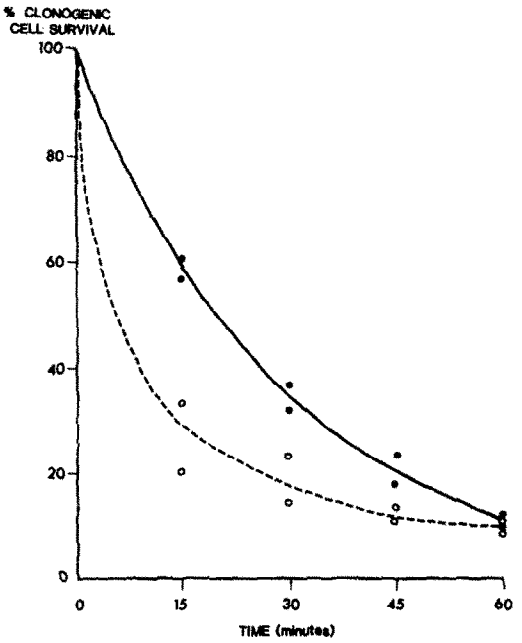


Fig. 8. The relationship between clonogenic cell survival and duration of exposure at constant drug concentration (●, adriamycin; ○, 4′-deoxy). The curves were computer fitted to data from 2 experiments.

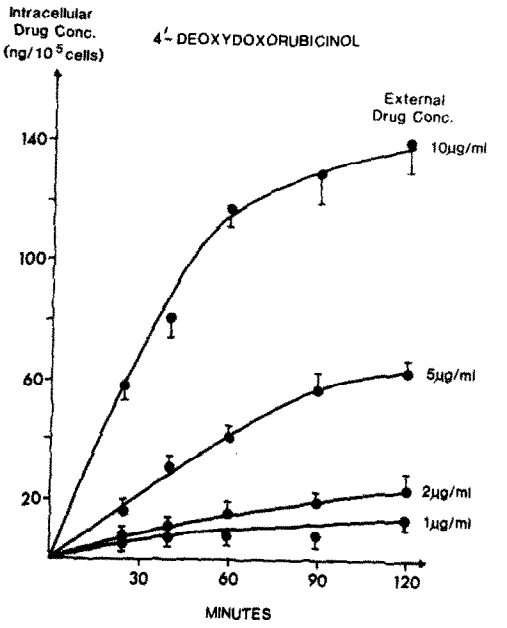


Fig. 9. Intracellular levels of 4′-deoxydoxorubicinol after exposure to 4′-deoxy.

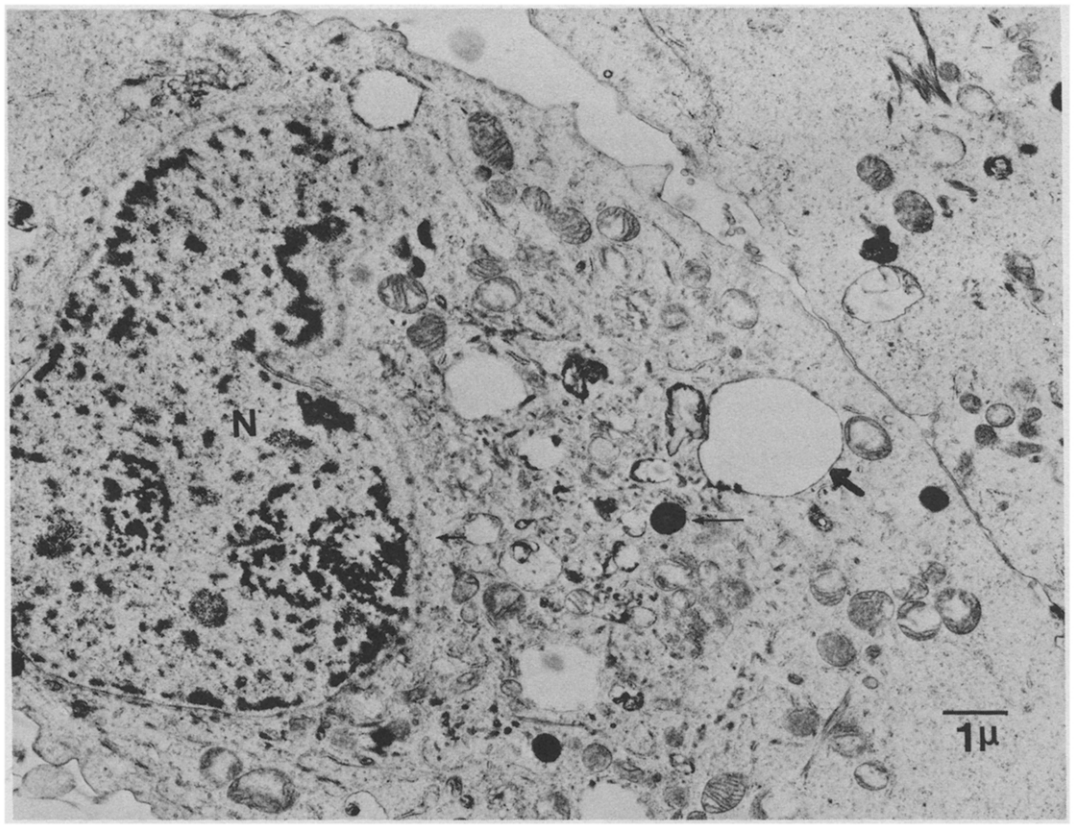


Fig. 10. Electron micrograph of a lung tumour cell after treatment with 4′-deoxy. There is nuclear damage with chromatolysis (arrowed) and large vacuoles, thought to be lysosomes are seen (arrowed).

mainly within the nucleus whereas 4′-deoxy was distributed both in the nucleus and in a granular fashion within the cytoplasm. Initially we considered the possibility that 4′-deoxy was binding to mitochondrial structures but conventional histochemical localization of mitochondria using Altmann's stain showed an entirely different distribution. Facchinetti *et al.* [12] studied the time course of intracellular distribution of adriamycin and *N*-trifluoroacetyl-adriamycin-14-valerate (NTAV) in mouse peritoneal macrophages. They found that adriamycin cytofluorescence, at higher extracellular concentrations than used in our experiments, was seen initially in the nucleus and later appeared in a granular distribution within the cytoplasm. The lipophilic derivative NTAV localized within the cytoplasm in a granular fashion with a similar distribution to 4′-deoxy. Ultrastructural studies showed that NTAV was bound within intracytoplasmic vesicles of an amorphous nature. Electron microscopy following cellular exposure to 4′-deoxy revealed large lysosomal vesicles, which could have been the fluorescent granular structures seen on light microscopy in this present study.

It would only require approximately 2–5% of total intracellular 4′-deoxy to bind to the nucleus to give levels approximate to total intracellular adriamycin (for a given concentration/time exposure). Fluorescent microscopy is a rather insensitive method of quantitating the amount of drug present within specific cell structures but it is possible that nuclear binding of 4′-deoxy is occurring to a significant extent although fluorescent light microscopy reveals a predominantly cytoplasmic distribution. Egorin *et al.* [13] have shown that DNA can quench the fluorescence of some anthracyclines more than that of others, which may be a further contributing factor.

These results indicate that the relationship between cytotoxicity and intracellular drug levels is complex when comparing a lipophilic derivative with its parent compound. The importance of anthracyclic cytofluorescence distribution is unknown. Egorin *et al.* [13] have examined the intracellular localization of a number of different anthracyclines and have shown that there is no correlation between the intracellular distribution of drug after exposure to intact cells and accumulation by isolated nuclei. It is possible that differential nuclear-cytoplasmic localization may not be related to different modes of drug action at cellular level but may represent intracytoplasmic storage for lipophilic drugs.

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