MODULATION OF ANTHRACYCLINE ACTIVITY BY A RANGE OF DRUG CARRIERS

by

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I dedicate this thesis to my wife, Penny.

AUTHOR'S STATEMENT

All of the work described in this thesis was performed personally by the author, except where specified above or in the text.

SUMMARY

Major advances in the treatment of leukaemia and lymphoma in the 1960s and 70s led to the euphoric belief that solid tumours would also yield to cytotoxic chemotherapy. Unfortunately this promise has remained largely unfulfilled. Many reasons are cited for this therapeutic failure, but it is generally accepted that drug resistance, either arising 'de novo' or aquired, is a major factor in this problem. The work detailed in this thesis looks at some possible methods of overcoming so-called 'pharmacological resistance' i.e. drug resistance based on an inability to deliver enough active drug to it's site of action.

The anthracycline class of anti-tumour agents form one of the cornerstones of modern chemotherapy of both leukaemias and lymphomas as well as solid tumours. Since the introduction of these drugs many attempts (mainly through analogue development) have been made to improve their therapeutic efficacy by increasing lipophilicity and thereby enhancing drug penetration into tumour. The data shown in chapter 2, using the in-vitro human multicellular tumour spheroid model, demonstrates the penetration limitation of anthracycline. In the in-vivo situation the intravenously administered cytotoxic drug has to traverse many penetration barriers before reaching the tumour micro-environment, this will exacerbate any penetration problems

encountered in the in-vitro test system which one could regard as the best possible scenario for penetration (since the cytotoxic is present in excess in the medium surrounding the tumour nodule). The data generated using other drugs also demonstrates that this is not a universal property of cytotoxics. Since the anthracyclines are already amongst the most efficacious cytotoxics it is possible that overcoming this problem may further improve on their therapeutic benefits.

Theoretically the problem of penetration may be overcome by a variety of methods. The work presented here concentrates on two possibilities; the first being delivery of a penetration enhancing agent together with the cytotoxic, the second is the targeting (or selective concentration) of drug to tumour tissue thereby achieving local concentration of cytotoxic not attainable by simple intravenous administration. If one assumes that penetration of drug into tumour is on the whole by passive diffusion, then a higher local concentration would lead to increased penetration into a nodule of tumour.

The data in chapter 3 looks specifically at the use of surfactants as penetration enhancing agents. The tested surfactants proved to have unexpected toxicities when given intravenously to rodents, and the simplistic approach of co-administration seemed to have no benefit. This theme is further developed in chapter 4, when the same surfactants are used to make vesicular formulations containing anthracyclines which are

used as a drug delivery vehicle. This approach may be an improvement in that the vesicle nature and composition may be manipulated to encourage a degree of tumour targeting. The results of this approach are a change in the pharmacokinetic behaviour of the anthracycline with some slow-release characteristics and some success in targeting. However, the in-vivo activity against a rodent tumour model is disappointing, probably because of bonding of the anthracycline to some component of the vesicle causing inactivation of the cytotoxic.

Historically, surfactant vesicles are at an early stage of development, but show some promise. Liposomes (which are lipid based vesicles) have a much longer history of investigation over the last 20 years, with various well established pharmaceutical applications. It is therefore not surprising that the results in chapter 5 dealing with a liposomal form of anthracycline show changes in pharmacokinetics, with a six fold increase in tumour AUC , which translates into enhanced anti-tumour activity in an in-vivo tumour model. This particular formulation is currently in early clinical trials in various centres.

A related method of drug targeting is the use of macro-molecular carriers to which drug can be attached, with subsequent release at the site of action. In chapter 6 the use of N-(2-hydroxypropyl)methacrylamide [HPMA] co-polymer bound anthracycline is explored. HPMA was originally developed as a

plasma expander, and is both non-toxic and non-immunogenic. This biodegradable substance is composed of a monomeric backbone with multiple carbon based side chains which lend themselves to chemical derivatisation. In addition, bonding of drug to the polymer can be achieved in such a way that only lysosomal enzymes can release drug from polymer. This has the dual purpose of targeting together with selective concentration within the cells and tissues with high endocytic activity such as certain tumour types. The results using this system are a high level of targeting with a marked increase in therapeutic efficacy, the pharmacokinetic results also imply an enhanced therapeutic ratio for this preparation. These results together with other pre-clinical data have led to a commit ment to develop this for human studies.

The novel intra-lysosomal route taken by the HPMA anthracycline copolymers raises the possibility that this may be a way of avoiding the classical multi-drug resistance phenotype of some experimental and human tumours, characterised by resistance to a number of 'natural product' cytotoxic agents, together with the presence of a 170 Kilodalton transmembrane peptide known as P-glycoprotein. This protein acts as an energy-dependent efflux pump, that reduces intracellular drug accumulation, thereby conferring resistance to many different drugs. This hypothesis is tested in chapter 7 using a naturally sensitive parent and resistant daughter pair of human ovarian

cancer cell lines. The resistant line was derived by serial passage in ever increasing drug concentrations and has been shown to express an abundance of P-glycoprotein by other workers using the same cell lines. When exposed to free or HPMA copolymer bound anthracycline these particular cell lines show only minor changes in the pattern or degree of response. This could be explained by the very high levels of P-glycoprotein, which may be far in excess of the amount encountered in-vivo, or by as yet undiscovered resistance mechanisms independent of this pump mechanism.

In conclusion, the work in this thesis shows that anthracyclines are limited by poor tumour penetration and that methods exist to improve on this situation, the most promising being the use of liposomes and HPMA copolymers.

CHAPTER 1

GENERAL INTRODUCTION

The 1960s and 70s brought major improvements in the treatment of the leukaemias and lymphomas. The advances made in these previously fatal illnesses were prompted by the introduction of new drugs; and with this a better understanding of the mechanism of action of existing drugs, allowing for logical drug combinations to be introduced. These changes resulted in major improvements in response rates and survival of patients with these malignancies. It appeared that the lessons from these disease states could be directly applied to the treatment of solid tumours, with similar results. Unfortunately, this promise has not been fulfilled. In the last decade few new cytotoxic drugs have become available in the clinic (other than analogues of existing drugs), and the widespread application of multi-agent treatment has failed to significantly alter the survival rates for the majority of patients with advanced forms of common solid tumours, especially lung, breast and colon cancers (DeVita, Hellman & Rosenberg, 1989).

In this rather depressing situation it is even more important that we look carefully at existing cytotoxic agents in a critical way. Therapeutic failure may be as important as success insofar as our understanding of the basic biology of cancer. Thus it is important that we strive to understand the mechanisms

of resistance either de novo or aquired, as applied to single cells or to intact tumours within the host. In attempting to explain these phenomena it is likely that we may 'stumble' upon mechanisms or strategies by which existing therapies can be improved, or by which the emergence of resistance may be circumvented.

The purpose of this short introduction is to highlight two (some others are given in Table 1) of the fundamental problems facing any cytotoxic drug. These are:-

1. Is it possible to achieve a sufficiently high concentration of drug in the region of the tumour, without causing unacceptable toxicities in other tissues?

2. Can the drug in the region of the tumour then diffuse through the tumour substance and into the neoplastic cells, with resultant death of these cells?

The first of these questions can only be answered by the use of in-vivo model systems, with all the attendant expense and complexity that these demand. It is clear from experiments using material derived from some types of human tumours that solid tumours are not inherently insensitive to all forms of currently available treatment. Therapeutic failure results in at least some cases from an inability to achieve therapeutically effective drug levels at the tumour (Stallard et al, 1989). It

Table 1

Potential problems or barriers to drug targeting

Tumour factors

- Cellular heterogeneity
- Phenotypic changes with time or metastases
- Membrane transport of drug

Host factors

- Plasma protein binding
- Degradation by intravascular enzyme systems
- Reticulo-endothelial system
- Immune system
- Vascular egress
- Diffusion to site of action
- Intracellular drug transport

is possible to measure the drug concentration within tumours, and then to design and test strategies to improve 'drug delivery'. It is also possible to measure drug concentrations in certain organs of toxicity for various drugs, and then to attempt to divert drug away from these organs and to enhance drug deposition in the tumour, so called 'drug targeting'. Specific methods to improve drug delivery or targeting are discussed in more detail in subsequent chapters.

The second question is more difficult to explore in-vivo because of inter-individual differences in pharmacokinetics of drugs, and because methods of 'tracking' the destination and intracellular fate of most cytotoxics are not available. Hence, an in-vitro approach employing the human multicellular tumour spheroid (Sutherland, McCredie & Inch, 1971) system has been used in some of the work within this thesis in an attempt to identify drugs for which tumour penetration is a problem, and to examine ways of modifying penetration. Details of the development and use of this system are presented in chapter 2.

Both of these limitations could theoretically be overcome by a system of drug delivery or targeting, since increasing the local concentration of drug in the vicinity of the tumour should facilitate diffusion of drug into the tumour (assuming that this is generally a passive process). It is therefore worth reviewing some of the criteria which an ideal drug targeting system should fulfil, and some of the potential obstacles.

THE TARGET

The target must be expressed by all tumour cells since even small numbers of remaining cells could cause recurrence. Since tumours are composed of a multitude of phenotypically distinct cells, and since not all tumours are alike, even metastases within the same host are distinct (Poste et al, 1982); it is unlikely that any one molecule will be the target. There are as yet few known unique differences between tumour cells and normal cells, but many quantitative differences exist which could be exploited (Hawkey et al, 1986). Furthermore, the cellular phenotypes which make up the tumour are not static, some sub-populations die as a result of selective pressure from the host or therapy, and are replaced by cells resistant to the selection process (Welch & Tomasovic, 1985). If the degree of heterogeneity is small enough, then the new sub-populations may still express the target, but if there is wide divergence the targeting strategy may fail. If it were possible to identify the molecular characteristics which define the malignant cellular phenotype, then a targeting system to some aspect of this should be possible. The closest candidates for this role so far are the oncogenes and their products (Bishop, 1987); but as yet no specific (and accessible) target has been defined, with many if not all of the oncogene products having pivotal roles in the regulation of proliferation and differentiation of normal cells. Some of the more promising potential candidates

for drug targets are given in table 2.

Rather than consider the biochemical differences between cell types, it may be possible to define functional attributes of tumours which lend themselves to targeting. The tumour vasculature is derived from the host in response to angiogenic factors released by the tumour (Jain, 1988). Microscopically this vasculature is rather abnormal, with dilated and tortuous vessels which may be partially lined by tumour cells (Gullino, 1975). These vessels also show ultrastructural anomalies with wide interendothelial junctions, fenestrae and discontinuous basal lamina (Jain, 1987). Thus the defective capillary networks in some tumours (Warren, 1970) may allow a degree of targeting through selective leakiness of the vasculature. Some tumour types also exhibit an increased endocytic rate (Sehested et al, 1987), which may allow targeting via this route. An alternative approach may be to target the drug to a particular organ or tissue in which the tumour resides, an example would be the reticulo-enothelial trapping (and hence hepato-splenic targeting), of most forms of liposomes (Kaye & Richardson, 1979).

Table 2

Potential cellular targets for drug targeting to tumour cells

- Oncogenes
- Oncogene products
- Cell surface:-
  - Tumour cell antigens
  - Adhesion or attachment molecules
  - Membrane receptors e.g. growth factor receptors

THE DELIVERY SYSTEM

It is possible to define certain requirements of a drug delivery system which would be necessary in order to overcome the barriers to drug targeting as listed in table 1. Table 3 outlines some of these basic properties.

The apparently simple concept of drug delivery is in reality a very complex problem. The problems for cytotoxic delivery are generally worse than for other drugs such as antibiotics, because most cytotoxics have major (if not life threatening) toxicities which may be exacerbated if drug is inadvertently delivered to an inappropriate site. Additionally, most cytotoxics are highly reactive molecules, which presents problems of drug bonding to the carrier, or of inactivation of drug. Even getting the drug to the tumour does not guarantee that the drug will be effective. The drug must then be transported into the cell as an active form or as a prodrug to be activated inside the cell.

The host immune system also represents a barrier to drug delivery. The delivery system itself may cause an immune response, with the reticulo-endothelial system sequestering the drug before it has a chance to reach the target.

Finally, the delivery system must be simple enough in chemical structure to allow for large scale production of material of suitable purity at reasonable cost.

Table 3

Requirements for a drug delivery system

Ability to incorporate a broad spectrum of drugs
Maintenance of activity of bound drugs
Protect drug from premature degradation
Non-immunogenic
Recognition of target
Delivery of therapeutic drug concentrations
Release of active drug at target
Biodegradable
Biologically inert
Large scale production feasible
Cost-effective

PURPOSE OF THE THESIS

This thesis examines the problems of drug penetration and drug delivery for one class of cytotoxic agents, namely the anthracyclines. It examines some approaches which have been around for many years e.g. liposomes; and others which are entirely novel such as polymeric carriers and surfactant vesicles. Each chapter is prefaced by a more detailed review of the literature pertinent to the particular approach employed.

The hypothesis to be tested is that the efficacy (therapeutic index) of anthracyclines can be significantly improved by the use of synthetic drug carriers; whose role may be to enhance drug penetration, alter host pharmacokinetics or promote selective endocytic tumour uptake.

CHAPTER 2

STUDIES USING THE HUMAN MULTICELLULAR TUMOUR SPHEROID MODEL

Multicellular tumour spheroids were first described by Sutherland, McCredie & Inch (1971). They are composed of tumour cells growing in a three dimensional spherical colony, and consist of an outer, fast growing cell layer, an intermediate cell layer with few proliferative cells, and a central region mainly containing degenerative cells (Sutherland et al, 1981). This 3-dimensional structure simulates the growth and microenvironmental conditions of malignant tumours in vivo (Sutherland, 1986). Thomlinson and Gray (1955) and Tannock (1968) showed that only tumour cells near capillaries were proliferative and that cells at a distance of about 100-200 um degenerated. Penetrating vessels and capillaries were surrounded by thin cords of viable cells. These two studies were carried out in tumours with a nodular appearance. This has given rise to the term "tumour nodule", describing a capillary with its surrounding layer of viable cells and an outer layer of necrotic cells. It may also be used for a peripheral network of capillaries surrounding a cell "spheroid" that may contain a central area of necrosis (Folkman & Greenspan, 1975). The latter pattern is almost identical with that seen in cultured multicellular tumour spheroids. This spatial arrangement leads

to diffusion gradients for nutrients into the tumour nodule which can be modelled in vitro by spheroids; oxygen being one of the most intensively investigated of such nutrients (Sutherland, 1986).

Spheroids have been used in a variety of research areas as an in-vitro tumour model system of intermediate complexity between standard monolayer cultures and tumours in-vivo. In particular, the similarity between spheroids and the avascular stage of micro-metastases has been emphasised (West, Weichselbaum & Little, 1980).

Spheroids are useful for drug cytotoxicity tests because the penetration barriers which exist in poorly vascularised parts of solid tumours (Goldacre & Sylven, 1962) can in part be simulated. The spheroids also contain extracellular matrix (Nederman et al, 1984) which may have an important influence on drug penetration both in spheroids (Nederman, Carlsson & Kuoppa, 1988), and in tumours (Jain, 1987). In addition, heterogeneity in other microenvironmental factors such as cell cycle growth phase can be demonstrated in spheroids and solid tumours in-vivo (Sutherland, 1986). Theoretically spheroids may provide a more sensitive model than monolayer systems for in-vitro cytotoxicity studies. However they present more technical difficulties, and such a conclusion would therefore have major practical implications. Thus it is important to examine in detail the differences in cytotoxicity data for individual drugs when the

same cell line is tested using both spheroid and monolayer systems.

Relative drug resistance in spheroids compared to the same cells in monolayer cultures has been demonstrated for many cytotoxics including m-AMSA (Wilson, Whitmore & Hill, 1981), methotrexate (West, Weichselbaum & Little, 1980), bleomycin (Toburen, 1981), vincristine (Wibe & Oftebro, 1981), vinblastine (Nederman, 1984; Nederman & Carlsson, 1984) and adriamycin (Sutherland et al, 1979; Durand, 1981; Erlichmann & Vidgen, 1984). In most cases this resistance is mainly based on poor drug penetration as shown by direct studies such as fluorescence, autoradiography or flow cytometry. However, microenvironmental factors such as oxygenation and cell cycle (Kwok & Twentyman, 1985), or pH changes (Kohn & Ewig, 1976) may also have a role in some cases (Sutherland et al, 1979). The only exception reported was 5-fluorouracil which seemed to penetrate efficiently at least through glioma spheroids (Nederman & Carlsson, 1984).

In this chapter are described experiments carried out to study the relative response to a variety of cytotoxic drugs of L-DAN human tumour cells treated either as multicellular spheroids or as monolayer cultures. The purpose of this being to establish which anti-tumour agents, if any, are subject to a 'penetration barrier', as judged by the spheroid/monolayer comparison.

MATERIALS AND METHODS

CELL LINE

L-DAN is a human non-small cell lung cancer line derived from a primary biopsy from one of our own patients (Merry et al, 1987). The cells are maintained in growth medium consisting of a mixture of equal volumes of Ham's F10 and Dulbecco's Modified Eagle's Medium supplemented with 10% bovine foetal calf serum and with a gas phase of 2% CO_2. The plating efficiency of this cell line is of the order of 15-20%.

DRUGS

The drugs used in this study were adriamycin (ADR, Farmitalia Carlo Erba Ltd, Barnet, Herts, UK), vincristine (VINC, trade name Oncovin, Eli Lilly and Co Ltd, Basingstoke, UK), methotrexate (MTX, Lederle Laboratories, Gosport, Hampshire, UK), 5-fluorouracil (5-FU, Roche Products Ltd, Welwyn Garden City, UK) and cisplatin (Lederle Laboratories, Gosport, Hampshire, UK). In each case the drugs were solubilised according to the manufacturer's instructions and diluted with growth medium to the desired final concentrations on the day of the experiment. The final solutions should all be stable under these conditions (Bosanquet, 1989), although this was not formally tested.

MONOLAYER CYTOTOXICITY ASSAY

L-DAN cells were grown as monolayers in 25 cm^2 flasks (Falcon) in growth medium. Three days after inoculation into flasks, when the cells were in exponential growth phase, they were exposed to drug diluted to the appropriate concentration in culture medium for 1 hour at 37^0C in a 2% CO_2 incubator. Drug containing medium was then removed and the monolayers washed twice with cold phosphate buffered saline (PBS = Dulbecco's A). A single cell suspension was prepared using 0.25% trypsin in PBS and 1mmol/LEthylene-diamine-tetra-acetic acid (EDTA). Cells were inspected microscopically to ensure a monodispersed population and counted using a model ZB Coulter counter (Coulter Electronics Ltd, Luton, UK). The cells were then diluted appropriately in growth medium and plated in 50mm Petri dishes (Nunc, Gibco, BRL, UK) at various dilutions in replicates of 12 for controls and 9 for drug treated cells.

The dishes were incubated for 14 days at 37^0C with 2% CO_2 and then removed, fixed with methanol for 10 minutes and stained with 0.5% dilution of Crystal Violet (BDH Chemicals, Poole, UK) for 20 minutes. Colonies of > 50 cells which were visible by naked eye were counted manually, and survival in drug-treated dishes expressed as a fraction relative to control survival. A value for log survival (log S) was also derived by the equation:-

$$\text{Log S} = \log (\text{colonies treated/colonies untreated})$$

SPHEROID CULTURE

Initiation of spheroids from monolayer L-DAN was performed by an adaptation of the method of Sutherland, McCredie & Inch (1971). Briefly, 10^6 cells were seeded into 200 ml of growth medium in glass spinner flasks (Techne, Cambridge, UK). The medium in addition contained 2.5 ug/ml of amphotericin, 100 IU/ml penicillin and 100 ug/ml streptomycin (Gibco Ltd, Paisley, Scotland) to reduce culture infections which are more common than in monolayer systems because of the repeated manipulations required in spheroid culture. Control experiments showed that the addition of these antibiotics did not affect parameters of growth in these spheroids (data not shown). The suspensions were maintained at 37^0C in a 5% CO_2 incubator and rotated at 40 revolutions per minute using a magnetic stirring device (Techne, Cambridge, UK). Aggregation of cells occurred in approximately 5 days and spheroids reached a diameter of approximately 200 um by day 8-9, at which time they were used for drug cytotoxicity experiments. In general, the spheroids grew with Gompertzian kinetics.

SPHEROID CYTOTOXICITY ASSAYS

10 ml aliquots of medium and spheroids were transferred into sterile plastic universal tubes for treatment of intact spheroids. The spheroids were allowed to settle by gravity and

the medium discarded and replaced by 10 ml of fresh medium containing the test drug at appropriate final concentration. The samples were incubated at $37^{0}C$ for one hour with intermittent agitation by hand to ensure even exposure of all spheroids to drug. After incubation the spheroids were allowed to settle, the drug containing medium was removed and the spheroids rinsed twice in cold PBS. Each rinse lasting 3 minutes in an attempt to eliminate the theoretical possibility of later diffusion of drug from a 'pool' trapped in the central necrotic zone of the spheroid. The spheroids were then resuspended in fresh medium and individual spheroids transferred by Pasteur pipette into agar based 24 well plates (Corning USA, supplied by J.Bibby science products Ltd, Stone, Staffs, UK) with 0.5 ml of medium in each well. Spheroids were then selected by eye with a tight size distribution (i.e. all spheroids between 150-250 um in diameter), so that growth curves for groups of spheroids should not be influenced by a wide variety of initial sizes.

Subsequent growth of the spheroids was measured using an inverted microscope coupled to a microcomputer based image analyser (Analytical systems model 40-10) which gives a cross-sectional measure of each spheroid, which can then be converted to volume assuming spherical geometry. Measurements were made at least twice-weekly, and the wells were topped up with 0.5 ml of fresh medium every 7 days. Between 20-24 spheroids/treatment group and control were utilised for each experiment. 2-4 experiments were performed for each drug treatment. Growth delay

was determined by measuring the time required for the spheroid volume to increase by a factor of ten for control and drug-treated spheroids. The growth delay was defined as the difference in time between drug-treated and control spheroids to reach this 10-fold increase in volume. We chose 10-fold increases in volume because initial studies had shown that the growth curves at this point were generally parallel to each other. Very few spheroids failed to regrow following treatment, so that no 'cure correction' was necessary to the growth data.

Clonogenic survival may be deduced from tumour regrowth data by (back-)extrapolation of regrowth curves to zero dose. These deductions have been attempted in-vivo but are complicated by possible immunological responses, the limited proliferation potential of so called 'doomed cells' and the practical difficulty of obtaining accurate measurements of the regrowing tumour (Wheldon, 1980). In the absence of most of these effects for the in-vitro situation pertaining to multicellular tumour spheroids, it seems reasonable to interpret the spheroid responses as being mainly due to sterilisation of clonogenic cells. Thus back-extrapolation of the regrowth curves has been attempted when the curves become parallel with control curves (see figure 2). By this method a 'surviving fraction' of clonogenic cells can be derived and directly compared with the results of the monolayer clonogenic assay (Wheldon et al, 1985).

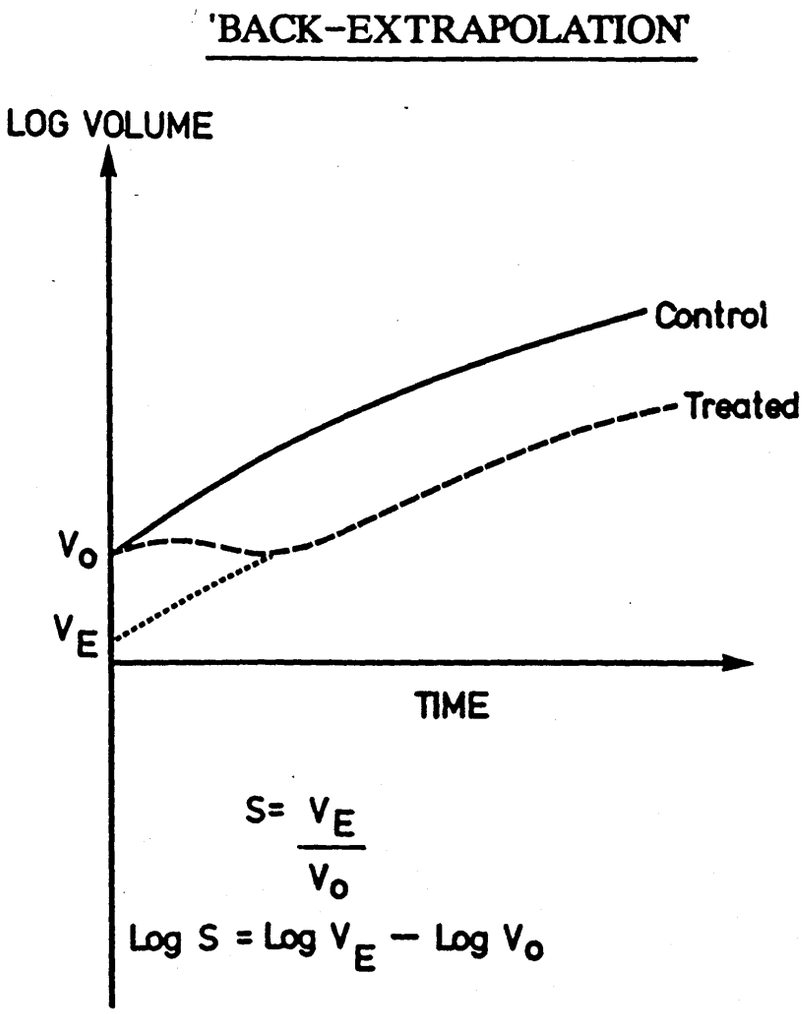


Figure 1: Illustration of method of back-extrapolation of spheroid regrowth curves.

RESULTS

CISPLATIN

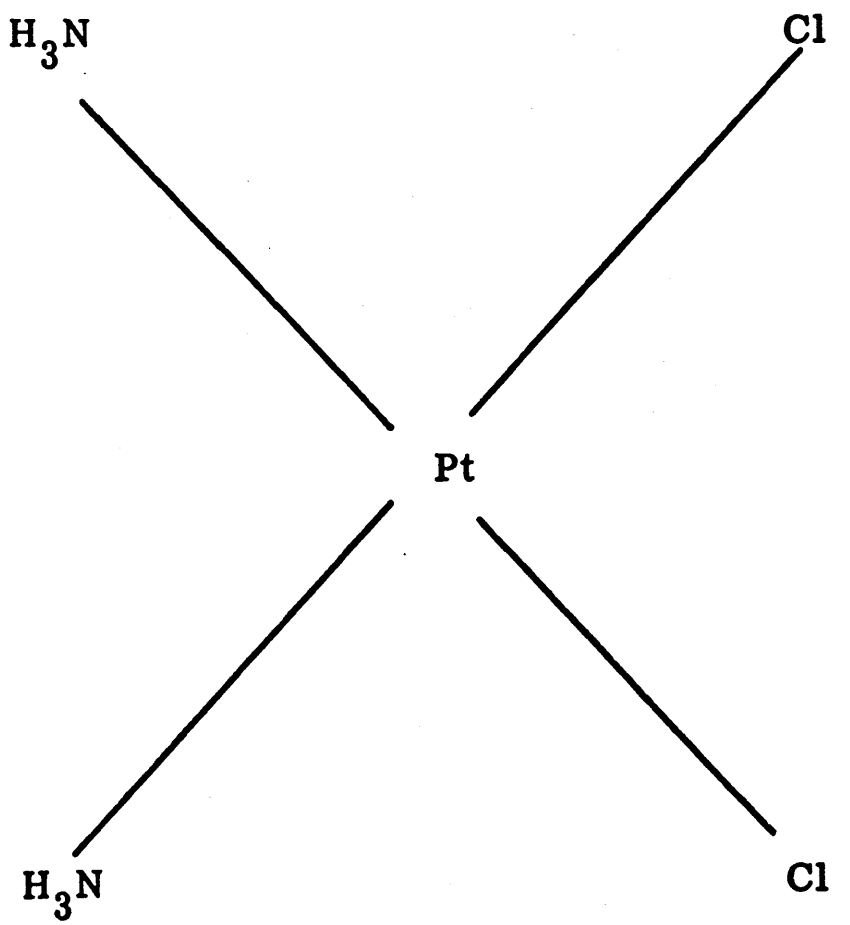


Figure 2; Structure of cisplatin. MW=300

Cisplatin is thought to exert its cytotoxic action by inhibition of DNA replication which results from complex co-ordination to bases in DNA, mainly at the N7 position of guanine

and adenine nucleotides (Roberts, 1986). There is no unique administration protocol for this inorganic compound, and its pharmacokinetics are dependent on both route and schedule of administration (Gouyette et al, 1986). A commonly used schedule in this country is 100mg/m^2 over 6 hours by intravenous infusion with saline diuresis. This schedule results in peak plasma levels of free platinum (thought to be the active form) in the region of 5-15 ug/ml (Ribaud et al, 1981). Free platinum half-lives of 30 minutes have been reported for bolus (Himmelstein et al, 1981) and infusion routes (Reece et al, 1985).

Figure 3 shows a dose-response curve for cisplatin against exponential phase L-DAN cells. The results are expressed as percentage of control growth versus cisplatin concentration. Each point is the mean of two experiments +/- standard error. The curve was drawn by eye as the best fit of observed data.

Figure 4 shows a representative experiment from a series of three in which L-DAN spheroids were exposed to cisplatin for one hour. Each set of symbols shows the median log volume from 20-24 individual spheroid measurements and the 95% confidence intervals about this median value. In the interest of clarity the figure does not show all of the tested doses of cisplatin, but these are included in table 4. Repitition of this experiment with spheroids of a smaller starting volume yielded similar results, as shown in figure 5. The growth delay data derived from both the 'small' and 'large' spheroids are shown in table 4.

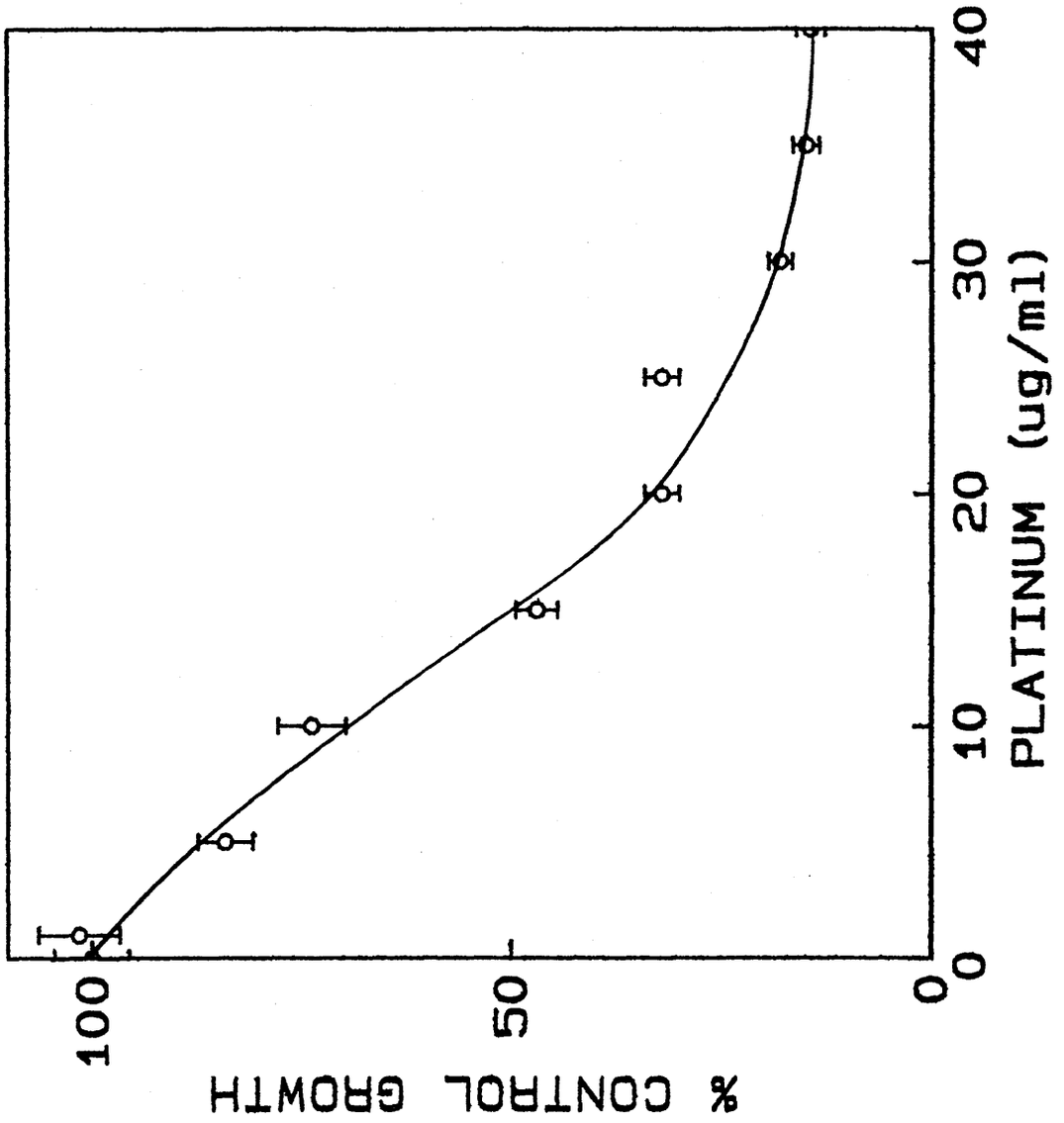


Figure 3: Dose-survival curve for L-Dan cells exposed to cisplatin in monolayer cultures

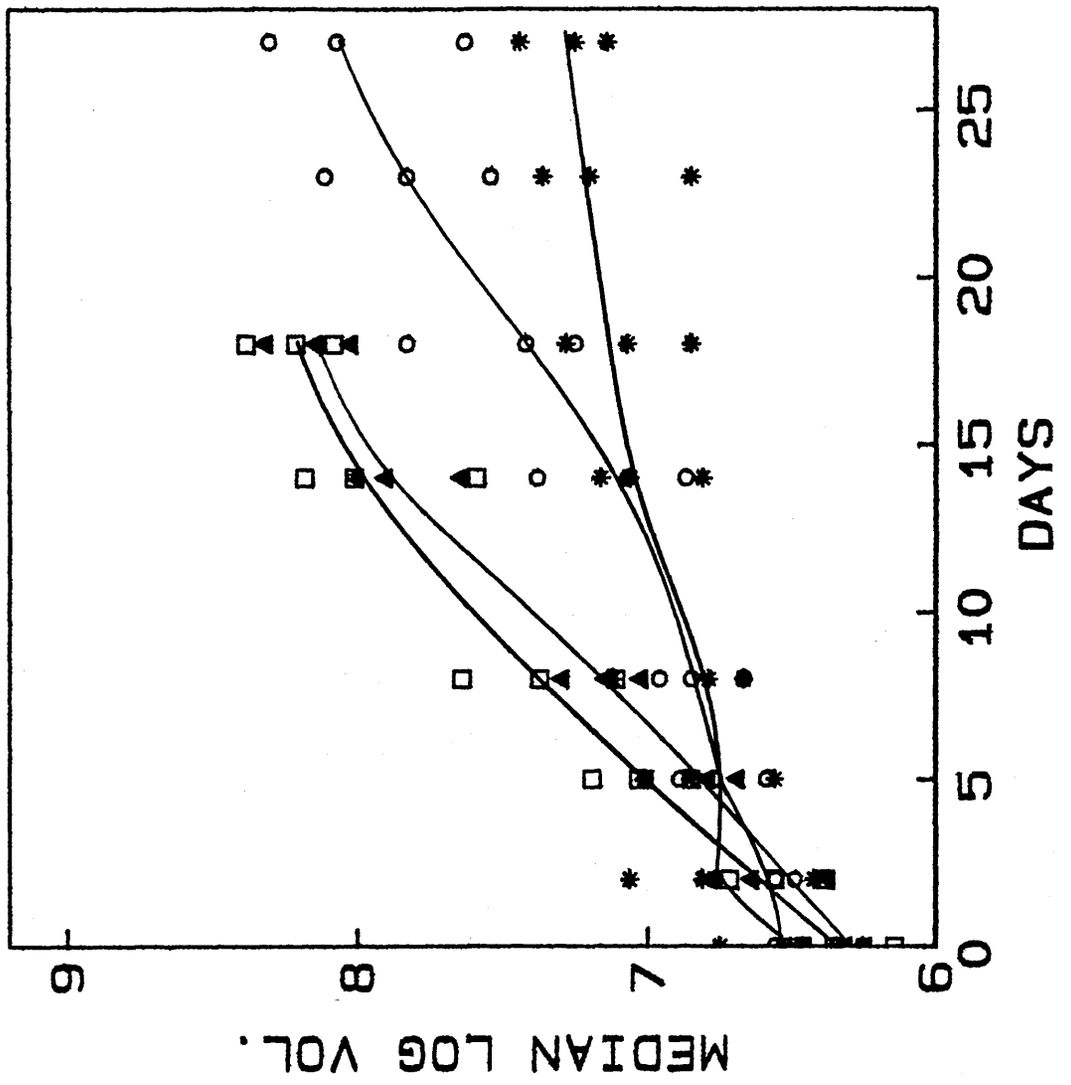


Figure 4: Regrowth curves of 'large' L-Dan spheroids following exposure to cisplatin. KEY: Control= open squares, 10 ug/ml= filled triangles, 20 ug/ml= open cirles, 40 ug/ml= stars.

TABLE 4

DOSE(ug/ml)	GROWTH DELAY (days)	
	SMALL	LARGE
0	0	0
5	0.9	0
10	4.4	1.6
20	11.2	9.8
30	14.7	13.2
40	ND	27.2

ND=not done.

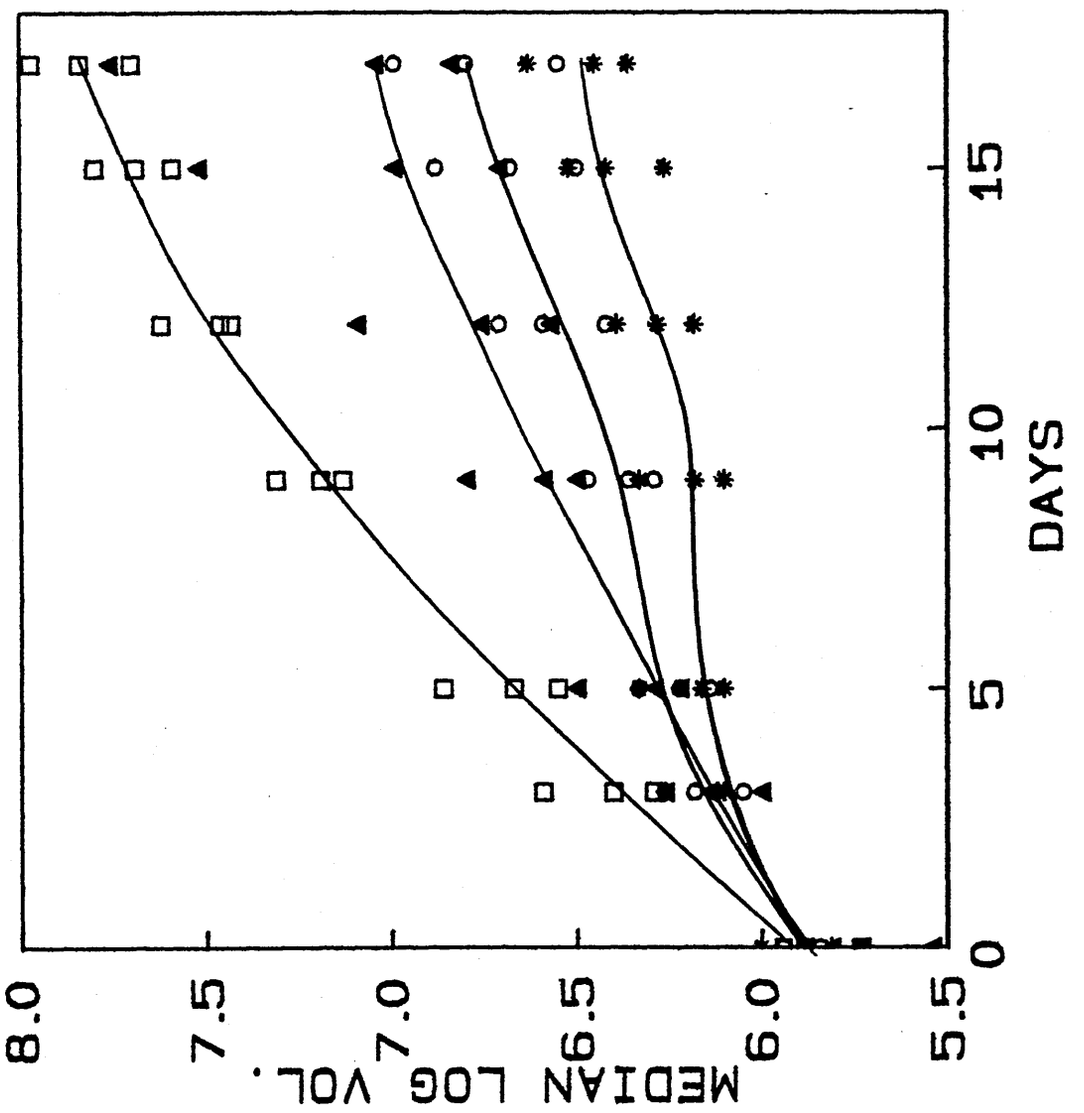


Figure 5: Regrowth curves of 'small' L-Dan spheroids following exposure to cisplatin. KEY: Control= open squares, 10 ug/ml= filled triangles, 20 ug/ml= open cirles, 40 ug/ml= stars.

The comparative log S versus dose plots are shown in figure 6. Back-extrapolation of the spheroid regrowth curves was only possible up to a dose of 20 ug/ml because at higher doses the curves failed to return to parallel with the control curve (this is a violation of one of the assumptions implicit in this model).

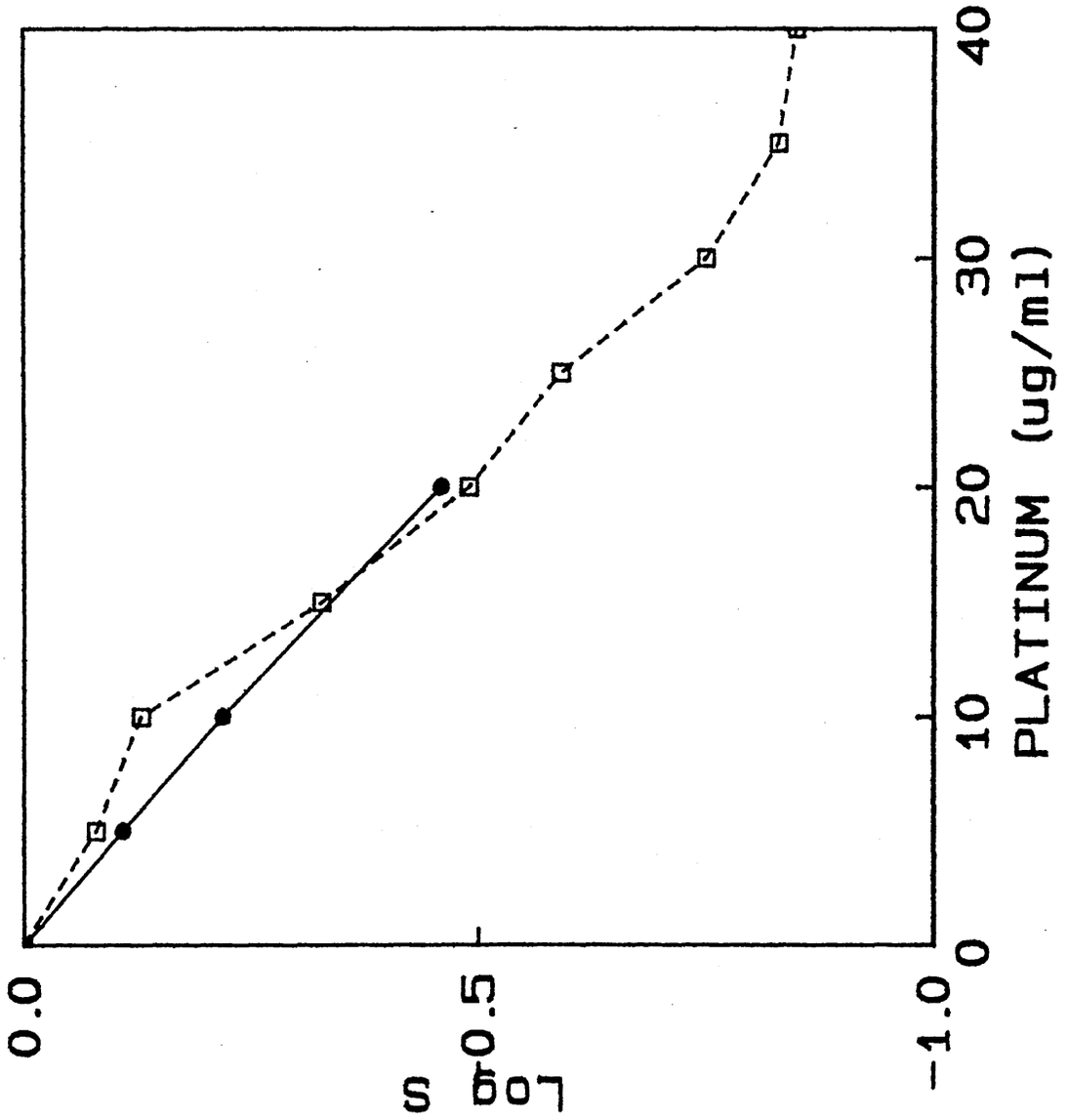


Figure 6: Log S versus dose of cisplatin plot for L-Dan spheroids and monolayer. KEY: Monolayers= open squares, spheroids= filled circles.

RESULTS

5-FLUOROURACIL

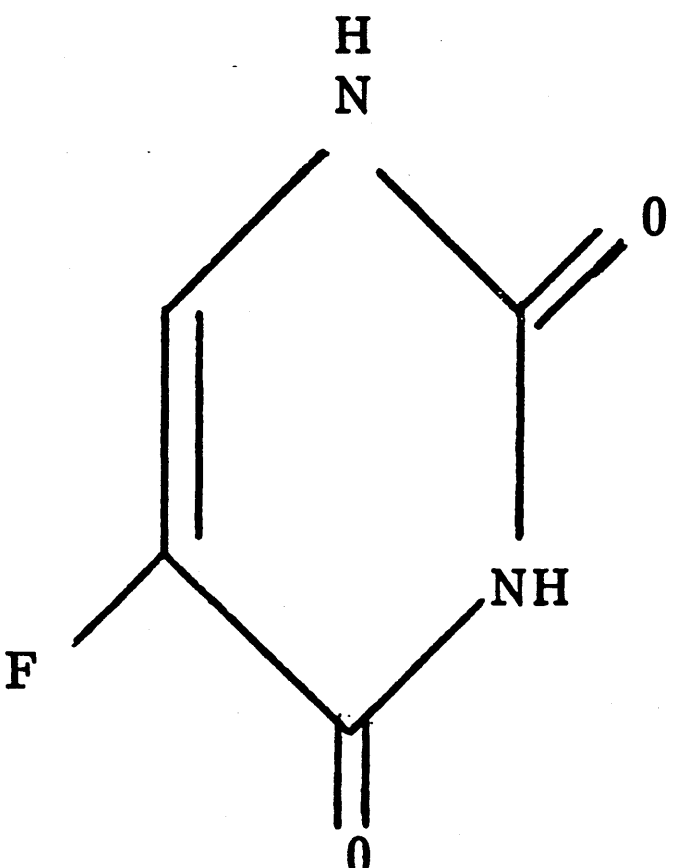


Figure 7; structure of 5-Fluorouracil. MW=130

5-Fluorouracil (5-FU) is a potent pyrimidine anti-metabolite which blocks thymidylate (and thereby DNA) synthesis and interferes with RNA synthesis, leading to numerous consequences for the affected cell (Mandel, Klubes & Fernandes, 1978. Pinedo & Peters, 1988). The main action of the drug is in the S-phase of the cell cycle, and the drug is most toxic to proliferating cells (Tannock, 1978). 5-FU is most commonly given as IV bolus

injections at doses ranging from 300-600mg/m^2, and exhibits nonlinear pharmacokinetics in humans (Collins, Dedrick & King, 1980). These IV doses give rise to peak plasma levels of 5-FU in the region of 60ug/ml (MacMillan, Wolberg & Welling, 1978).

Figure 8 shows a dose response curve for 5-FU against exponential phase L-DAN cells in monolayer cultures. The results are expressed as percentage of control growth versus 5-fluorouracil concentration. Each point is the mean of two experiments +/- standard errors. The curve was drawn by eye as the best fit to the data. The derived values of log S are given in table 5.

Figure 9 shows representative regrowth curves of L-DAN spheroids exposed to the stated concentrations of 5-FU. In the interest of clarity only a limited dose range is illustrated, but log S values for all concentrations tested are given in table 5. Each set of symbols shows the median log volume from 20-24 individual spheroid measurements and the 95% confidence intervals about this median value. Since the regrowth curves are parallel with the control curve back-extrapolation has been performed giving the results shown in table 5 and plotted in figure 10.

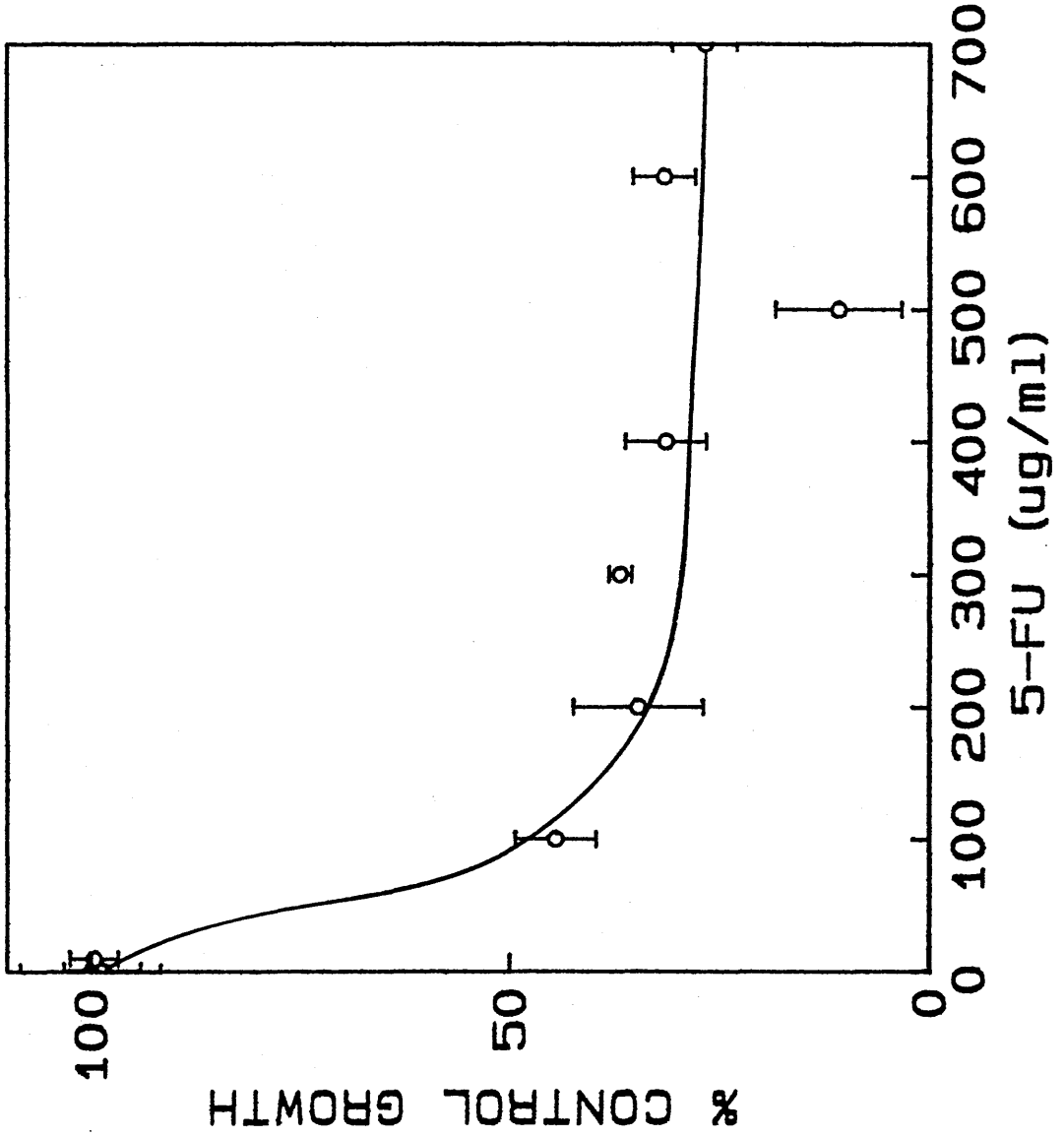


Figure 8: Dose-survival curve for L-Dan cells exposed to 5-fluorouracil in monolayer cultures

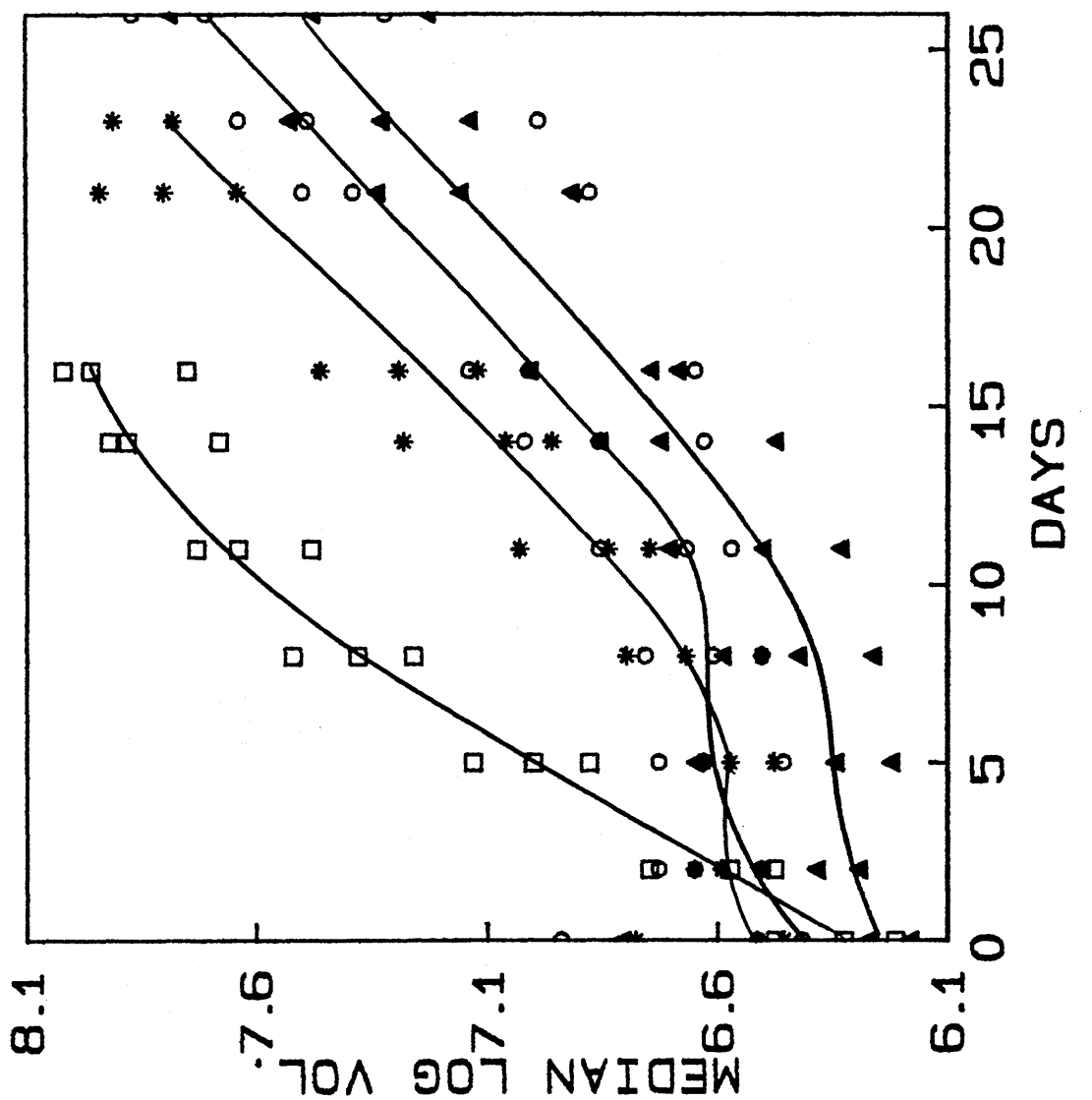


Figure 9: Regrowth curves of L-Dan spheroids following exposure to 5-fluorouracil. KEY: Control= open squares, 200ug/ml= filled triangles, 400ug/ml= open circles, 600ug/ml= stars.

TABLE 5

DOSE	LOG S	
(ug/ml)	Monolayer	Spheroids
10	-0.002	0
100	-0.35	-0.6
200	-0.46	-1.07
300	-0.43	-0.98
400	-0.5	-1.2
600	-0.5	-1.0
700	-0.57	-1.35

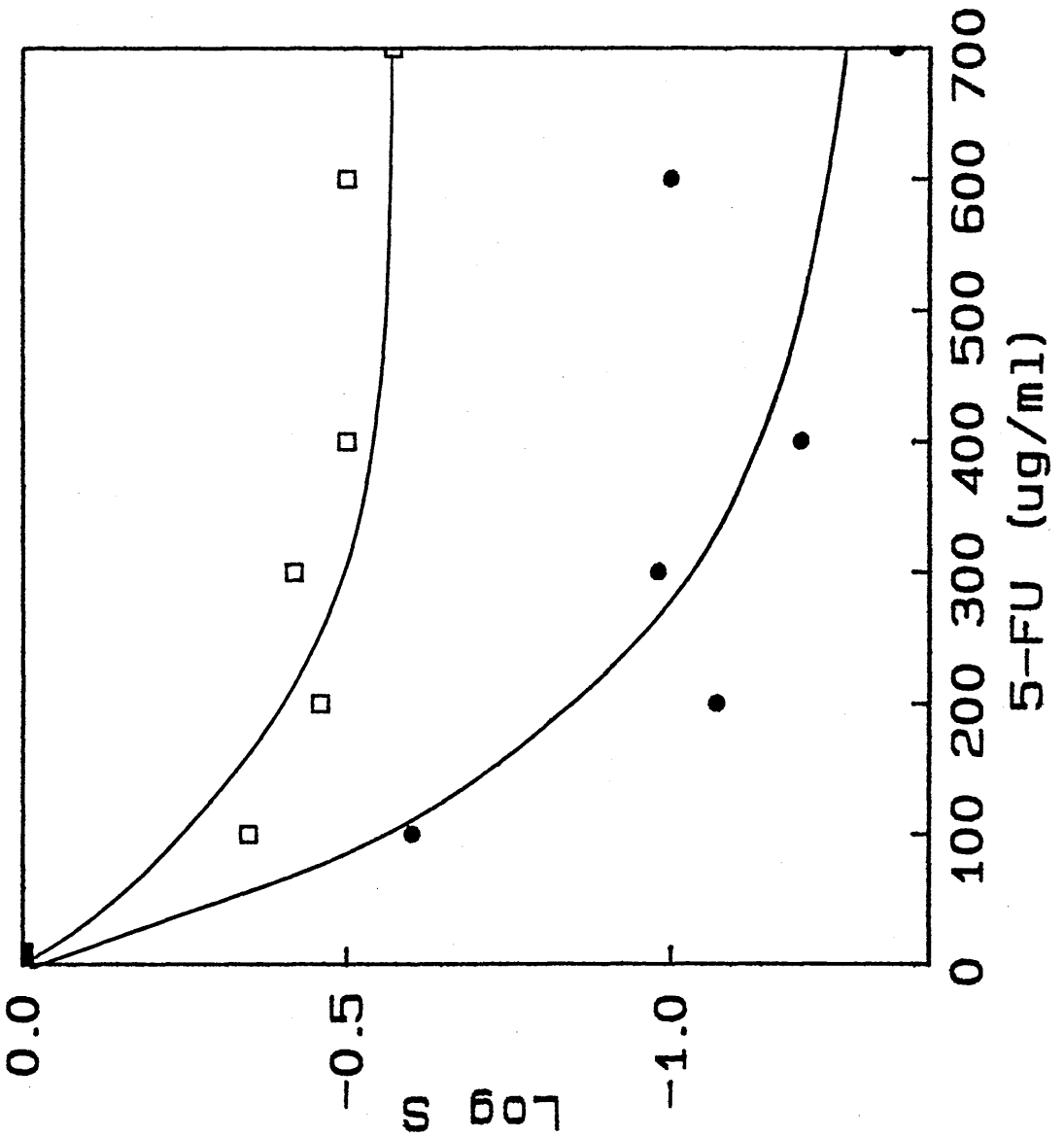


Figure 10: Log S versus dose of 5-fluorouracil plot for L-Dan spheroids and monolayers. KEY: Monolayers= open squares, spheroids= filled circles.

RESULTS

ADRIAMYCIN

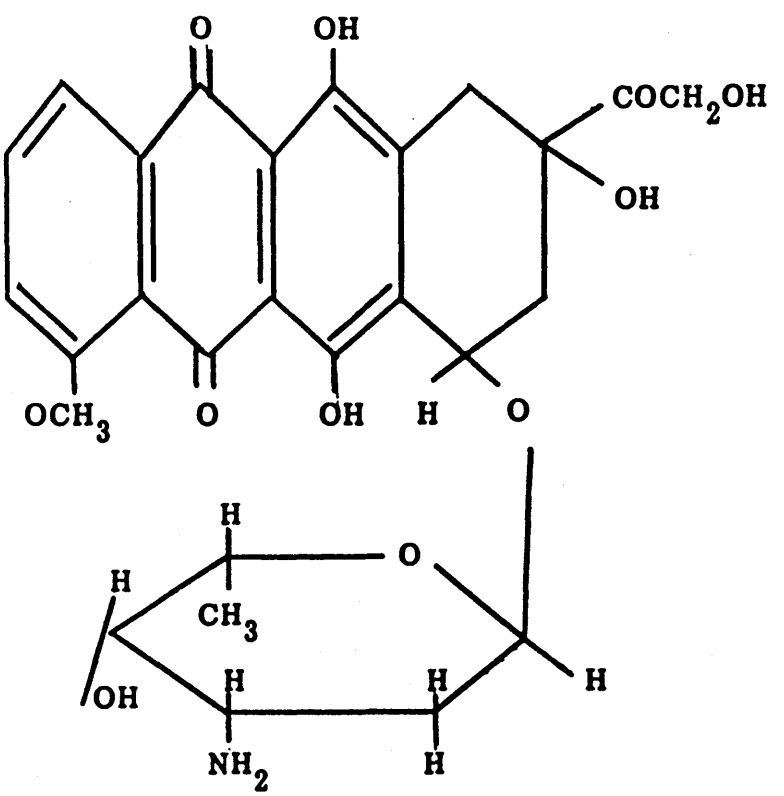


Figure 11; Structure of Adriamycin. MW=580

Adriamycin is an anthracycline antibiotic with a wide spectrum of clinical activity in both solid tumours and haematological malignancies. It is probably the single most active agent available at present.

Despite the clinical importance of this group of drugs and

major research efforts, the mechanism of action of the anthracyclines is still uncertain. Most would support a nuclear site of action, with DNA as the prime target (Capranico et al, 1989), and this is supported by cytofluorescent localisation studies (Egorin et al, 1974). The possible mechanisms of DNA damage include :-

1) production of free radicals (Sinha & Gregory, 1981) though this appears not to be of importance in-vivo (Potmesil, Israel & Silber, 1984).

2) direct intercalation of DNA through the aglycone moiety (Pigram, Fuller & Hamilton, 1972), which can be demonstrated by a variety of techniques including nuclear magnetic resonance (Feigon et al, 1984).

3) binding through ternary complexes of iron, adriamycin and DNA (Eliot, Gianni & Myers, 1983).

4) stabilisation of topoisomerase II-DNA complex in an open (cleaved) conformation of DNA (Pommier et al, 1985).

The situation is made more complex by the ability of anthracyclines to inhibit RNA synthesis (Kriebardis, Meng & Aktipis, 1984); to damage nuclear membranes (Mimnaugh et al, 1985); and cell membranes (Goormaghtigh & Ruysschaert, 1984. Hickman, Chachwala & Thompson, 1985) leading to alterations in ion permeability (Santone et al, 1986). Some would even claim that they can be cytotoxic without entering the cell (Tritton & Yee, 1982. Rogers, Carr & Tokes, 1983).

The dose schedule in most common use is 40-60 mg/m^2 by IV injection every 3 weeks. This results in plasma levels of the order of 1-2 ug/ml (Kerr et al, 1986. Benjamin, Riggs & Bachur, 1977).

Figure 12 shows a dose response curve for adriamycin against exponential phase L-DAN cells in monolayer culture. The results are expressed as percentage of control growth versus adriamycin concentration. Each point is the mean of 2-3 experiments +/- standard errors. The curve was drawn by eye as the best fit to the data. Corresponding values for log S are given in table 6.

Figure 13 shows a series of representative regrowth curves for L-DAN spheroids after 1 hour exposure to the stated concentration of drug. Each set of symbols is the median log volume from 20-24 spheroids and the 95% confidence limits about this value. Log S values for these curves and all other tested concentrations (data not shown) derived by back-extrapolation of the curves are given in table 6, and plotted in figure 14.

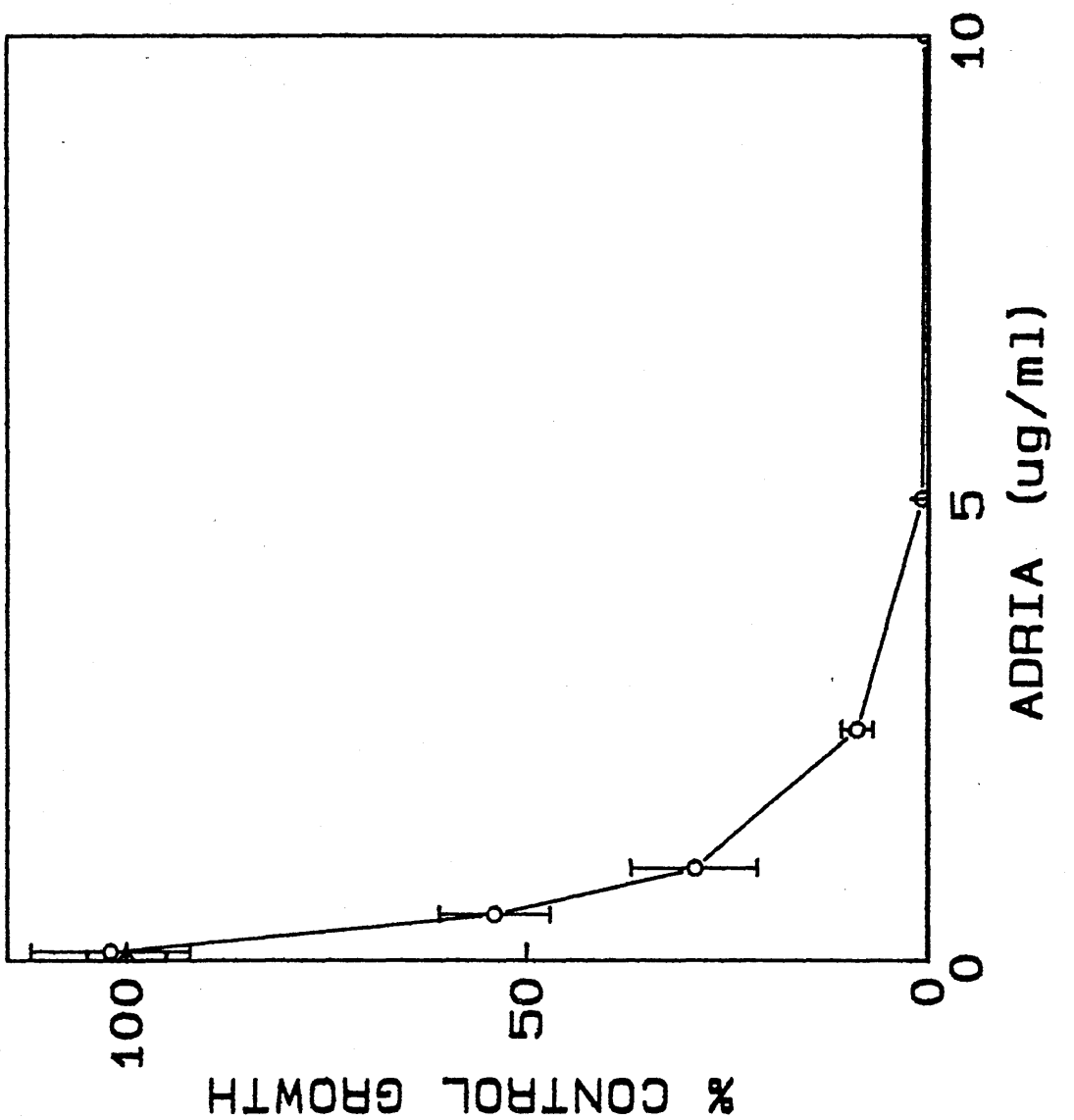


Figure 12: Dose survival curve for L-Dan cells exposed to adriamycin in monolayer cultures.

TABLE 6

Dose (ug/ml)	Log S	
	Monolayer	Spheroids
0	0	0
0.1	-0.02	-0.0004
0.5	-0.27	ND
1.0	-0.54	-0.15
2.5	-1.4	-0.4
5.0	-2.15	-0.41
10.0	-2.44	-0.43

ND - not done.

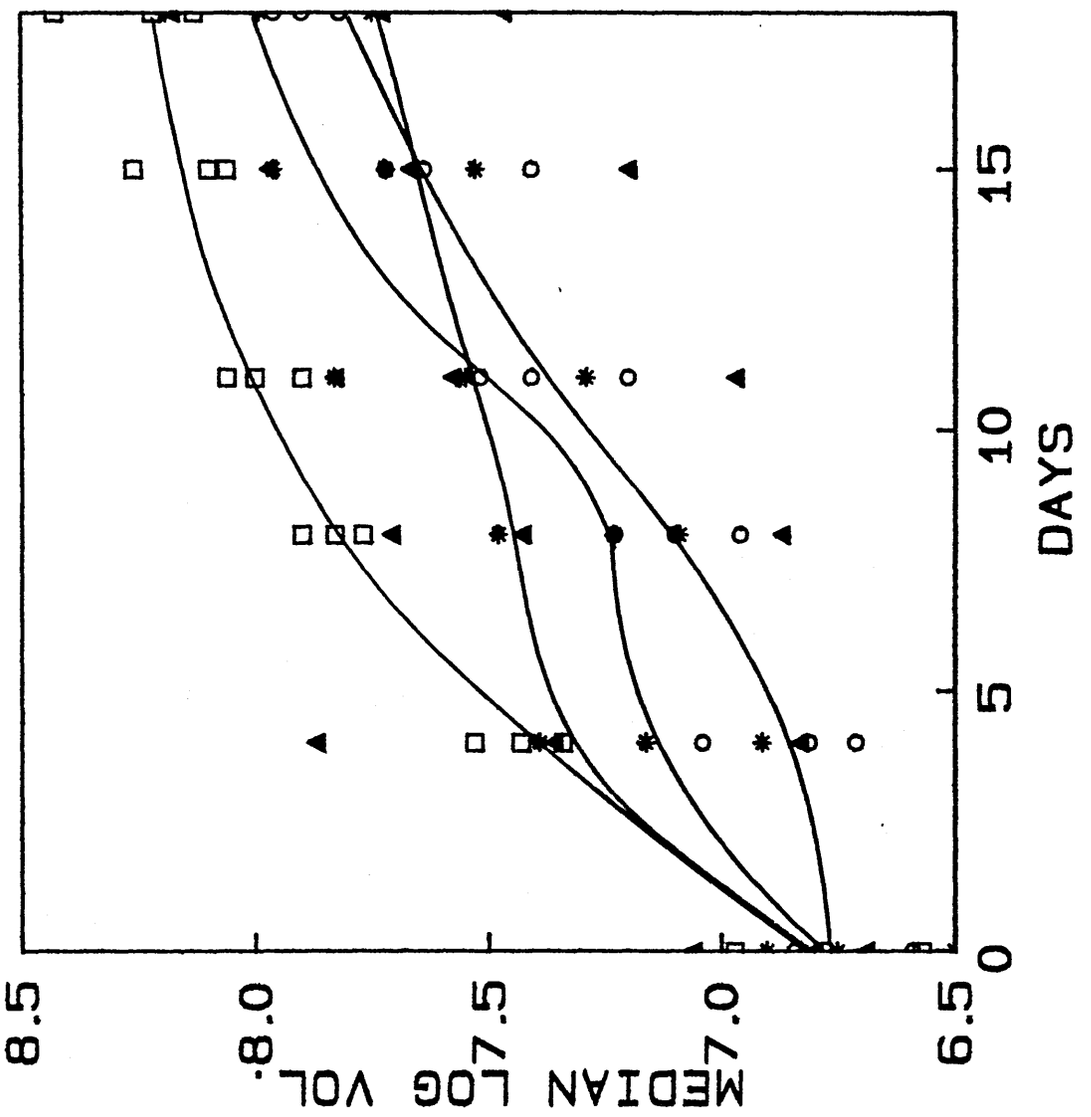


Figure 13: Regrowth curves of L-Dan spheroids following exposure to adriamycin. KEY: Control= open squares, 10ug/ml= filled triangles, 2.5ug/ml= open circles, 5ug/ml= stars.

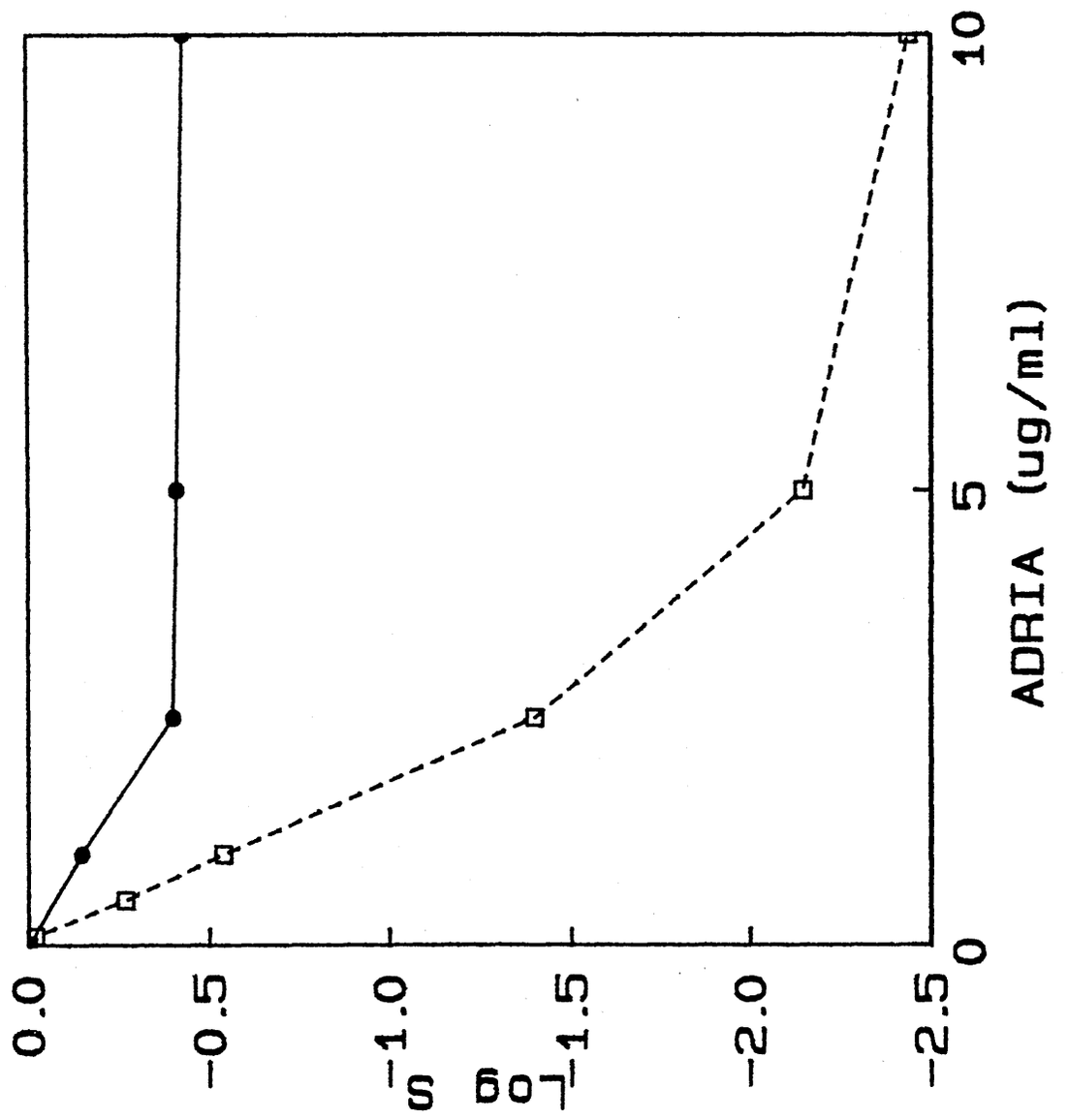


Figure 14: Log S versus dose of adriamycin plot for L-Dan spheroids and monolayers. KEY: Monolayers= open squares, spheroids= filled circles.

RESULTS

METHOTREXATE

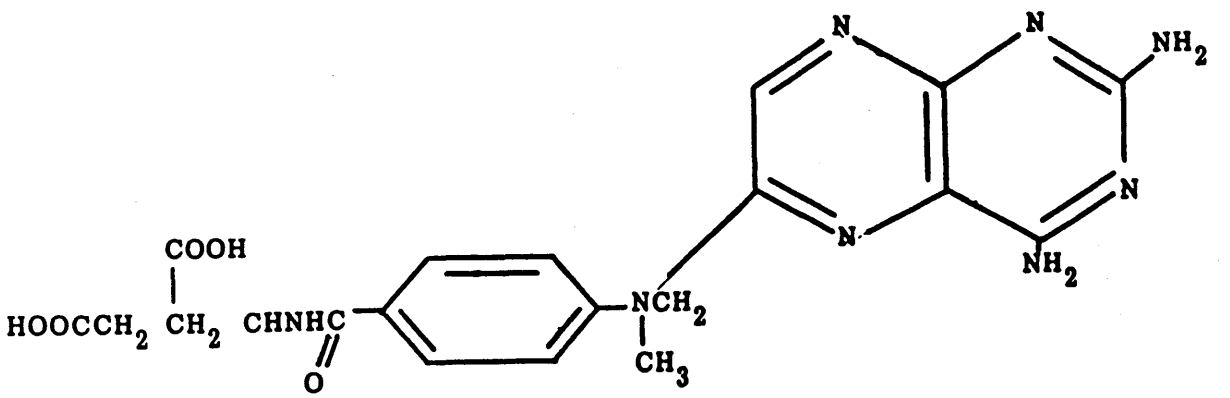


Figure 15: Structure of methotrexate. MW=472

Methotrexate is believed to produce cytotoxicity by depletion of reduced folates which are necessary for the synthesis of thymidylate, purines and certain amino acids. Depletion of these substrates results from the tight binding inhibition of dihydrofolate reductase. Li and Kaminskas (1984) reported a linear correlation of DNA strand breaks and cell death in cells exposed to methotrexate. They hypothesised that the DNA strand

breaks were the ultimate cause of cell death, and since the strand breaks occurred in mature DNA, they suggested that the breaks were the result of ineffective repair mechanisms.

The spectrum of clinical activity of methotrexate is wide. The use of the biochemical antidote, 5-formyltetrahydrofolate (leucovorin, citrovorum factor), has allowed the use of very high doses (Goldman & Matherly, 1985). Doses in clinical usage range from 0.5mg to >30g/m^2, and there is wide inter-individual variation in drug handling, emphasising the need for monitoring of plasma methotrexate concentrations during high dose treatment (Bertino, 1981). Plasma levels greater than 0.1umolar (approx 0.5 ug/ml) would be expected to be cytotoxic. A dose of 100 mg/m^2 will result in peak plasma levels of around 10 umolar; a dose of 1.5 g/m^2 results in a peak of 0.1-1 mmolar (Chabner, Donehower & Schilsky, 1981). Figure 16 shows a dose response curve for methotrexate (one hour exposure) against exponential L-DAN cells. The results are expressed as percentage of control growth versus methotrexate concentration. Each point is the mean of 3 experiments +/- standard error. The derived values of log S are given in table 7.

Figure 17 shows representative regrowth curves of L-DAN spheroids exposed for one hour to the stated concentrations of methotrexate. Each point shows the median log volume from 20-24 individual spheroid measurements (the 95% limits are too small to plot accurately). Log S values for all the tested drug concentrations are given in table 7.

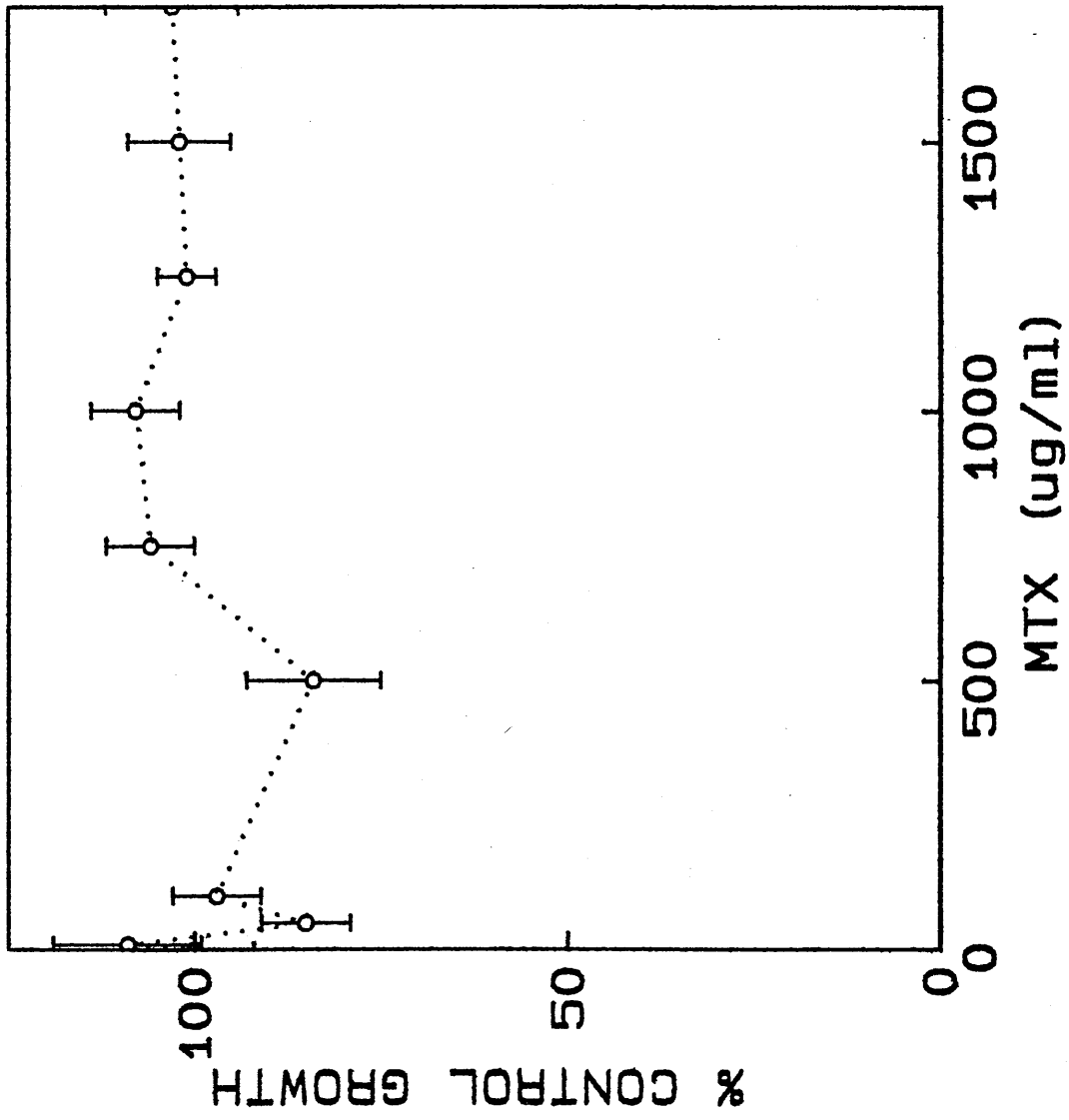


Figure 16: Dose survival curve for L-Dan cells exposed to methotrexate in monolayer cultures

TABLE 7

Dose (ug/ml)	Log S monolayers	Log S spheroids
1	0.12	ND
2	-0.08	ND
3	-0.004	ND
4	-0.1	ND
5	-0.02	-0.23
10	0.04	-0.09
50	-0.07	-0.37
100	-0.01	-0.38
500	-0.08	-0.34
750	0.03	ND
1000	0.03	-0.37
1250	0.004	ND
1500	0.009	ND
1750	0.01	ND

ND - not done.

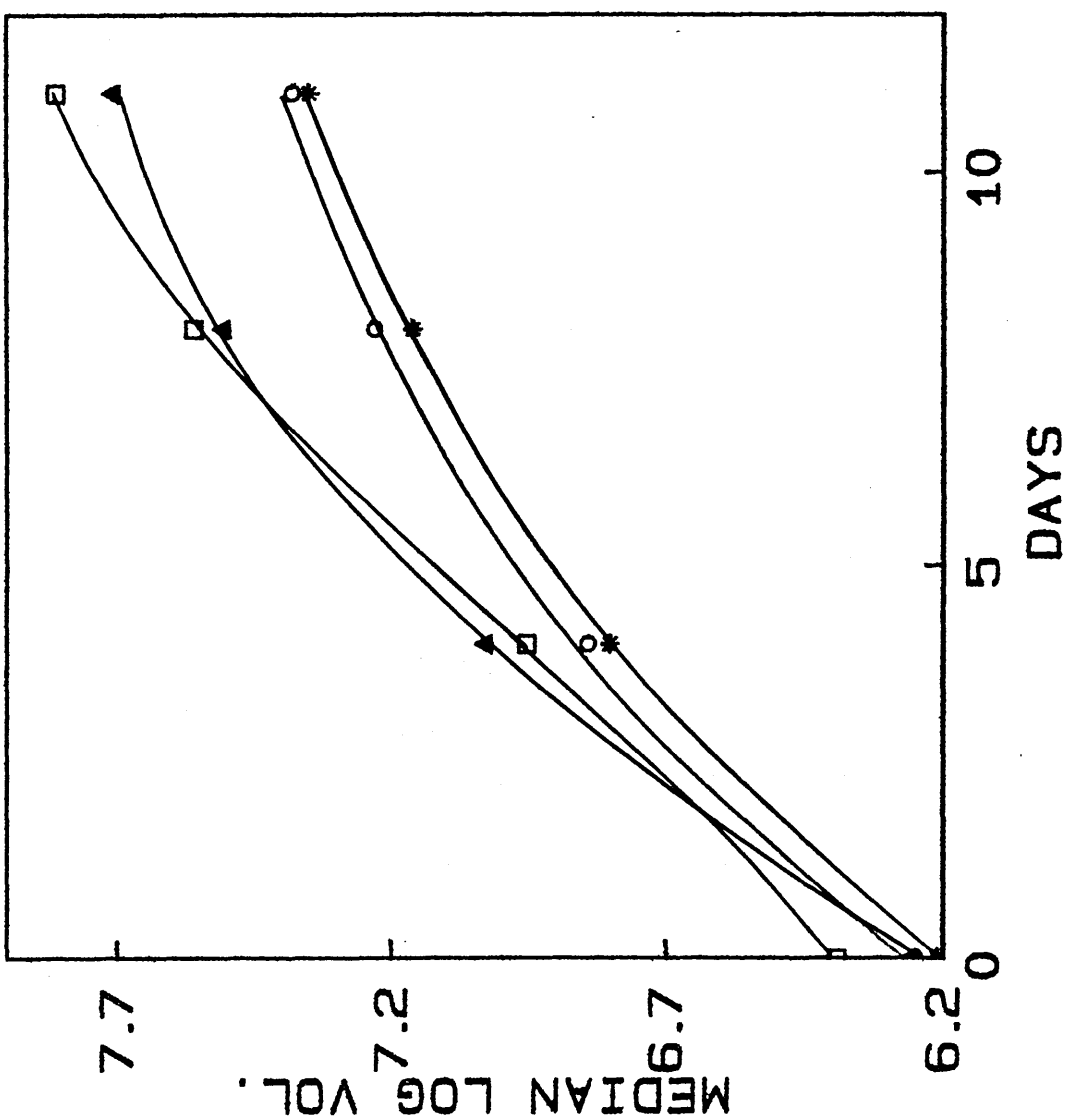


Figure 17: Regrowth curves of L-Dan spheroids following exposure to methotrexate. KEY: Control= open squares, 10ug/ml= filled triangles, 100ug/ml= open circles, 1000ug/ml= stars.

Figure 18 shows the comparative log S values for L-DAN spheroids and monolayers when exposed to the stated concentrations of methotrexate for one hour.

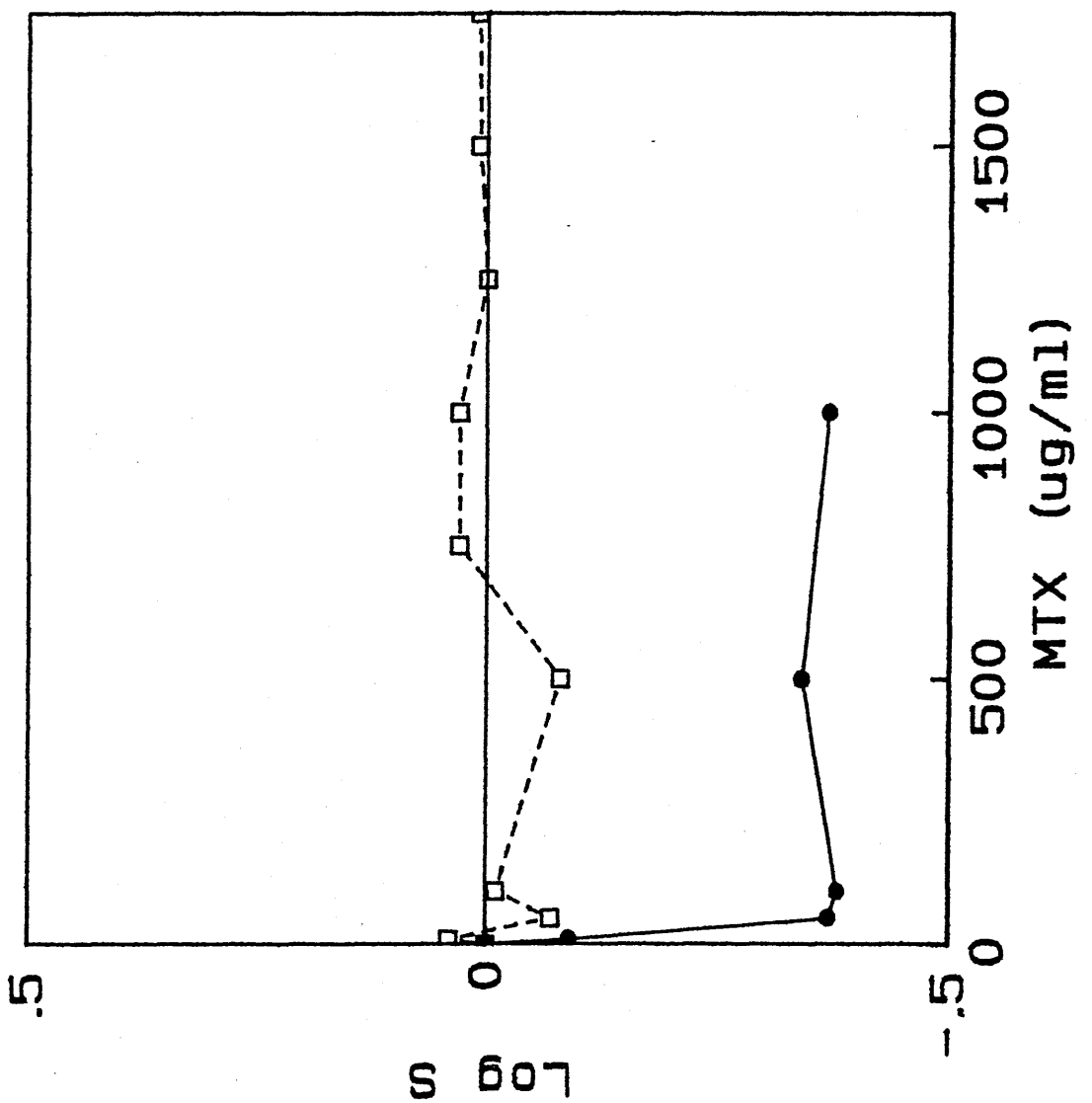


Figure 18: Log S versus dose of methotrexate plot for L-Dan spheroids and monolayers. KEY: Monolayers= open squares, spheroids= filled circles.

RESULTS

VINCRISTINE

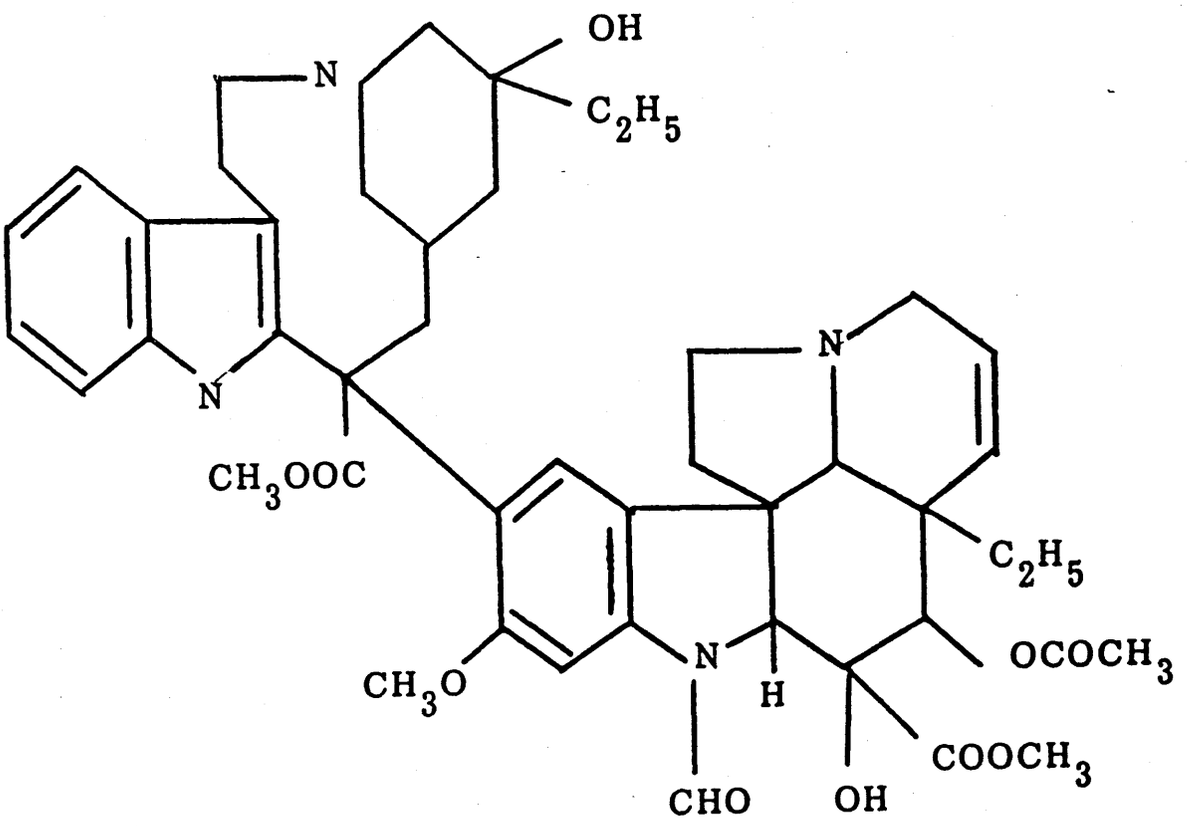


Figure 19: Structure of Vincristine MW=922

Vincristine is a member of the vinca alkaloid family of cytotoxic drugs. These agents kill cells by disrupting microtubular structures by binding to tubulin (Owellen, Owens & Donigian, 1972) and preventing its polymerisation (Owellen et al, 1976). This results in defective mitotic spindle formation, impaired cellular motility (Mareel & DeMets, 1984) and perturbed intracellular transport systems (Ruzzier et al, 1984. Bennett, Parsons & Carlet, 1984).

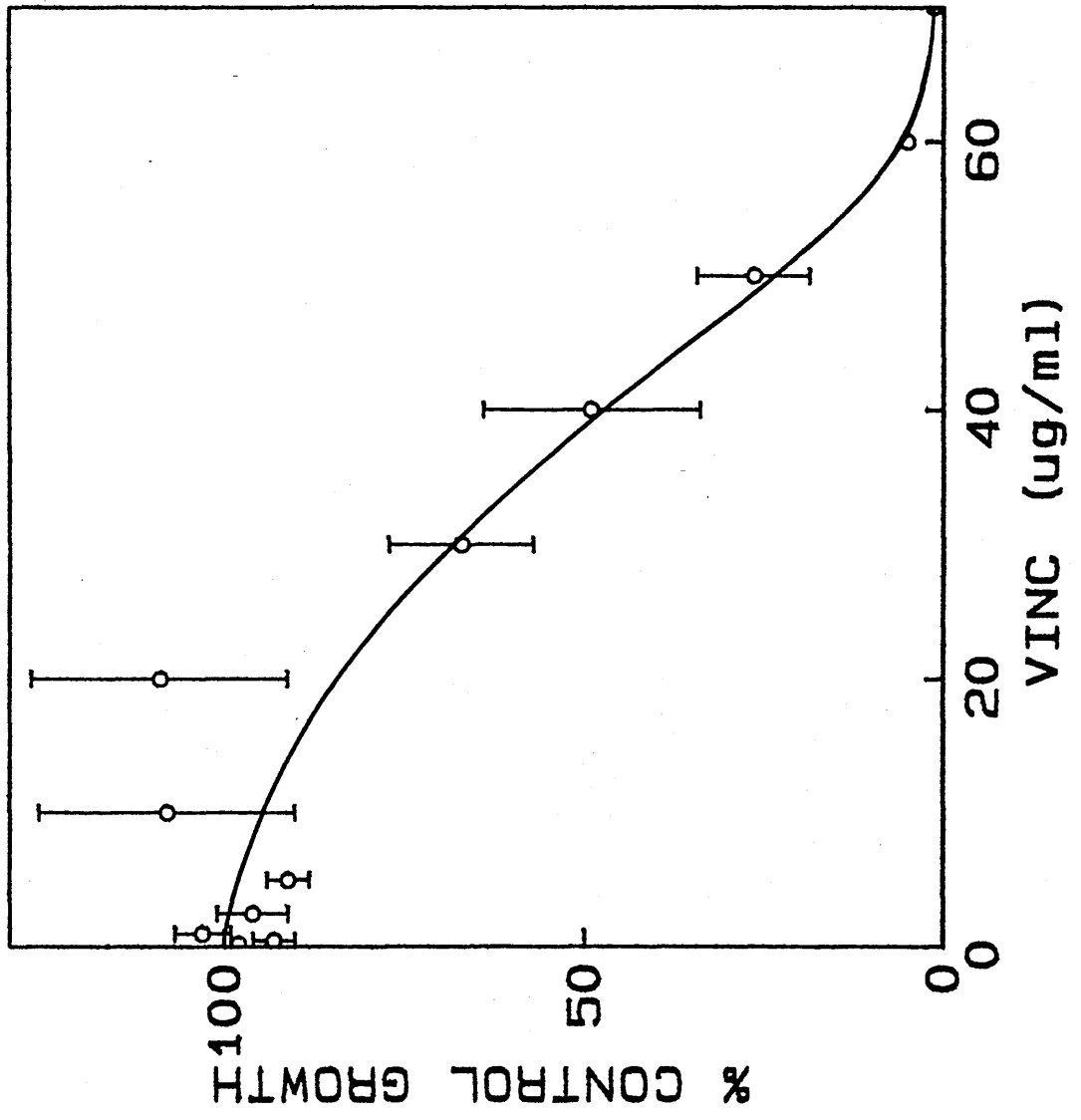


Figure 20: Dose survival curve for L-Dan cells exposed to vincristine in monolayer culture.

The standard clinical dosage of vincristine in adult practice is 1.4 mg/m^2 intravenously every 3 weeks. This dosage results in peak plasma concentrations of around 100 nmol/L = 90 ng/ml (Owellen, Root & Hains, 1977). Vincristine has a large volume of distribution and a long biological half-life indicating avid tissue binding and slow drug release from the body tissues (Sethi et al, 1981).

Figure 20 shows a dose response curve for vincristine against exponential L-DAN cells in monolayer culture. The results are expressed as a percentage of control growth versus vincristine concentration. Each point is the mean of 3-9 experiments +/- standard error (more replicates were required at some concentrations because of wide variability between experiments). The derived values of log S are given in table 8.

Figure 21 shows representative regrowth curves of L-DAN spheroids exposed for one hour to the stated concentrations of vincristine. Each set of symbols is the median of 20-24 individual spheroid measurements and the 95% confidence limits about that value. Log S values for all the tested drug concentrations are given in table 8.

Figure 22 shows the comparative log S values for L-DAN spheroids and monolayers when exposed for one hour to the stated vincristine concentrations.

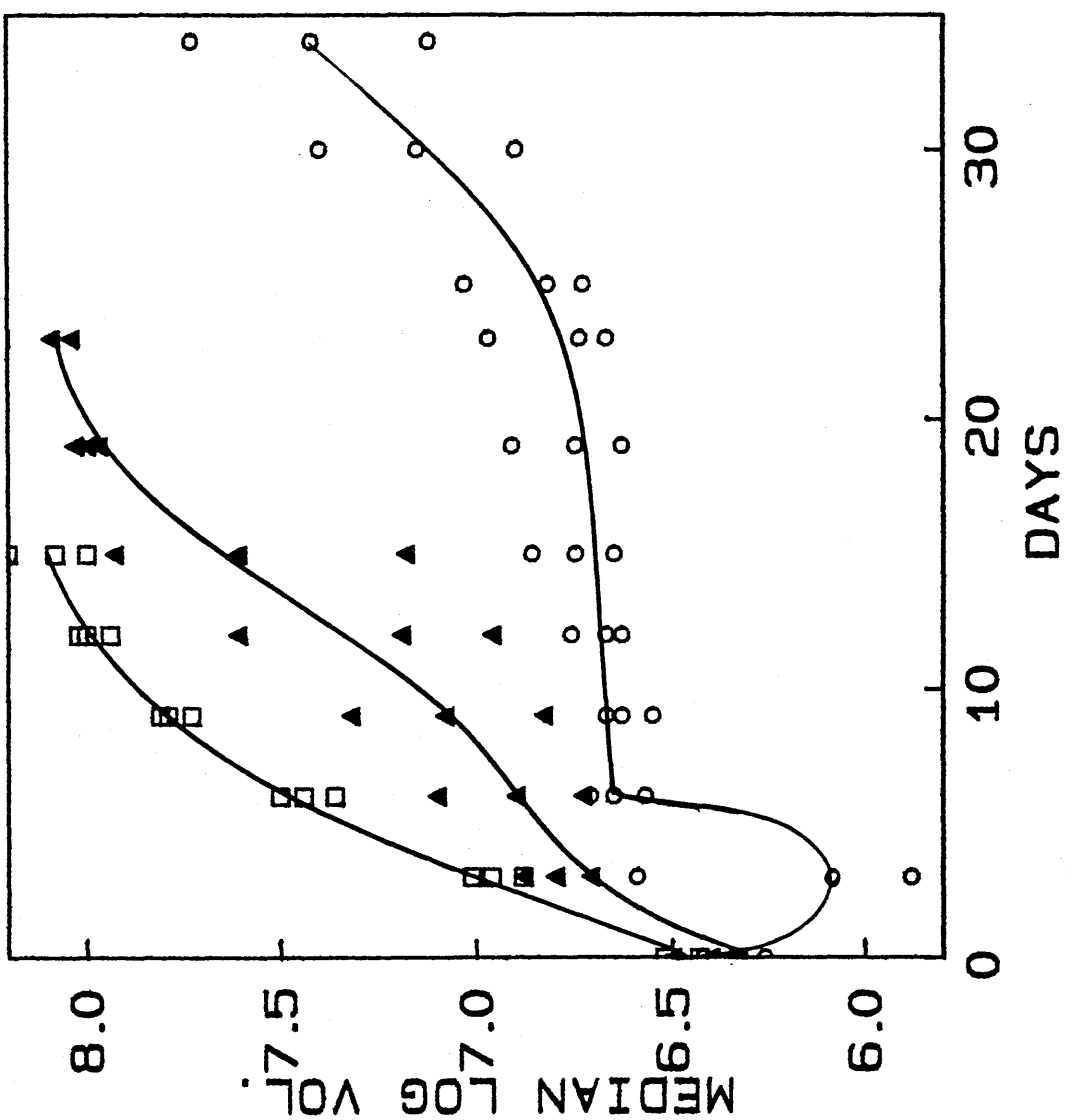


Figure 21: Regrowth curves of L-Dan spheroids following exposure to vincristine. KEY: Control= open squares, 10ug/ml= filled triangles, 50ug/ml= open circles.

TABLE 8

DOSE (ug/ml)	Log S	
	Monolayer	Spheroid
0.25	-0.009	-0.02
0.5	-0.03	-0.07
1.0	0.013	-0.07
2.5	-0.018	-0.24
5.0	-0.04	-0.36
10.0	0.03	-1.3
20.0	0.04	ND
30.0	-0.17	-2.3
40.0	-0.3	ND
50.0	-0.58	-3.33
60.0	-1.3	ND
70.0	-1.9	ND

ND - not done.

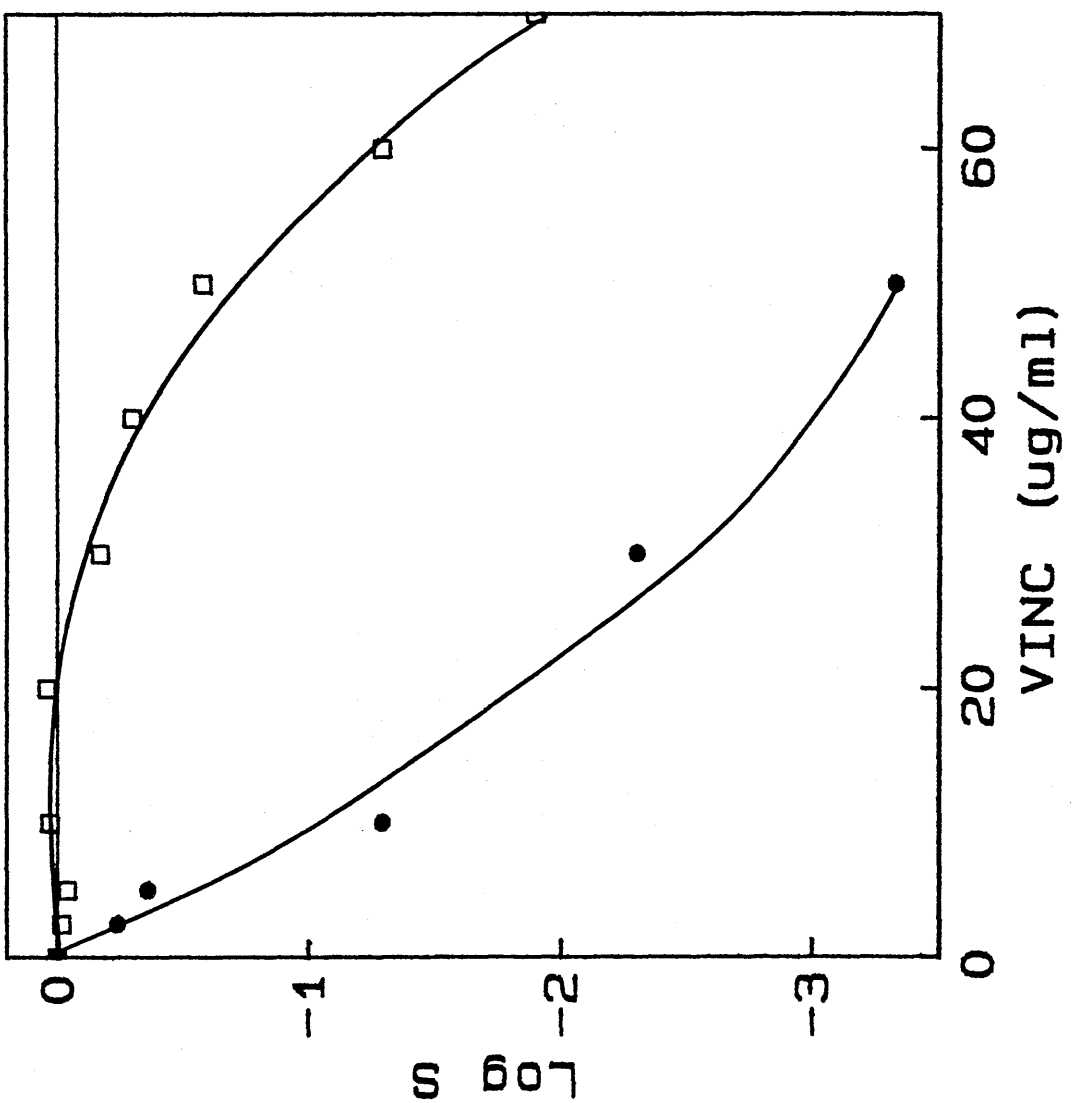


Figure 22: Log S versus dose of vincristine plot for L-Dan spheroids and monolayers. KEY: Monolayers= open squares, spheroids= filled circles.

DISCUSSION

This series of experiments confirms and extends the observations of other groups that when comparisons of cytotoxicity in monolayer or spheroid systems are made, tumour cells differ, and that 3 groups can be recognised (Erlichman & Vidgen, 1984).

Clearly cisplatin has similar effects in both growth systems for this cell line, implying that penetration into the spheroid is not a problem for this drug. This is contradictory to data generated using proton induced x-ray emission as a measure of cisplatin penetration into intraperitoneal nodules of tumour (Los et al, 1989), but is compatible with data generated for the platinum analoque paraplatin (Durand, 1979). It is possible that this apparent contradiction is due to more close 'packing' of the cells in-vivo, or that the extracellular matrix permeability is different in the two cell types.

Methotrexate had little effect in both growth systems, even at concentrations only clinically achievable using high dose protocols with forced diuresis and leucovorin rescue. In the spheroids there is an apparently small growth inhibition at high concentrations of methotrexate, but no effect at all in the monolayers. If this difference reflected the percentage of 'cycling' cells in each system, then the exponentially growing monolayers should be most sensitive. It seems likely that this

apparent effect in the spheroids is an artefact of the methodology employed. In the spheroids the drug is presented in only 10 ml of culture medium, whereas in the monolayer each flask of cells is treated with 25 ml of drug containing medium, as a result on a cell-for-cell basis the spheroids will suffer from a degree of nutrient deprivation at the very high concentrations of methotrexate used.

The data on adriamycin are a confirmation of the results of other authors, showing relative resistance in the spheroid model based on poor drug penetration into the spheroid. This has been demonstrated in the same cell line by fluorescent microscopy (Kerr et al, 1988), and in V79 Chinese hamster cells by flow cytometry (Durand, 1981). Similar penetration problems have been demonstrated in-vivo following intraperitoneal administration of adriamycin to mice bearing a transplantable ovarian cancer (Ozols et al, 1979).

Fluorouracil shows the opposite of the adriamycin data, in other words the spheroids appear to be more sensitive than the monolayers. As stated in the introduction to this chapter other workers have shown that 5-fluorouracil penetrates with ease through glioma and thyroid cancer cellular spheroids (Nederman, 1984). The apparent increased sensitivity of the spheroids is therefore surprising, as one would expect equal sensitivity rather than enhanced cytotoxicity in the spheroid if drug penetration were the only consideration. As the central cells of

the spheroid are non-cycling one would also expect a degree of resistance to a drug with its main action in the S-phase of the cycle (Mandel, Klubes & Fernandes, 1978), as has been demonstrated for Chinese Hamster V79 multicellualr spheroids by Durand (1986). However Tannock (1987) has shown equal sensitivity between aerobic and hypoxic cells to fluorouracil exposure in two murine tumours, despite the difference in cell cycle. A similar situation was found comparing intact with disaggregated spheroids for chlorambucil, mitomycin, thiotepa and bleomycin (Durand, 1986). One explanation of this finding would be the possibility that fluorouracil having found its way into the necrotic core of the spheroid has remained there through the washes after drug exposure , and has then diffused out from this central 'reservoir' to cause further cytotoxicity. Alternatively this may represent an artefact of the system used, Twentyman (1980) described a phenomenon of excessive growth delay for the amount of cell kill for some drugs in the multicellular spheroid system.

Vincristine shows a similar pattern to fluorouracil. In this case penetration into the core of the spheroid is probably limited. The penetration of vinblastine (which differs from vincristine by only one carbonyl group) has been demonstrated to be poor (Nederman & Carlsson, 1984) after short duration of exposure. Vincristine also has the highest molecular weight of all the drugs tested in this series of experiments, which one

would intuitively expect would be detrimental to tissue penetration. It is therefore unlikely that a central 'reservoir' of drug could form in this case. Both the monolayer and spheroid experiments using vincristine were subject to a higher than expected inter-experiment error, and this is the reason for the higher numbers of replicates. In particular the spheroid experiments were difficult to interpret because of changes in the shape and configuration of the cell aggregates; some would disintegrate after drug exposure, some would degenerate into monolayers, others would become large islands of cells rather than spheres making measurements of area invalid. Stephens & Peacock (1978) noted that when tumours were treated with vincristine in-vivo then excised and dissaggregated extensive cell lysis had occurred. This effect has not been noted in monolayers, and presumably reflects an increased susceptibility to damage of relatively hypoxic cells. This could explain our data both in terms of the cytotoxicity and the morphological changes observed.

These experiments also highlight some of the limitations of the multicellular spheroid system. The data presented looks at cytotoxicity indirectly as a function of growth delay. The validity of this approach is questionable; for irradiation studies there does seem to be a correlation between growth delay and cell survival (Moore, West & Hendry, 1987), but for cytotoxic drugs the situation is less clear with a breakdown in

this correlation at least for some drug classes (Twentyman, 1980. Yuhas, Tarleton & Harman, 1978). Possible alternative end-points such as disaggregation or outgrowth on plastic also have theoretical limitations. In the case of disaggregation with trypsin, it appears that the timing of this procedure with respect to drug exposure is crucial (Twentyman, 1980). In the case of failure of outgrowth or other so-called 'cure' experiments, usually very high (and clinically unachievable) concentrations of drug are required to come close to this successful radiobiological technique (Durand, 1975).

Even accepting the limitations of regrowth delay as an end-point it was difficult to use back-extrapolation in those situations when the regrowth curves do not return to parallel the controls, or when changes occur in the morphology of the cellular aggregates (as was the case with vincristine).

A further limitation, common to most in-vitro systems is that the cells are exposed to specified drug concentrations for a short time. In reality the cells in a tumour would be exposed to an oscillating drug concentration over a time which is dependant on drug handling by the host. Attempts to get round this problem have been made by exposing intraperitoneally implanted spheroids to intravenously administered drug, but again this approach is severely limited by additional immunological reaction to the spheroids (Yuhas, Tarleton & Harman, 1978).

In conclusion, adriamycin is the only agent from this selected group of drugs for which penetration into the central

region of the spheroid seems to be a limiting consideration. A similar conclusion was made from a recent series of elegant experiments using fluorescence activated cell sorting techniques, followed by clonogenic assay. This study also revealed an apparent dissociation between cellular drug levels and activity, implicating a major role for the cellular microenvironment in modulating drug toxicity (Durand, 1989).

Despite this problem with penetration, the anthracyclines are clinically the most active of cytotoxic agents. It is therefore logical to attempt to improve the penetration of anthracyclines to enhance this anti-tumour activity, and studies along these lines are described in the following chapters.

CHAPTER 3

SURFACTANT STUDIES

One possible method of increasing the anti-tumour efficacy of adriamycin would be to co-administer an agent which would in some way improve the penetration of the cytotoxic. Optimally this would occur only in the target tissue (tumour) and not in organs of toxicity e.g. bone marrow. Surfactants are molecules with hydrophilic areas and hydrophobic (usually hydrocarbon) chains, as such they act in a similar fashion to detergents. Surfactants are present in many foodstuffs and are used commercially as emulsifiers, solubilisers, defoamers and detergents in the pharmaceutical, cosmetic and other industries. Non-ionic (or ionic) surfactants have been shown to be capable of altering the absorption of drugs. These effects have been attributed to an increase in membrane fluidity in the presence of the detergent. Some of the factors determining the effects observed include the size and polarity of the drug molecule, the concentration of the surfactant, the nature of the surfactant and drug-surfactant interactions (Florence, 1981).

The effect of the non-ionic surfactant polysorbate 80 (Tween 80) on methotrexate absorption and distribution has been most extensively investigated. This non-vesicle forming surfactant enhances the oral bioavailability of methotrexate and resulted

in enhanced delivery of methotrexate to the brain of treated animals (Azmin et al, 1982. Azmin, Stuart & Florence, 1985), the mechanism of this change probably involves changes in cell membrane structure and permeability (Kay, 1965). This could have therapeutic implications in the therapy of childhood leukaemia with the need for penetration of drug into the 'sanctuary site' of the CNS. In addition, the same surfactant has been reported to potentiate the anti-neoplastic effect of adriamycin (Riehm & Biedler, 1982), is able to restore adriamycin sensitivity in a cell line with in-vitro resistance to this drug (Seeber, Meshkov & Schmidt, 1978) and causes a reduction in peak plasma levels of adriamycin following co-administration in cancer patients (Cummings et al, 1986a).

The non-ionic polyoxyethylated lauryl ether surfactant, Brij 30 has also been shown to enhance the cytotoxicity of adriamycin in-vitro (Kerr et al, 1987). This compound has not previously been tested in-vivo.

The toxic effects of parenterally administered surfactants are largely unknown. High doses can be tolerated orally (especially of the non-ionic surfactants),which is the most common route of administration (R.T.E.C.S., 1979. #JR5960000. Kabara,1978). Information on intravenous toxicology is scant for most surfactants though Tween 80 is tolerated by this route in rats (R.T.E.C.S., 1979. #WG2932500) and is administered in a

dose of 400mg/m^2 as an aid to solubility in Etoposide (VP16) as supplied by Bristol-Myers for use in cancer patients.

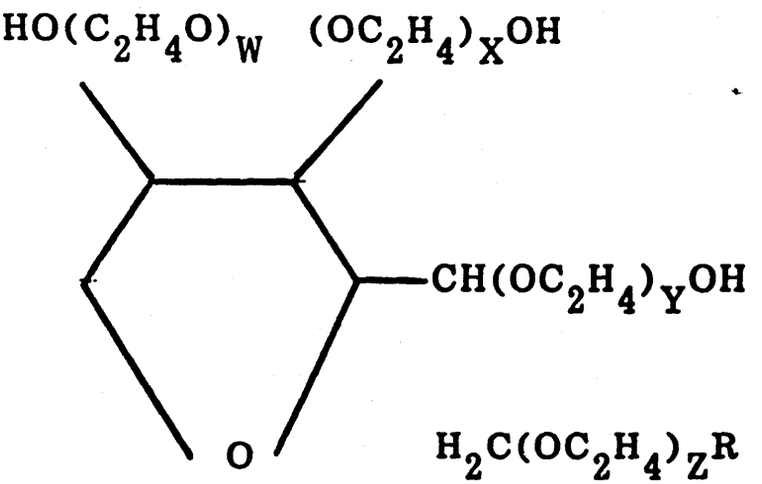
In this chapter are described experiments which attempt to extend some of these observations, and particularly to investigate the feasibility of intravenous co-administration of surfactants with the anthracycline cytotoxics.

MATERIALS AND METHODS

SURFACTANTS

The structure of Tween 80 (polyoxyethylene 20 sorbitan monooleate) is shown in figure 1. That of Brij 30 is given in figure 2. Both were supplied by B.D.H. Ltd, Broom Rd, Poole, Dorset, UK.

Surfactants I and II were the gift of the French drug company L'Oreal in collaboration with Prof. A.T. Florence, Department of Pharmacy, University of Strathclyde, UK. Their available chemical structures are shown in figures 3 and 4 respectively.



Sum of W,X,Y,Z is 20;

R is $(C_{17}H_{33})COO$

Figure 1: Structure of Tween 80

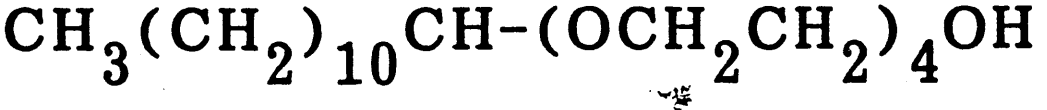


Figure 2: Structure of Brij 30

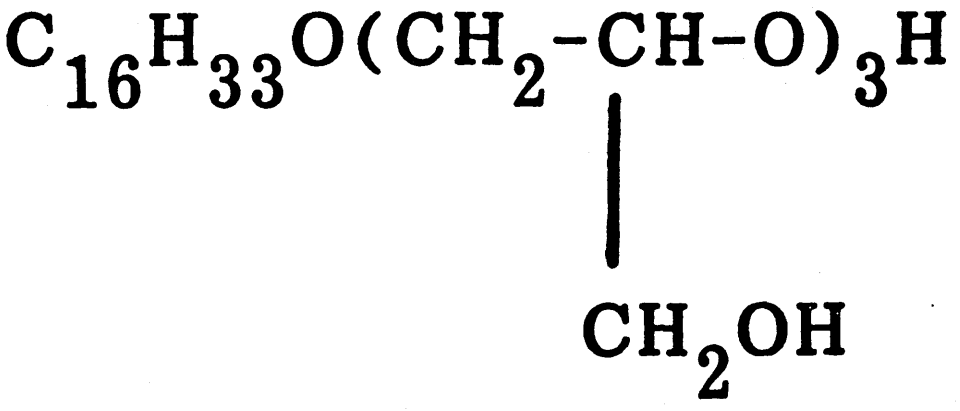


Figure 3: Structure of L'Oreal surfactant I

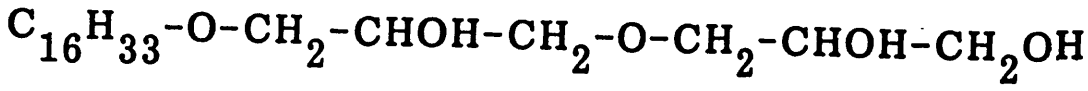


Figure 4: Structure of L'Oreal surfactant II

IN-VIVO TOLERANCE

The surfactant solutions were diluted with 0.9% Sodium chloride to appropriate concentration in a final volume of 0.2 ml for intravenous or intraperitoneal administration. Animals were restrained (no anaesthesia) and the surfactant injected IV or intraperitoneally (IP) through a sterile fine bore needle (25 g Microlance).

The animals used in each case were non-tumour bearing mice (MF1 Nu^{+}) from our own breeding colony. The animals were fed standard laboratory chow and allowed water ad libitum throughout the experimental period. After injection the animals were observed for one hour and thereafter checked daily for 28 days. Animals exhibiting lethal toxicities were dissected post-mortem and the tissues fixed in formaldehyde and submitted for histological examination (Dr Reid, Western Infirmary, Glasgow). Each dose was injected into a minimum of 3 mice by each route, in some experiments a larger number of mice were used. A tolerable dose was arbitrarily defined as that which allowed at least 75% of animals to survive without ill effect.

IN-VITRO CYTOTOXICITY

The cell line used throughout these experiments was a human non-small cell lung cancer line derived in our own laboratory

(Merry et al, 1987) known as L-DAN. Details of the growth conditions and the clonogenic assay used have been presented in detail elsewhere (chapter 2). In addition, because the surfactants often cause the cells to lose their adhesion to the plastic surface of the culture dish, after trypsinisation the cultures were centrifuged at 100 G for two minutes to ensure that no cells were lost. In each case a dose response curve was constructed for adriamycin alone or adriamycin together with the surfactant at the chosen concentrations. All drug exposures were for one hour, and each assay was performed in triplicate.

RESULTS

BRIJ 30

At a concentration of 3×10^{-5} Molar Brij 30 reduced clonogenic survival of L-DAN by approximately 10% (Kerr et al, 1987). An 0.2ml dose of this concentration was immediately fatal in 3/3 mice treated IP, and 3/3 mice treated IV. The same immediate fatal toxicity was exhibited at a concentration of 3×10^{-6} M. At post-mortem dissection histological evidence of pulmonary oedema was found, probably due to disruption of the alveolar capillary membranes thus allowing major and rapid extravasation of extracellular fluid. After a further 10 fold

dilution (3 x 10^{-7}M) this toxicity was not seen. All treated animals surviving without apparent ill-effect to the end of the observation period (28 days). Unfortunately, at this concentration an enhancement of adriamycin cytotoxicity could no longer be demonstrated as shown in figure 5 (and table 10).

TWEEN 80

This agent was well tolerated by all animals treated with concentrations up to 0.8 Molar given IV or IP. This surfactant was cytotoxic in its' own right against L-DAN, even at 10^{-7} Molar clonogenic survival was consistently less than 0.3% of control. This high level of cytotoxicity made it impossible to discern any additional effect of the surfactant-drug combination treatment.

SURFACTANT I

At concentrations greater than 10^{-5} M given IV or IP to mice this compound produced acute fatal toxicity similar to Brij 30. At 10^{-6} M, 4/5 mice treated intravenously survived and it was decided to use this concentration to investigate in-vitro cytotoxicity. Figure 5 shows the dose response curve to adriamycin with and without surfactant I. It is clear that at

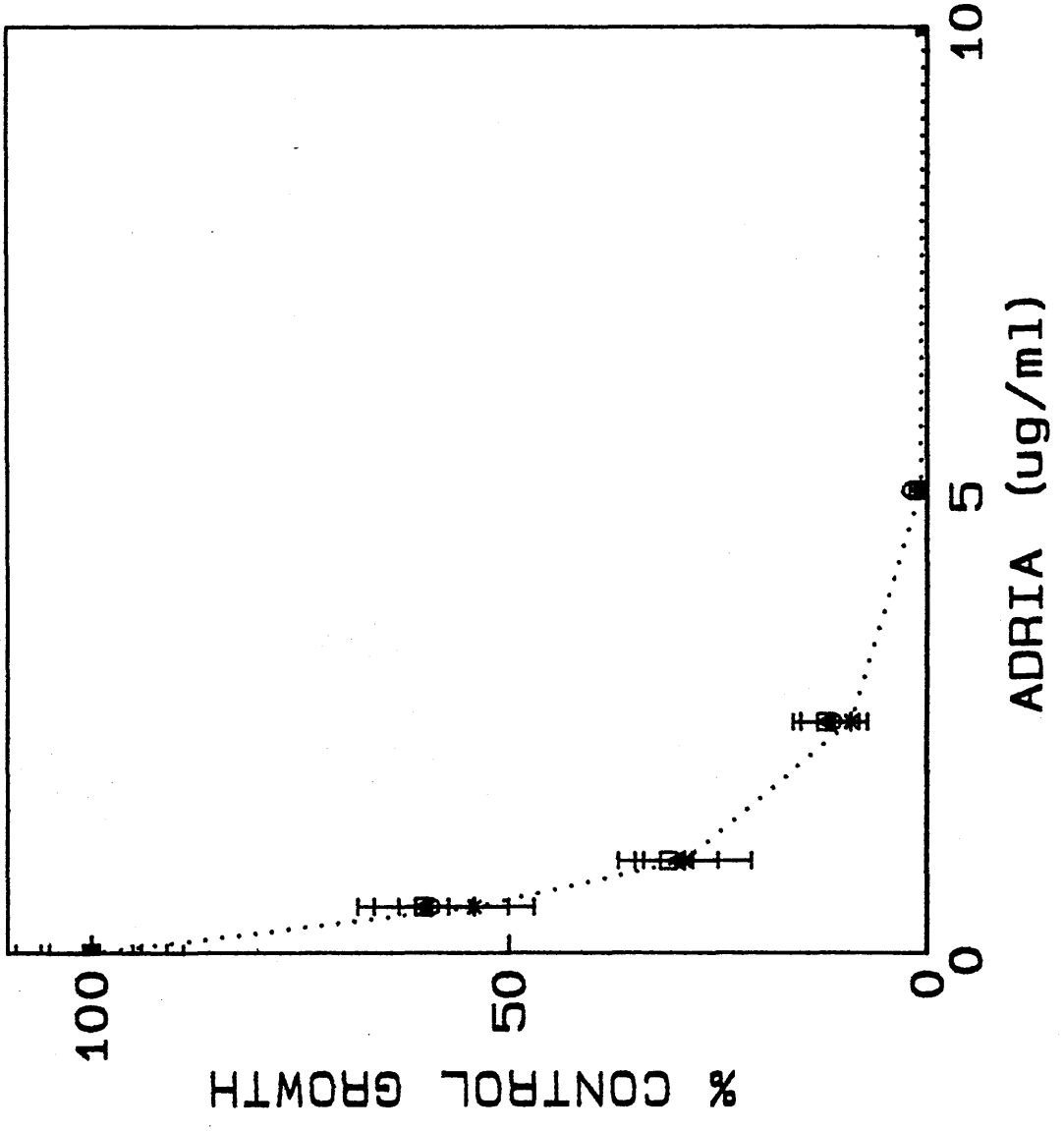


Figure 5: Dose survival curve for L-Dan monolayer cells exposed to adriamycin alone (controls) together with various surfactants. KEY: Control= stars, Brij 30= open circles, surfactant 1= open squares, surfactant 2= filled triangles.

this non-toxic concentration there is also no enhancement of adriamycin cytotoxicity.

SURFACTANT II

At concentrations greater than 10^{-7} M given IP or IV to mice this compound again exhibited acute lethality similar to Brij 30. Table 9 describes the experience of all treated mice with this surfactant. In each case the deaths of animals occurred within five minutes of injection.

TABLE 9

Dose (M)	No. treated	No. of 10 day survivors
10^{-5}IP	3	0
10^{-5}IV	3	0
10^{-6}IP	3	1
10^{-6}IV	3	0
10^{-7}IP	5	5
10^{-7}IV	5	4

Thus a concentration of 10^{-7}M was selected for in-vitro experiments. Figure 5 shows a dose response curve for adriamycin with and without surfactant II. Again this shows no enhancement

of adriamycin cytotoxicity by this concentration of the surfactant.

Table 10 summarises the results of the in-vitro cytotoxicity assays using adriamycin in combination with the non-toxic concentrations of surfactants as defined above.

TABLE 10

Adriamycin dose (ug/ml)	Control	Brij 30 $3x10^{-7}M$	Surfactant 1 $10^{-6}M$	Surfactant 2 $10^{-7}M$
0	100 (5)	100 (11)	100 (6)	100 (9)
0.1	102 (10)	87 (10)	93 (4)	96 (12)
0.5	54 (7)	59 (9)	60 (5)	60 (3)
1.0	29 (8)	ND	31 (3)	30 (5)
2.5	9 (2)	11 (4)	12 (3)	12 (4)
5.0	0.7 (.2)	2 (.7)	1.1 (.6)	1 (.6)
10.0	0.4 (.2)	0 (0)	0.2 (.2)	0.4 (.9)

Results expressed as mean (standard deviation)

ND = not done.

DISCUSSION

The increase in membrane transport observed at low concentrations of surfactant is attributed to the amphipathic nature of the surfactant and its' ability to penetrate, and thus disrupt, normal biomembrane structure. Many authors have attempted to define a structure-function relationship for this effect with varying degrees of success. It appears that a hydrocarbon chain length of 12 is optimal in this respect (Walters, Duggard & Florence, 1981). This chain length is also associated with maximal effects on DNA synthesis and cytolysis in guinea-pig kidney fibroblasts in vitro, on release of histamine from rat peritoneal mast cells in vitro (Ferguson & Prottey, 1976), as anti-microbial agents (Kabara, 1978), and was also found to be optimal in a series of surfactants tested in-vitro for enhancement of anthracycline activity (Kerr et al, 1987).

Brij 30 (a C_{12} compound) produced unexpected toxicity when administered intravenously. The timing of this toxicity and the histological findings suggest that this ability to disrupt biomembranes extents to the alveolar-capillary membrane, resulting in rapidly fatal pulmonary oedema. Other workers have shown a decrease in plasma volume after IP administration of Tween 80, and it is possible that this was a contributory factor to the toxicity observed in this study (Harrison, Cusic & McAfee, 1981).

On the other hand Tween 80 was well tolerated by the intravenous route in the experimental animals, and in human subjects (Cummings et al, 1986a). It also had a profound cytotoxic effect in its' own right against exponential phase L-DAN cells in-vitro, and it proved impossible to determine if it had a penetration enhancing effect on adriamycin.

The other two surfactants used are C_{16} compounds. At concentrations which were tolerable to the mice neither of these surfactants showed any enhancement of adriamycin cytotoxicity.

Our studies, and others quoted above, suggest that not only is the C_{12} compound the optimum for enhancement of anthracycline activity, but is also highly toxic following IV administration. Using isolated gastric mucosal preparations a similar association between histological damage and the ability to enhance drug penetration has been demonstrated (Walters, Duggard & Florence, 1982). It is possible that both effects share a common mechanism.

One limitation of this model system is that the actual concentration of surfactant in the tissues was not determined. The concentrations in the tissues would neccesarily be less than those given to the whole animal (probably by at least one order of magnitude), and as enhancement of cytotoxicity could not be demonstrated at the in-vitro concentrations it is unlikely that an effect would be apparent in-vivo, though this was not formally tested.

In addition, it is unlikely, due to whole animal variations in drug handling, that by simple mixing and IV co-administration of adriamycin and surfactant both agents would get to the target tissue at the correct concentration and at the same time to allow any additive or synergistic effect. However it was necessary to perform these experiments as a pre-requisite to consideration of further use of surfactants, and they have helped to identify surfactants for which further study may be appropriate.

A possible way of alleviating the toxicity of the surfactant and delivering drug and surfactant at the same site and time would be to form vesicles from the non-ionic surfactant (similar to the more commonly used liposomes) which could then be loaded with drug. These so-called 'niosomes' are the subject of the following chapter.

CHAPTER 4

STUDIES USING NON-IONIC SURFACTANT VESICLES (NIVS)

Liposomes, which are constructed of uni-lamellar or multi-lamellar lipid bilayers have shown some promise in drug delivery, particularly if the target tissue is the reticulo-endothelial (macrophage) system which actively removes macromolecular materials from the circulation, though problems still remain in tumours because of the many penetration barriers interposed between the circulation and the target. The use of liposomal formulations of adriamycin (and some other cytotoxics) is currently in Phase I - II clinical evaluation in a number of centres (Treat et al, 1989. Cowens et al, 1989). A more detailed discussion of liposomes is presented in chapter 5.

Similarly, NIVs (formerly known as 'niosomes') are uni- or multi-lamellar structures composed of non-ionic surfactants, usually in a mixture with cholesterol. Drug can be loaded into the aqueous phase of the vesicles during the process of formation, or more lipophilic drugs may associate within the bilayers or more loosely with the surface of the vesicles (Baillie et al, 1985).

The advantages of this approach to a 'drug carrier' is that a number of critical factors can be altered by variation of the chemical composition of the vesicles, for example; degree of cholesterol incorporation controls permeability (Rogerson, Cummings & Florence, 1987), positive or negative charge of the

vesicle may be important in targeting, size and stability may be altered to give a degree of control of vesicle destination. One theoretical advantage of NIVs over liposomes is that one may be delivering a penetration enhancing agent together with the drug payload (see previous chapter), though it remains to be proven if this mechanism would be active in a selective fashion at the target tissue (tumour), but not at the major sites of anthracycline toxicity (marrow, heart), which would result in an improvement in therapeutic ratio.

The use of surfactant micelles has been shown to enhance intestinal absorption (Franz & Vonderscher, 1981) and to result in significant changes in drug handling when used intravenously (Azmin et al, 1986. Cable et al, 1988). NIV entrapment of adriamycin may enhance the therapeutic ratio of that drug by a reduction in cardiotoxicity, as implied by reduced cardiac uptake of this form of adriamycin whilst maintaining anti-tumour activity (Kerr et al, 1988).

The experiments detailed in this chapter describe our experience with two new formulations of NIVs, and were performed in collaboration with Dr.Cable and Prof.Florence, Dept. of Pharmacy, Strathclyde University. Some of the work has been previously reported in abstract form (Cable et al, 1988).

In the course of these experiments, adriamycin metabolites were measured as well as the parent drug. in order to establish if significant changes occurred in biotransformation as a result of changes in tissue distribution. Cummings, Merry &

Willmott (1986) have suggested that the degree of biotransformation may have an effect on both anti-tumour efficacy and the toxicity profile. However, the significance of serum metabolite concentrations on the therapeutic index remains a disputed area (Dessypris et al, 1988).

MATERIALS AND METHODS

PREPARATION OF NIVS

Surfactants I and II were used as supplied by L'Oreal, France (see previous chapter for structures). Cholesterol was obtained from Sigma Chemicals, Dorset, U.K. All other reagents were of highest purity available (Analar or HPLC grade).

150 umol of surfactant or surfactant mixture was dissolved in diethyl ether or chloroform (10ml) in a round bottom flask (50ml). The solvent was evaporated under reduced pressure in a water bath at 50-60^{0}C, using a rotary evaporator (Buchli), leaving the starting material as a thin film on the wall of the flask. The hydrating solution (2-5ml) was heated to 50^{0}C and added to the round bottom flask, with gentle agitation, until no material remained on the wall of the flask. The flask was left in the water bath for a further 30 minutes, with gentle agitation every 10-15 minutes. The resulting niosomal suspension was sonicated with an MSE PG100 probe sonicator with a titanium probe (150Watt) for a specified time (usually 5-15 minutes) at

15% of maximum output. In drug loaded NIVs the hydrating solution was 2-5 mg/ml adriamycin. In 'empty' NIVs the hydrating solution consisted of 200 mM glucose(both in aqueous solution).

NIVs of two different chemical compositions were used in the following experiments. NIV I contained surfactant I (50%), cholesterol (25%) and a cholesteryl poly(24)oxyethylene ether (Solulan C-24) (25%) and had an average size of 420 nm diameter as determined by photon correlation spectroscopy. NIV II were composed of a 50:50 mixture of surfactant II and cholesterol to which was added 5% stearyl amine. These NIVs had a mean diameter of 186nm by photon correlation spectroscopy.

SEPARATION OF FREE AND ENTRAPPED ADRIAMYCIN

This was achieved by equilibrium dialysis of samples against 200 mM glucose. A Cuprophane No.3 dialysis membrane was used, and dialysis performed for 48 hours at 4^0C in the dark, with six changes of solution (6 x 800ml).

MEASUREMENT OF ENTRAPPED ADRIAMYCIN (Rogerson et al, 1987).

Five ml of NIV suspension was adjusted to pH 5 to ionise the adriamycin, shaken for 30 seconds in 10 ml of chloroform in a 50 ml separatory funnel, and after 5 minutes the two phases were separated. The upper aqueous phase contained adriamycin and the lower chloroform the surfactant. The aqueous phase was clarified

by gentle addition of 5 ml of double distilled water, then collected and analysed directly. The chloroform layer was evaporated to dryness, avoiding heat, and was reconstituted in 1 ml of methanol prior to immediate analysis. Cholesterol-containing NIVs were reconstituted in 2 ml of chloroform:methanol (50:50 V/V) prior to analysis.

DRUG ANALYSIS

Adriamycin and its metabolite concentrations were measured in serum and tissues using an established reverse-phase isocratic high-performance liquid chromatographic technique developed in our laboratory (Cummings, Stuart & Calman, 1984).

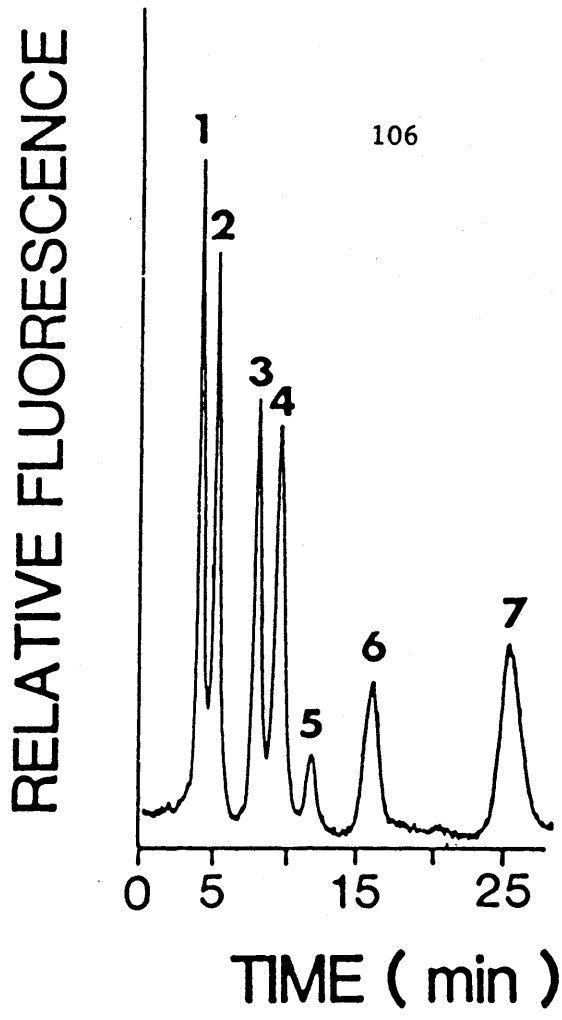
HPLC was performed using an Altex Model 100A pump amd a Milton Roy 'promis' autosampler programmed for a 20 ul injection volume (Milton Roy, Co.Clare, Ireland), and a Gilson Spectro-glo filter fluorimeter with narrow band interference filters at 480 nm (excitation) and 560 nm (emission) and a 10 ul quartz micro flow cell (Gilson, Villiers-le-Bel, France); a Shimadzu CR-1B integrator (supplied by Scotlab Instrument Sales, Bellshill, U.K.) and a 250 mm x 4 mm internal diameter stainless steel column packed by ourselves with u-Bondapak C_{18}.

All methanol, propan-2-ol and chloroform were HPLC reagent grade (Fisons Scientific, Loughborough, U.K.). Orthophosphoric acid and all other solvents and chemicals were of analytical reagent grade (Analar, BDH, Poole, U.K.). Water was double-

distilled and deionised in a quartz glass still. The mobile phase for the chromatographic separation of adriamycin and its metabolites consisted of 74% 5mM (final concentration) orthophosphoric acid, 26% propan-2-ol (pH 3.2). The mobile phase was degassed by purging with helium for one minute. Pure adriamycin and adriamycinol were gifts from Dr. S. Penco (Farmitalia, Milan, Italy). 7-Deoxy-aglycones of adriamycin were synthesised as reported for validation of this assay* (Cummings, Stuart & Calman, 1984). Elution was isocratic at a flow-rate of 1.2 ml/min. The column was at ambient room temperature (normally 25^{0}C). Quantitation of extracts was by peak area and was always by reference to standard calibration curves run on the same day. Corrections to allow for differences in the relative molar fluorescence of the metabolites were made to all metabolite concentrations according to Cummings, Stuart & Calman (1984). A sample chromatogram illustrating the separation of metabolites and parent drug from serum is shown in figure 1.

To 200ul of mouse serum were added 100 ng daunorubicin (for adriamycin analysis, or adriamycin in the case of daunorubicin analysis) in 10 ul of methanol as an internal standard. The sera were then mixed with 5 volumes of chloroform - propan-2-ol (2:1) and whirlimixed for twenty minutes in 25 ml PTFE-lined screw capped test-tubes. After mixing, the samples were centrifuged at 2000g for 15 minutes at 4^{0}C to separate two phases. The upper aqueous phase was discarded by aspiration, the lower organic phase was transferred to clean 10 ml tapered centrifuge tubes

* purity was checked by TLC and structure by mass spectrometry.



1 ADRIAMYCINOL
2 ADRIAMYCIN
3 ADRIAMYCINOL AGLYCONE
4 DAUNORUBICIN (I.S.)
5 ADRIAMYCIN AGLYCONE
6 ADRIAMYCINOL 7-DEOXYAGLYCONE
7 ADRIAMYCIN 7-DEOXYAGLYCONE

STATIONARY PHASE: micro-Bondapak C18
MOBILE PHASE: 5mM-phosphoric acid, 74%
propan-2-ol 26%

Figure 1: Sample chromatographic separation of adriamycin and its metabolites from human serum.

and was evaporated in the Buchler vortex evaporator at $40^{0}C$ and 30 mm Hg of vacuum. The residue was redissolved in either 50 or 100 ul of methanol by whirlimixing for 1 minute and 20 ul were injected onto the HPLC column. This extraction technique is capable of almost equal recovery (>77%) of adriamycin, metabolites and daunorubicin (the internal standard).

The drug was extracted from tissues by the method of Cummings, Merry & Willmott (1986), which involved tissue homogenisation followed by treatment with $AgNO_3$ solution (33%, W/V) to remove adriamycin intercalated to DNA, then extraction with 5 volumes of Chloroform - propan-2-ol as described for sera.

In all samples attempts were made to measure adriamycin (ADR), adriamycinol 7-deoxyaglycone (7-AOL), adriamycin 7-deoxyaglycone (7-ADR), adriamycinol (AOL) and adriamycinol aglycone (AOLONE). The lower limit of detection for this assay is 3 ng/ml for adriamycin and 1 ng/ml for adriamycinol and the 7-deoxyaglycones. The inter and intra-assay coefficients of variation for the assay are both <10%.

ANIMALS AND TUMOUR MODEL

In the NIV I experiments non-tumour bearing mice (MF1 $\hat{Nu}^{+}$) from our own breeding colony were used. For NIV II experiments AKR mice supplied by Harlan Olac Ltd, Shaws Farm, Blackthorn, U.K. bearing subcutaneously implanted ROS (Ridgway Osteogenic Sarcoma) tumour were used. In both cases the animals were fed on

standard laboratory chow, allowed water ad libitum and kept in a 12 hour light/dark cycle throughout the duration of the experiments.

The ROS tumour spontaneously arose as an inguinal mass in a male AKM mouse in 1948 in the laboratory of Dr. J. H. Burchenal at the Sloan-Kettering Institute in New York. This tumour has been found to respond to a wide range of drugs (including anthracyclines) used in the treatment of solid tumours in man. Furthermore it generally exhibits an orderly and reproducible dose response to active drugs (Laster, 1975). The tumour was subcutaneously implanted (taking full antiseptic precautions) as $1mm^3$ fragments into a surgically created subcutaneous pouch on the right flank of lightly anaesthetised 6-8 week old mice, and the wound then closed by surgical skin clips. After approximately 12-14 days the tumours were easily palpable and measurable in two dimensions using calipers. The median life span of ROS-implanted mice is about 25-35 days.

MURINE PHARMACOKINETIC STUDIES

Groups of animals were restrained without anaesthetic and tail-vein injected with the following combinations: saline as control, adriamycin in solution at a dose of 5mg/kg (for formulation I experiments; for formulation II 10mg/kg was used), adriamycin entrapped in NIVs at a dose sufficient to deliver 5 mg/kg or 10mg/kg respectively of the anthracycline, or adriamycin in solution (5 mg/kg or 10mg/kg) plus empty NIVs. In all experiments the same total quantity of NIVs were administered in the drug loaded or empty state. In

all groups the total volume of injection was less than 0.2ml.

At specified times over the next 48 hours groups of mice (n=4) were sacrificed by cervical dislocation. They were exsanguinated from the inferior vena cava and tissues of interest (heart, liver, kidneys, spleen, lungs and tumour) dissected out, blotted dry, and rapidly frozen in liquid nitrogen. The blood was centrifuged at 100 g for 2 minutes and the serum then removed and rapidly frozen. All samples were maintained at -20^{0}C until the day of analysis.

MATHEMATICAL ANALYSIS

The area under the concentration time curve (AUC) was calculated by the log trapezoidal rule from time zero to the last measured time point (48 hours unless stated otherwise). Clearance was determined using the model-independent equation; Clearance = Dose/AUC (Gibaldi & Perrier, 1982).

Statistical comparisons were made by two way analysis of variance or Student's T-test with Bonferroni correction where appropriate. As it was impossible to take multiple plasma samples from single mice, it is impossible to statistically compare AUCs as this parameter is derived from single samples from multiple animals.

TUMOUR GROWTH RESPONSES

On the 10th day after subcutaneous implantation of tumours 4 groups of mice (n=8 for control, n=4 for experimental) were

administered combinations of drugs as detailed above at doses calculated to deliver 10mg/kg of the anthracycline component. The resultant tumours were measured bi-dimensionally by calipers every 2 days thereafter until the tumour burden became intolerable to the animals. Tumour volume (assuming spherical geometry) was then calculated by the following formula:-

$$\text{Volume} = 4/3\pi(a+b/4)^3$$

were a is the largest diameter and b is the diameter at a perpendicular to a.

FLUORESCENT MICROSCOPY

Six mice with established subcutaneous ROS tumour were treated with NIV entrapped-adriamycin (5 mg/kg, formulation II only) or saline as controls. At 5 minutes, 4 hours and 24 hours after dosing groups of 2 animals were sacrificed and tissues of interest removed as detailed above. The tissues were divided into two; one part was rapidly frozen in liquid nitrogen, the other was immediately placed into a fixative composed of 80% propanol:20% water. The fixed samples were processed to paraffin wax, 5 nm sections cut, dewaxed and mounted using glycerol PSB mountant containing PPD (p-Phenyldiamine) which retards fading of fluorescence. The snap frozen samples were cut using a cryotome, sections were examined using a Leitz Lablux K Epifluorescent microscope. Photography was performed using

Kodak Ektachrome film P800/1600. The final magnification for the photographs was eyepiece (x10) x objective (x 50 water immersion). I gratefully acknowledge the help of the Department of Pathology Southern General Hospital and Dr. Margaret Burgoyne in the expert processing and interpretation of these sections.

RESULTS

MURINE PHARMACOKINETICS (FORMULATION I)

Peak drug levels and tissue AUC's are outlined in table 11. Figures 2 to 7 illustrate the concentration time profiles of adriamycin or its' metabolites in plasma or tissues following administration of adriamycin in solution (A), empty NIVs with adriamycin in solution (EN) or NIV entrapped adriamycin (NIO). Each point is the mean of at least 3 observations with associated standard errors.

Attempts were made to measure adriamycin (ADR), adriamycinol 7-deoxy aglycone (7-AOL), adriamycin 7-deoxyaglycone (7-ADR), adriamycinol (AOL) and adriamycinol aglycone (AOLONE) in all samples. In tissues other than liver the metabolite concentrations were extremely variable, often being measurable in only one of 3 apparently identical samples, possibly reflecting inter-individual differences in drug handling between the mice. Such wide inter-individual variation has also been observed in humans, but the therapeutic significance of these observations are unclear (Cummings et al, 1986 b). These data are therefore not discussed in any further detail.

Figure 2 shows plasma concentration-time profiles for adriamycin, the peak concentration following drug-loaded NIVS is statistically significantly higher than adriamycin in solution and subsequent plasma levels decline relatively slowly

TABLE 11

Tissue	Formulation	Measured	Peak	+/- S.E.	(units)	P*	AUC**
Plasma	Nio	ADR	4786	496	(ng/ml)	0.013	8330
Plasma	EN	ADR	947	414			3919
Plasma	A	ADR	397	58			1283
Heart	Nio	ADR	5.6	1.1	(ug/g)	0.2	54.8
Heart	EN	ADR	12.6	2.3			155.7
Heart	A	ADR	7.8	0.4			59.6
Liver	Nio	ADR	15.6	3.5	(ug/g)	0.17	275.3
Liver	EN	ADR	19.4	1.4			189.0
Liver	A	ADR	24.6	2.4			145.1
Kidney	Nio	ADR	13.1	3.5	(ug/g)	0.53	104.6
Kidney	EN	ADR	38.5	7.3			462.7
Kidney	A	ADR	33.7	27			240.6
Liver	Nio	7-ADR	65.1	24.9	(ng/g)	0.01	1298
Liver	EN	7-ADR	210	10			1482
Liver	A	7-ADR	270	40.1			2306
Liver	Nio	7-AOL	51.8	23	(ng/g)	0.006	1459
Liver	EN	7-AOL	540	180			3250
Liver	A	7-AOL	560	100			4038

* value from two-tailed T-test of niosomal vs. adriamycin in solution. ** in corresponding units x hours.

KEY: A= adriamycin in solution, EN= empty NIVs with adriamycin in solution, Nio= NIV entrapped adriamycin.

for this form of drug but decline exponentiallly as expected for adriamycin in solution or the mixture of adriamycin solution and empty NIVs. Since the first plasma samples were taken at 10 minutes it is likely that the entire dose of NIV entrapped drug would still be intravascular, whereas for adriamycin in solution the drug is avidly and rapidly taken up by tissues, resulting in a lower measured 'peak' level. The prolonged higher plasma levels with NIV adriamycin could be interpreted in two ways: the NIVs may be stable and still in the circulation or they may have been deposited in tissues with drug slowly leaching out of the vesicles with at least a portion of the released drug finding its way back into the circulation i.e. a 'depot'.

Figure 3 illustrates heart concentrations, and shows a reduction in peak levels for the NIV preparation (although not statistically significant, see table 1). Figure 4 shows liver concentrations with the expected increase in AUC for the NIV entrapped drug, due to reticulo-endothelial uptake of the vesicles. Figure 5 shows renal concentrations, with a reduction of 50% in AUC for the NIV preparation in comparison with adriamycin in solution. Figures 6 and 7 show concentrations of hepatic metabolites with a significant ($P<0.05$) reduction in peaks and AUCs for the NIV form of adriamycin.

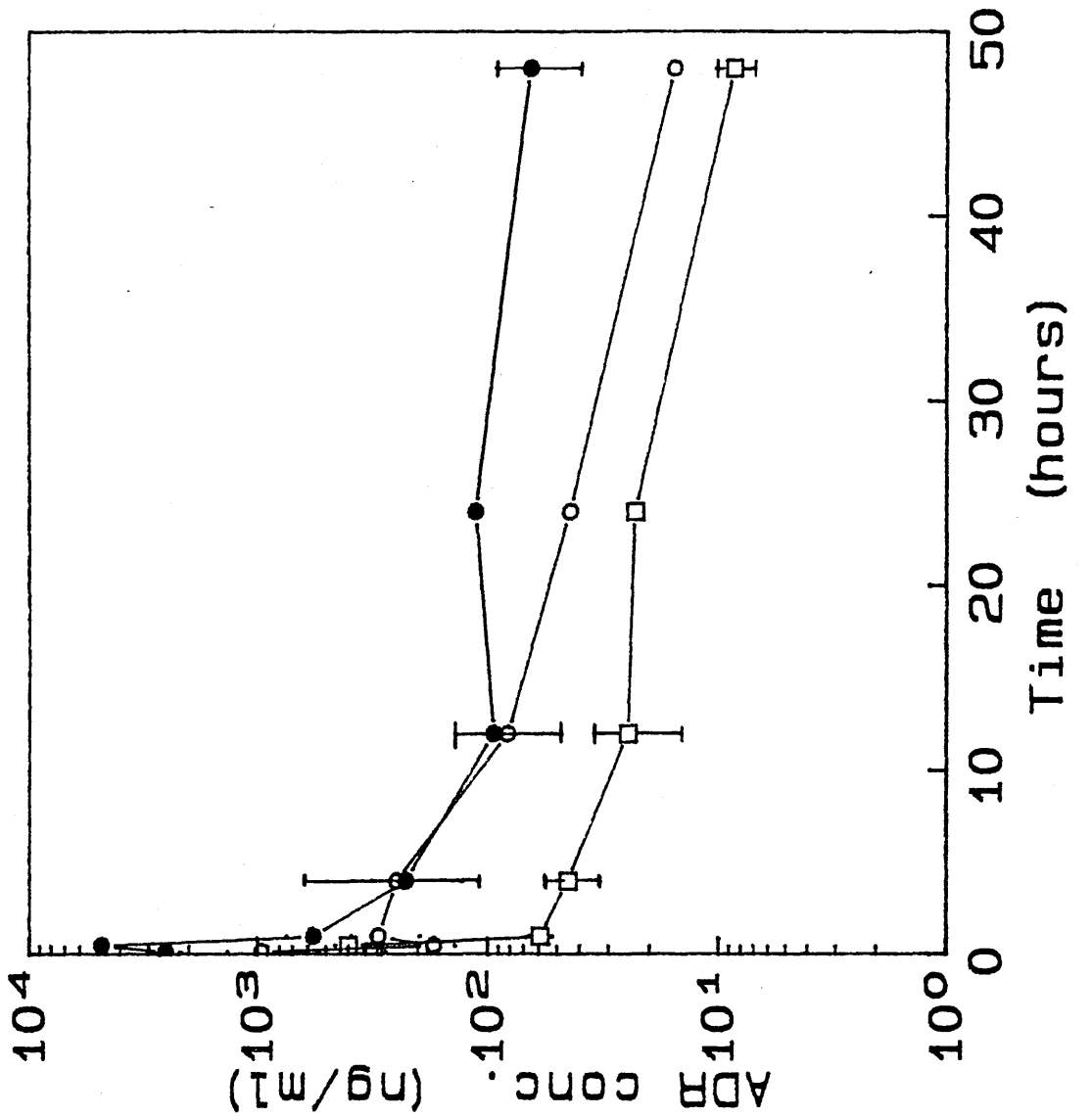


Figure 2: Plasma adriamycin concentration versus time following IV administration of formulation 1 niosomal adriamycin (filled circles), adriamycin in solution (open squares) or a mixture of empty niosomes plus adriamycin in solution (open circles).

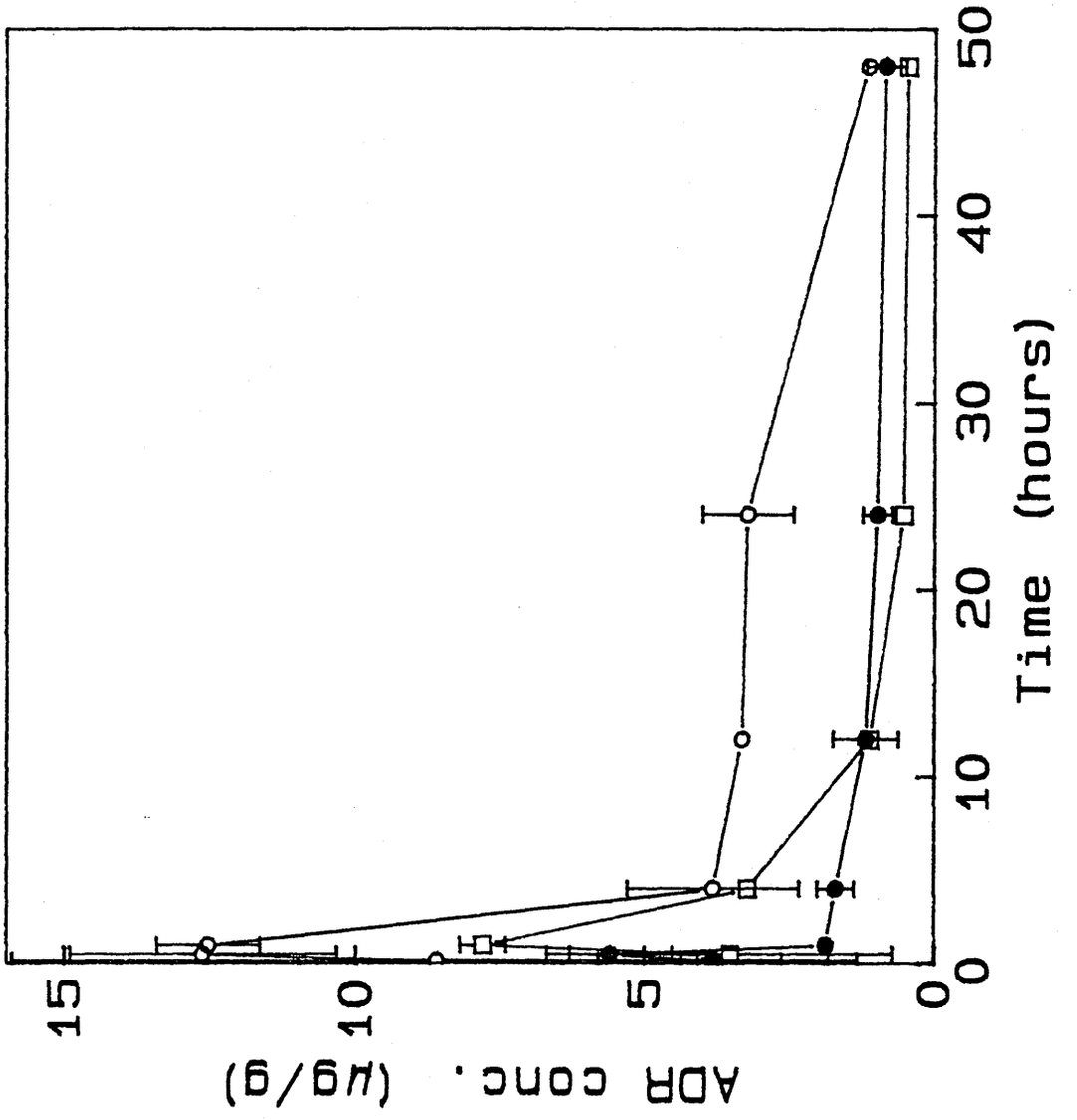


Figure 3: Heart adriamycin concentration versus time following IV administration of formulation 1 niosomal adriamycin (filled circles), adriamycin in solution (open squares) or a mixture of empty niosomes plus adriamycin in solution (open circles).

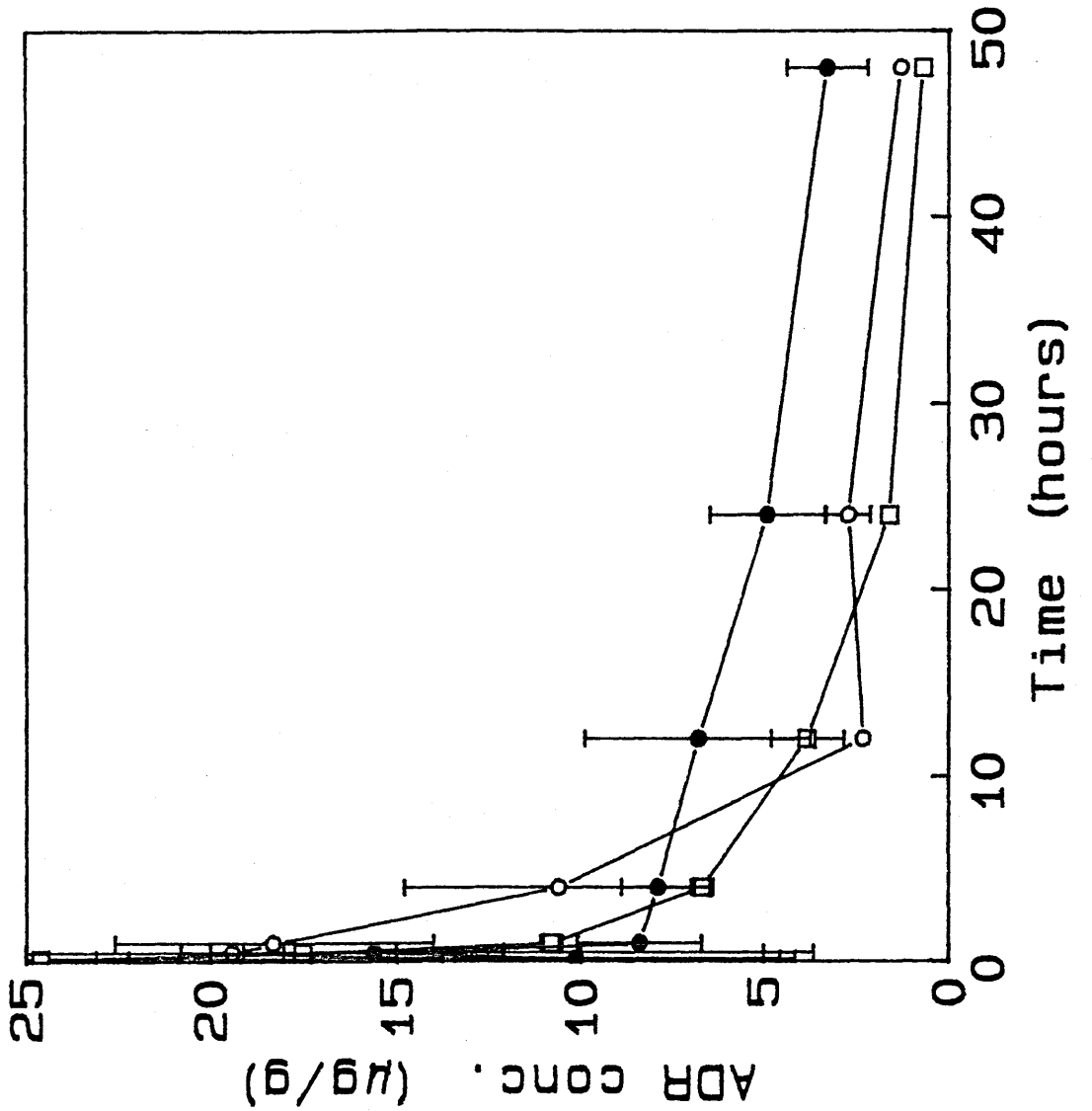


Figure 4: Liver adriamycin concentration versus time following IV administration of formulation 1 niosomal adriamycin (filled circles), adriamycin in solution (open squares) or a mixture of empty niosomes plus adriamycin in solution (open circles).

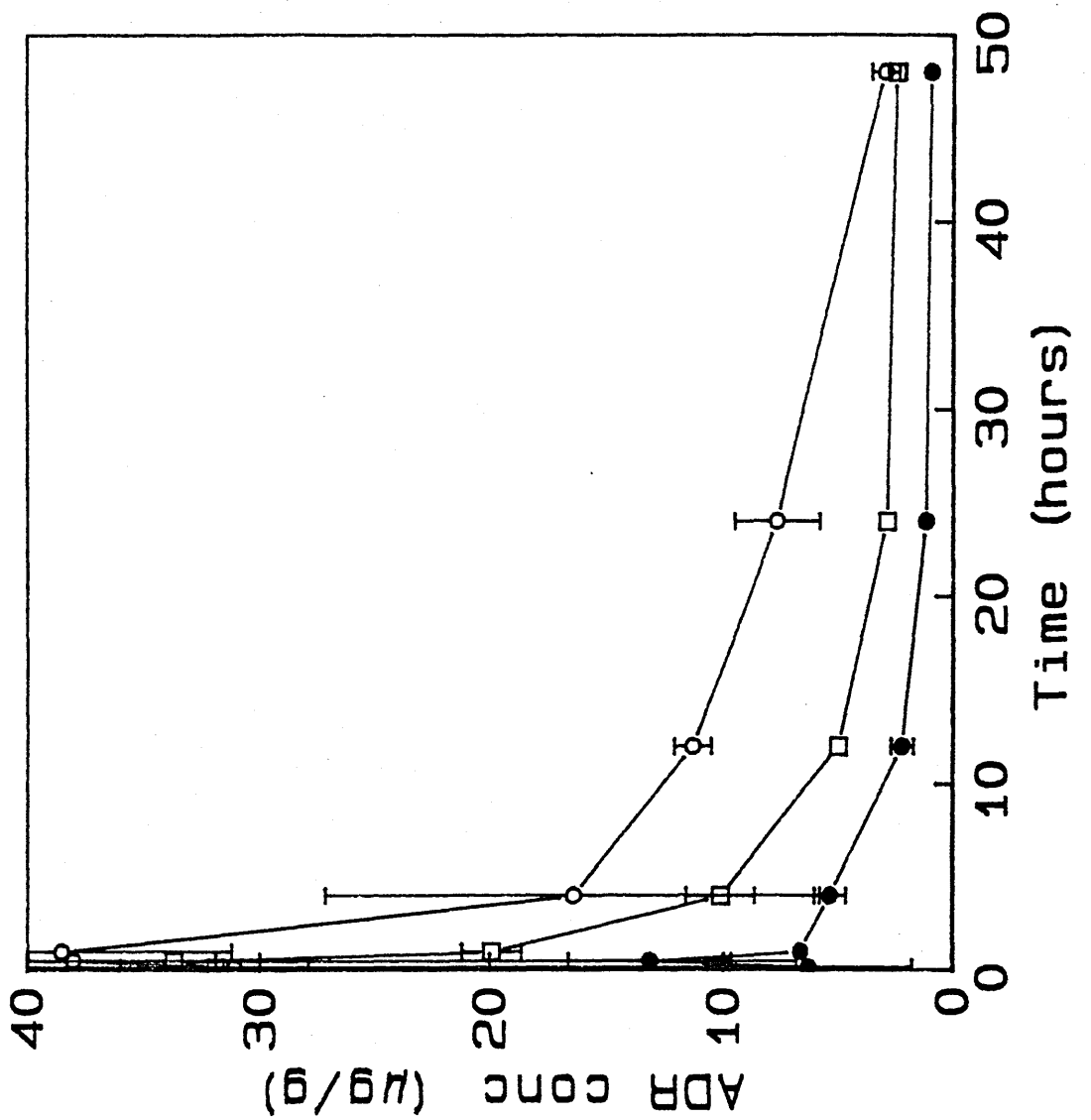


Figure 5: Renal adriamycin concentration versus time following IV administration of formulation 1 niosomal adriamycin (filled circles), adriamycin in solution (open squares) or a mixture of empty niosomes plus adriamycin in solution (open circles).

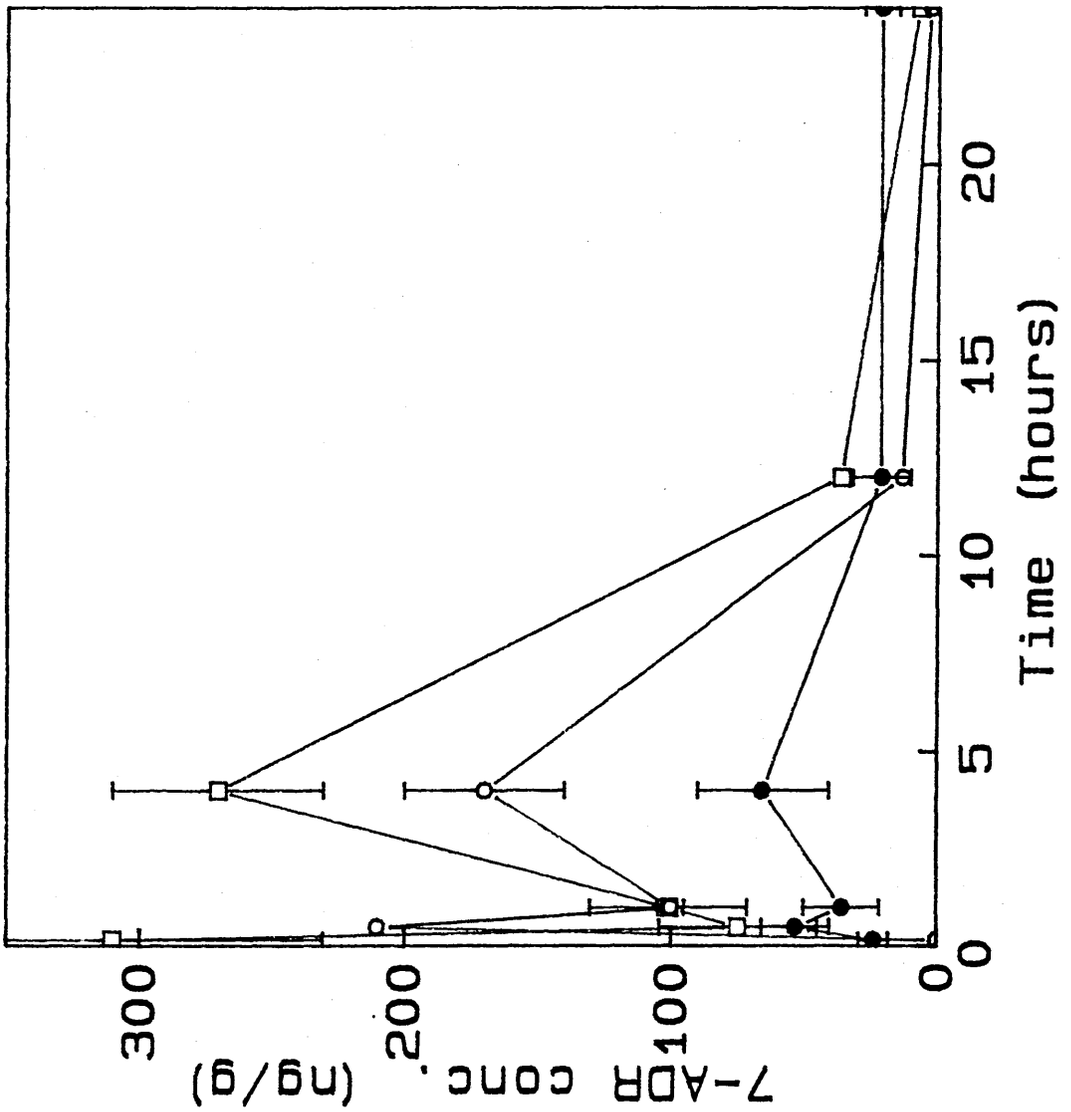


Figure 6: Hepatic concentration of adriamycin 7-deoxy-aglycone versus time following IV administration of formulation 1 niosomal adriamycin (filled circles), adriamycin in solution (open squares) or a mixture of empty niosomes plus adriamycin in solution (open circles).

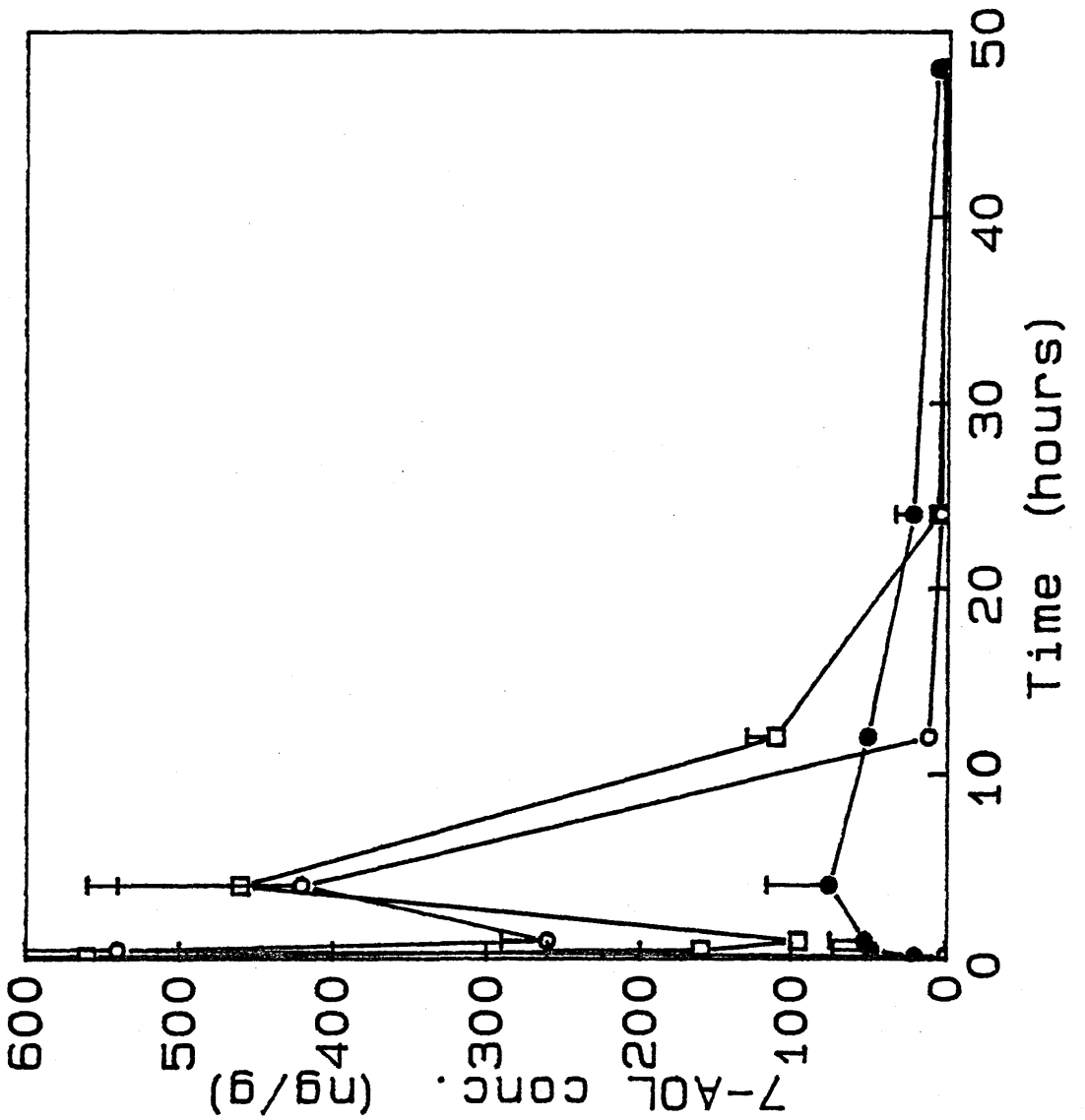


Figure 7: Hepatic concentration of adriamycinol 7-deoxy-aglycone versus time following IV administration of formulation 1 niosomal adriamycin (filled circles), adriamycin in solution (open squares)or a mixture of empty niosomes plus adriamycin in solution (open circles).

MURINE PHARMACOKINETICS (FORMULATION II)

Peak drug levels and tissue AUC's are given in table 12. Figures 8 through 13 illustrate the concentration of adriamycin in various tissues following administration of NIV II as previously described. Each point is the mean of 4-5 observations with associated standard errors.

All of the adriamycin metabolites were again measured as described in the last section. Figures 14 to 19 illustrate concentration time profiles for metabolite levels in those tissues in which they were measurable in a consistent fashion. Once more occasional animals appeared to have alternative drug handling, but the data points shown represent the means of at least 3 observations.

Figure 8 shows plasma concentration-time profiles. In contrast to formulation I the measured 'peak' levels are very similar for each of the formulations, but in common with formulation I there are sustained relatively higher plasma concentrations following administration of the NIV entrapped drug. Figures 9 to 13 all show a similar pattern of low concentrations of adriamycin following NIV II administration. These low levels are particularly surprising in the liver and spleen, which are the main sites of the reticulo-endothelial system. Figures 14 and 16 illustrate hepatic levels of metabolites and are in keeping with the low levels of parent drug in the liver. In contrast, figures 15 and 17 show tumour

TABLE 12

Tissue	Formulation	Measured	Peak+/-S.E.(units)		AUC(units.h)
Plasma	Nio	ADR	146	43	5915
Plasma	EN	ADR	312	94	3551
Plasma	A	ADR	246	20	3582
Tumour	Nio	ADR	1.1	0.3	24.2
Tumour	EN	ADR	18.2	10	263.4
Tumour	A	ADR	7.8	3.2	217.8
Heart	Nio	ADR	6.4	3.2	20.2
Heart	EN	ADR	19.6	1.6	222.5
Heart	A	ADR	17.2	1.8	155.0
Liver	Nio	ADR	40.7	14.3	91.4
Liver	EN	ADR	26.9	2.1	191.0
Liver	A	ADR	18.3	0.6	166.2
Spleen	Nio	ADR	2.0	0.75	58.8
Spleen	EN	ADR	14.5	3.9	564.3
Spleen	A	ADR	9.5	1.2	521.4
Lung	Nio	ADR	0.6	0.08	20.6
Lung	EN	ADR	24.1	2.6	430.9
Lung	A	ADR	20.0	1.6	331.1

Continued overleaf

Units are as given in corresponding figures 8 to 19.

TABLE 12 (continued)

Tissue	Formulation	Measured	Peak+/-S.E.(units)		AUC(units.h)
Liver	Nio	7-ADR	44.0	7.7	1594
Liver	EN	7-ADR	351	170	2872
Liver	A	7-ADR	173	30	2144
Tumour	Nio	7-ADR	147	47.6	5166
Tumour	EN	7-ADR	46.3	18.4	not done
Tumour	A	7-ADR	0		0
Liver	Nio	7-AOL	48	14.5	2075
Liver	EN	7-AOL	597	204	5790
Liver	A	7-AOL	682	176	6275
Tumour	Nio	7-AOL	143	72	4330
Tumour	EN	7-AOL	111	27	3079
Tumour	A	7-AOL	272	71	4738*
Plasma	Nio	7-AOL	48.4	2.5	1870
Plasma	EN	7-AOL	56.8	23.6	1062*
Plasma	A	7-AOL	59.0	10.0	886
Tumour	Nio	AOLONE	100.5	25.4	3594
Tumour	EN	AOLONE	470.2	45.2	5210
Tumour	A	AOLONE	141.0	24.0	2813*

* AUC to 24 hours only calculated.

KEY: A= adriamycin in solution, EN= empty NIVs with adriamycin in solution, Nio = NIV entrapped adriamycin

Units are as given in corresponding figures 8 to 19.

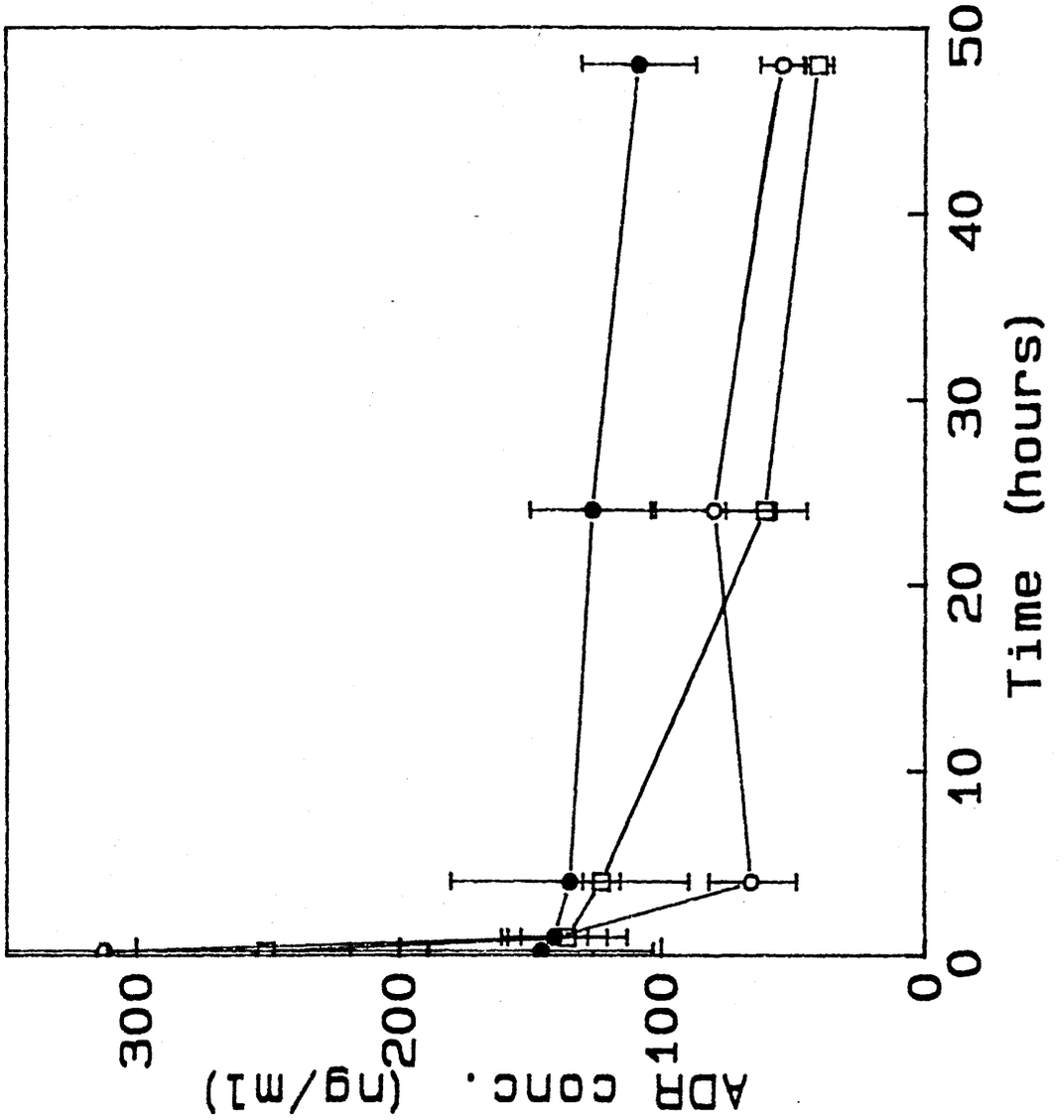


Figure 8: Plasma adriamycin concentration versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

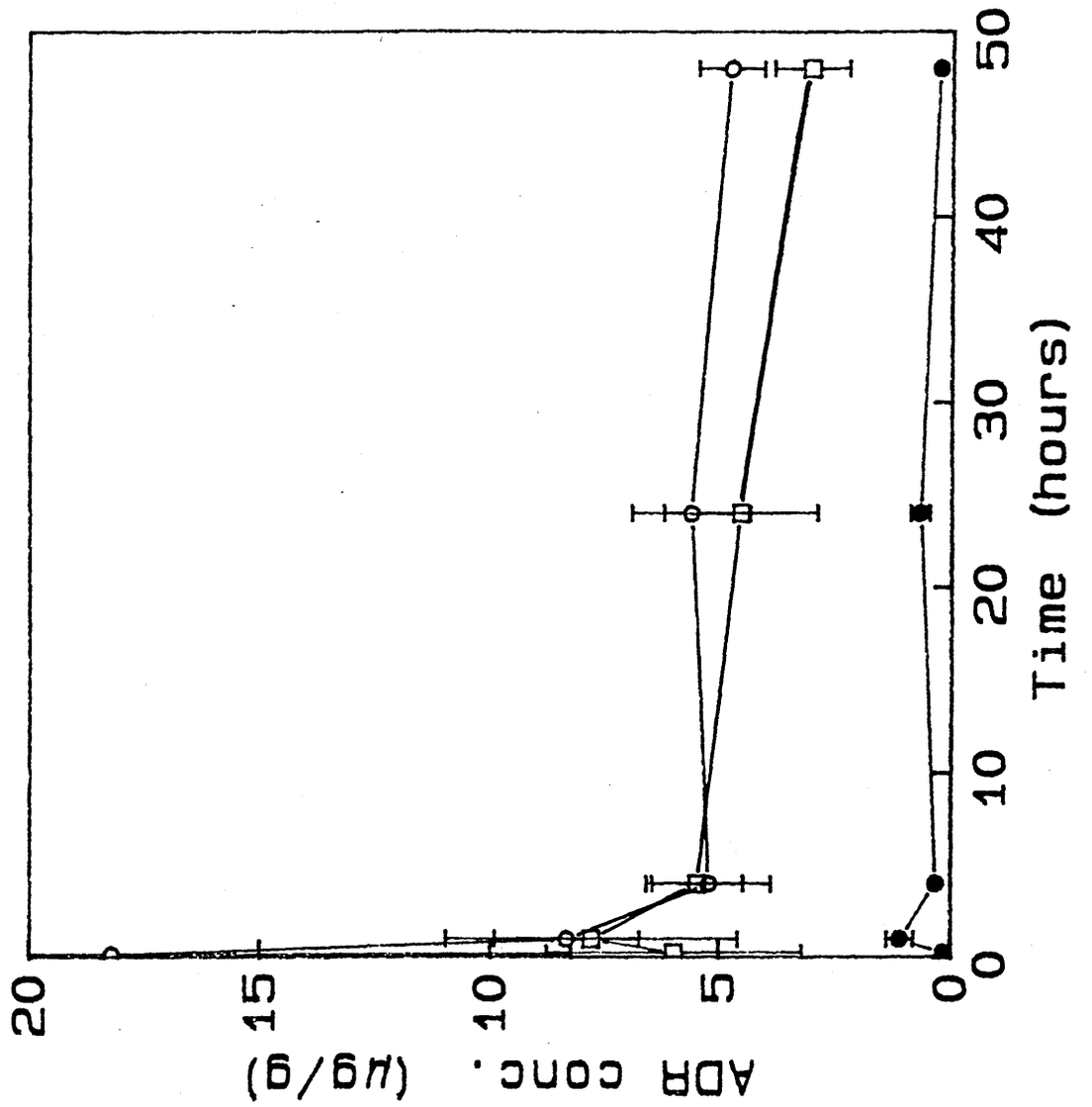


Figure 9: Tumour adriamycin concentration versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

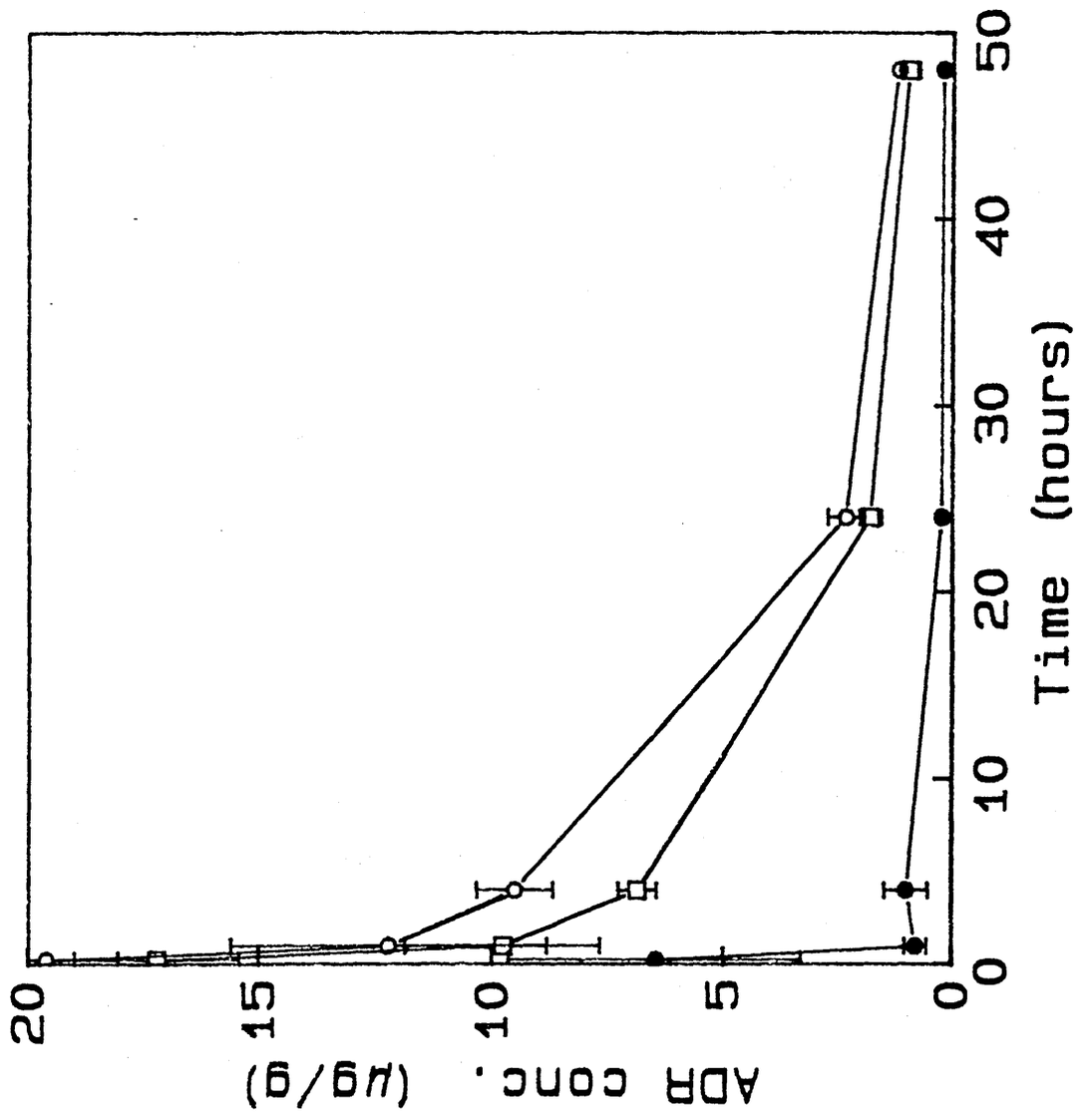


Figure 10: Heart adriamycin concentration versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

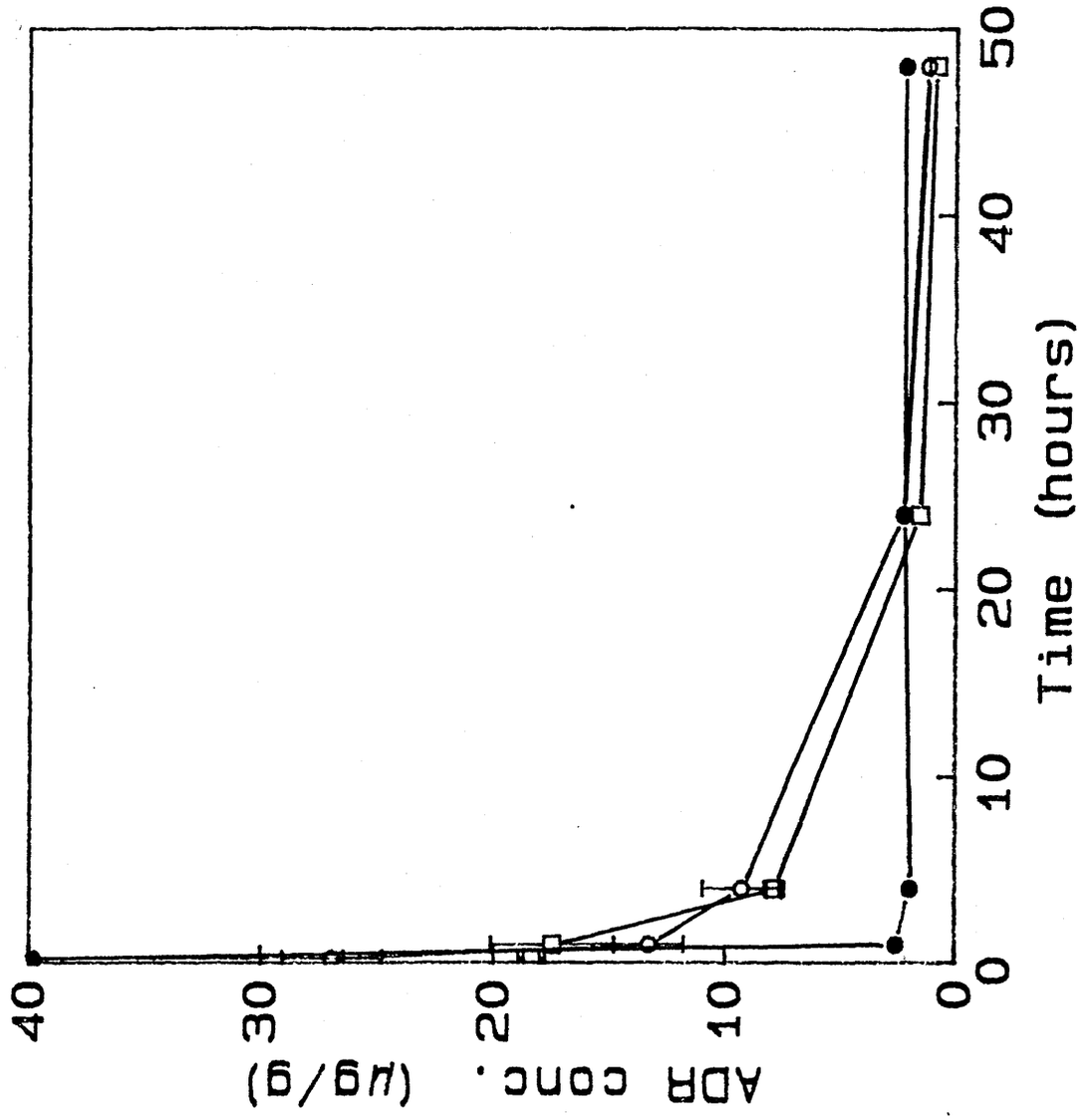


Figure 11: Liver adriamycin concentration versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

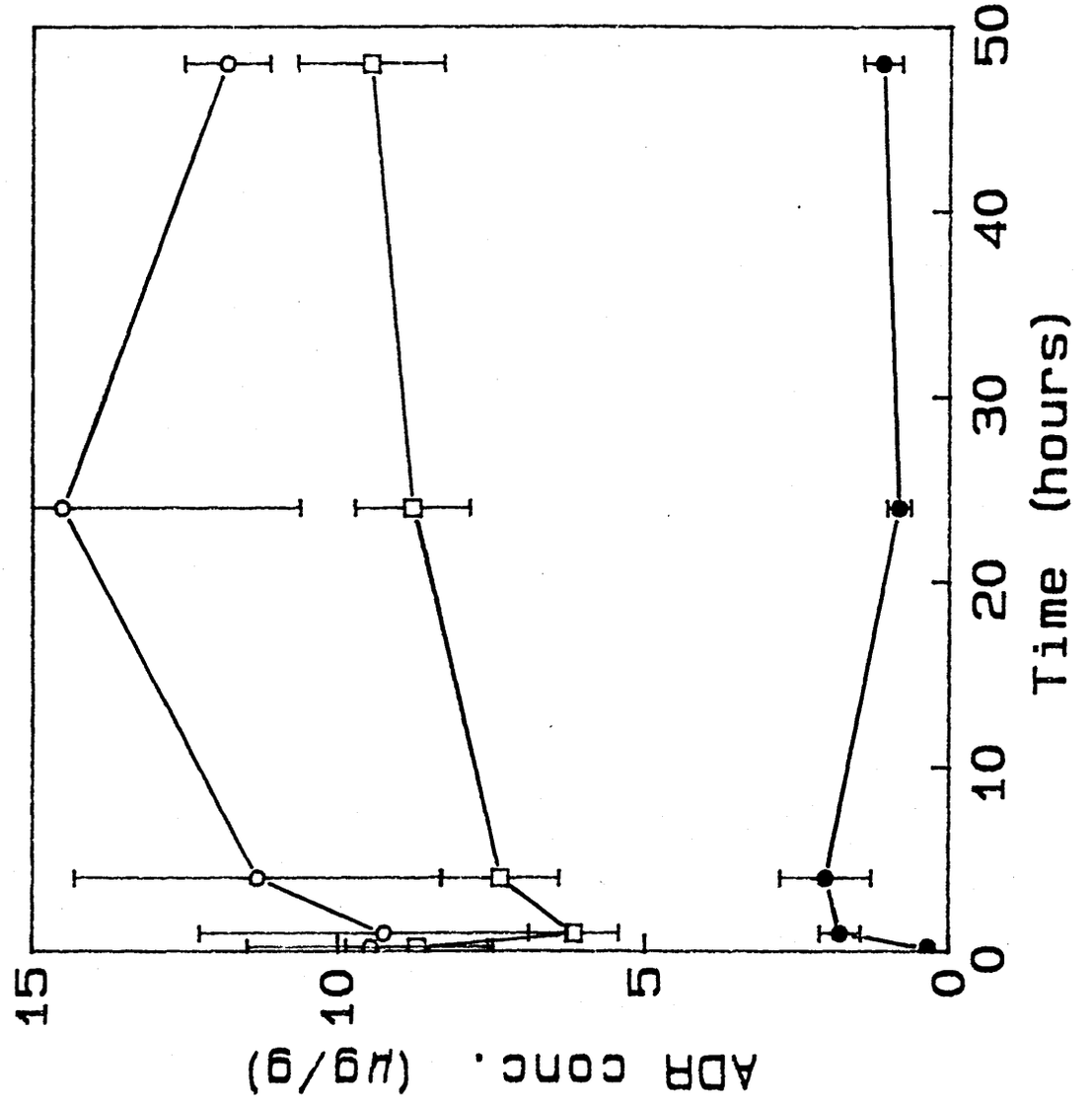


Figure 12: Spleen adriamycin concentration versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

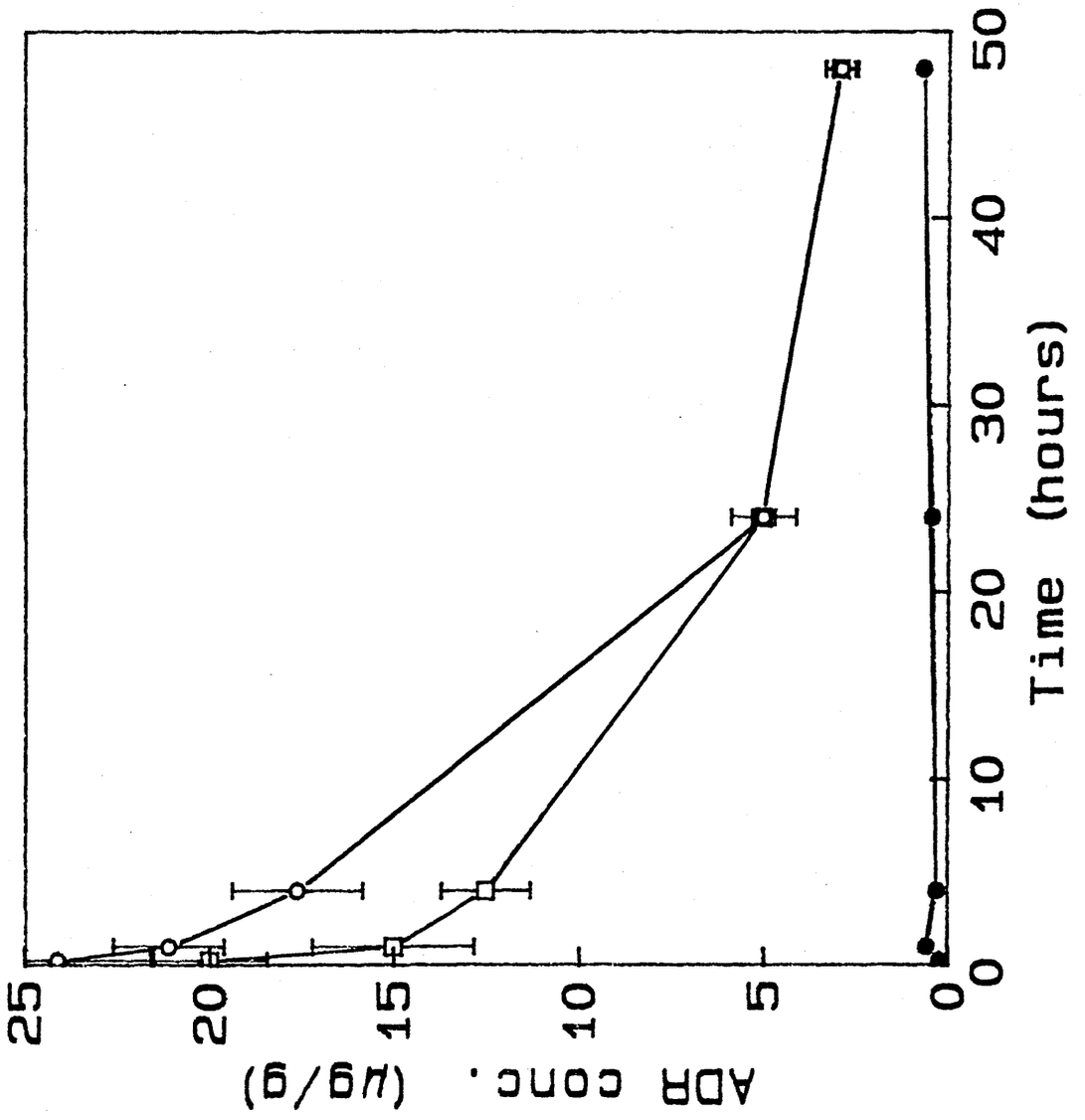


Figure 13: Lung adriamycin concentration versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

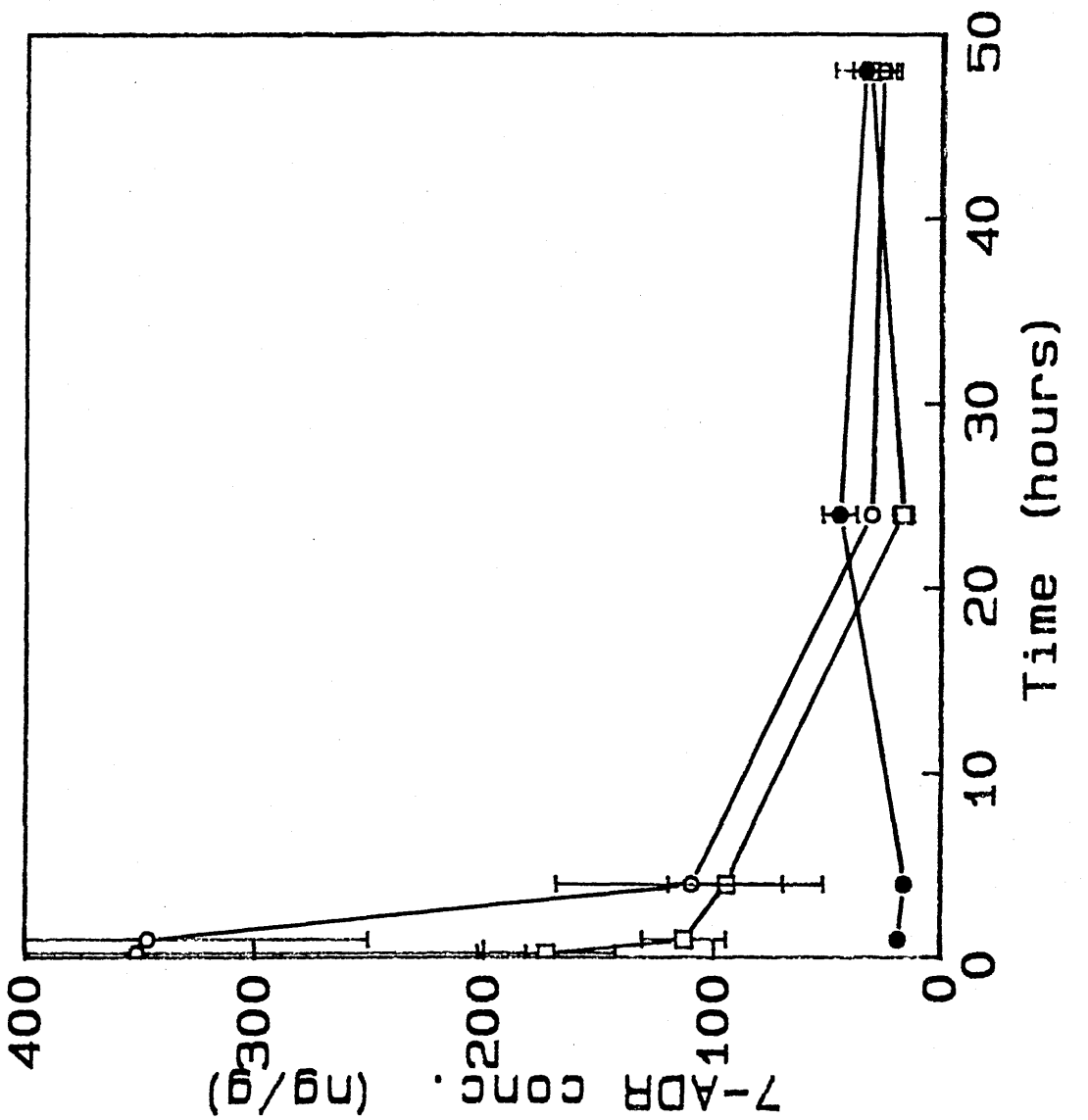


Figure 14: Liver concentration of adriamycin 7-deoxy-aglycone versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

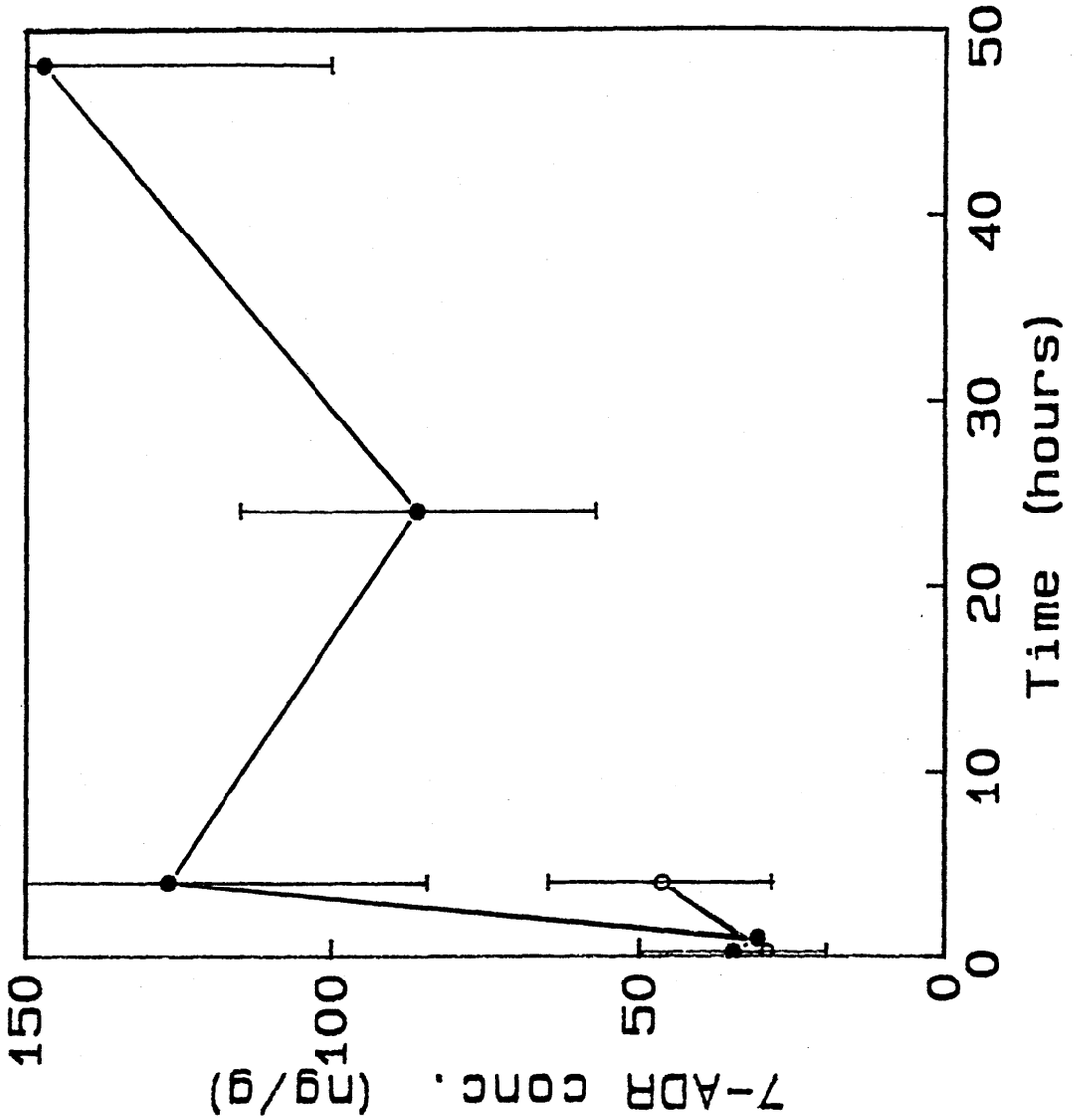


Figure 15: Tumour concentration of adriamycin 7-deoxy-aglycone versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

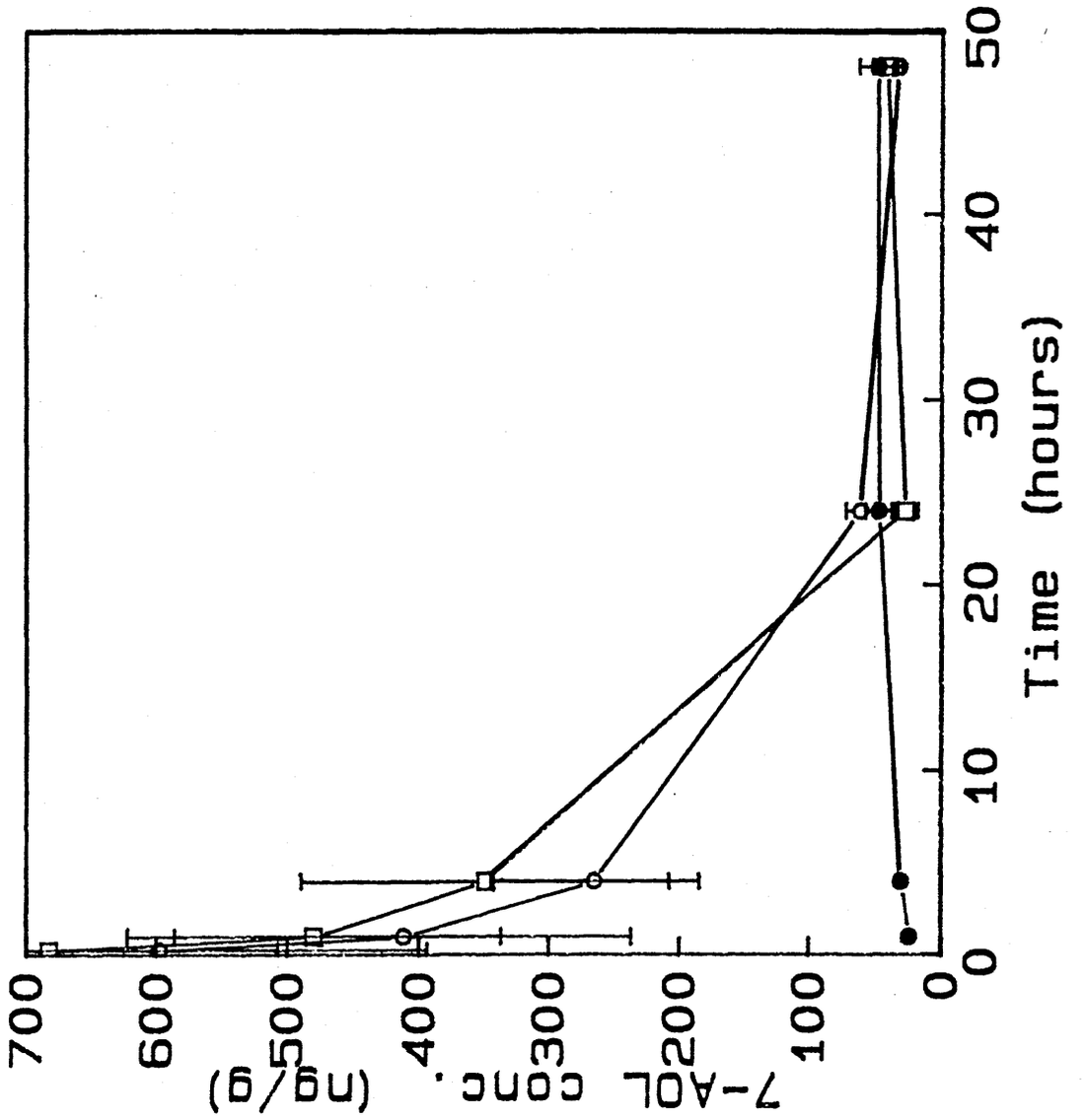


Figure 16: Liver concentration of adriamycinol 7-deoxy-aglycone versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

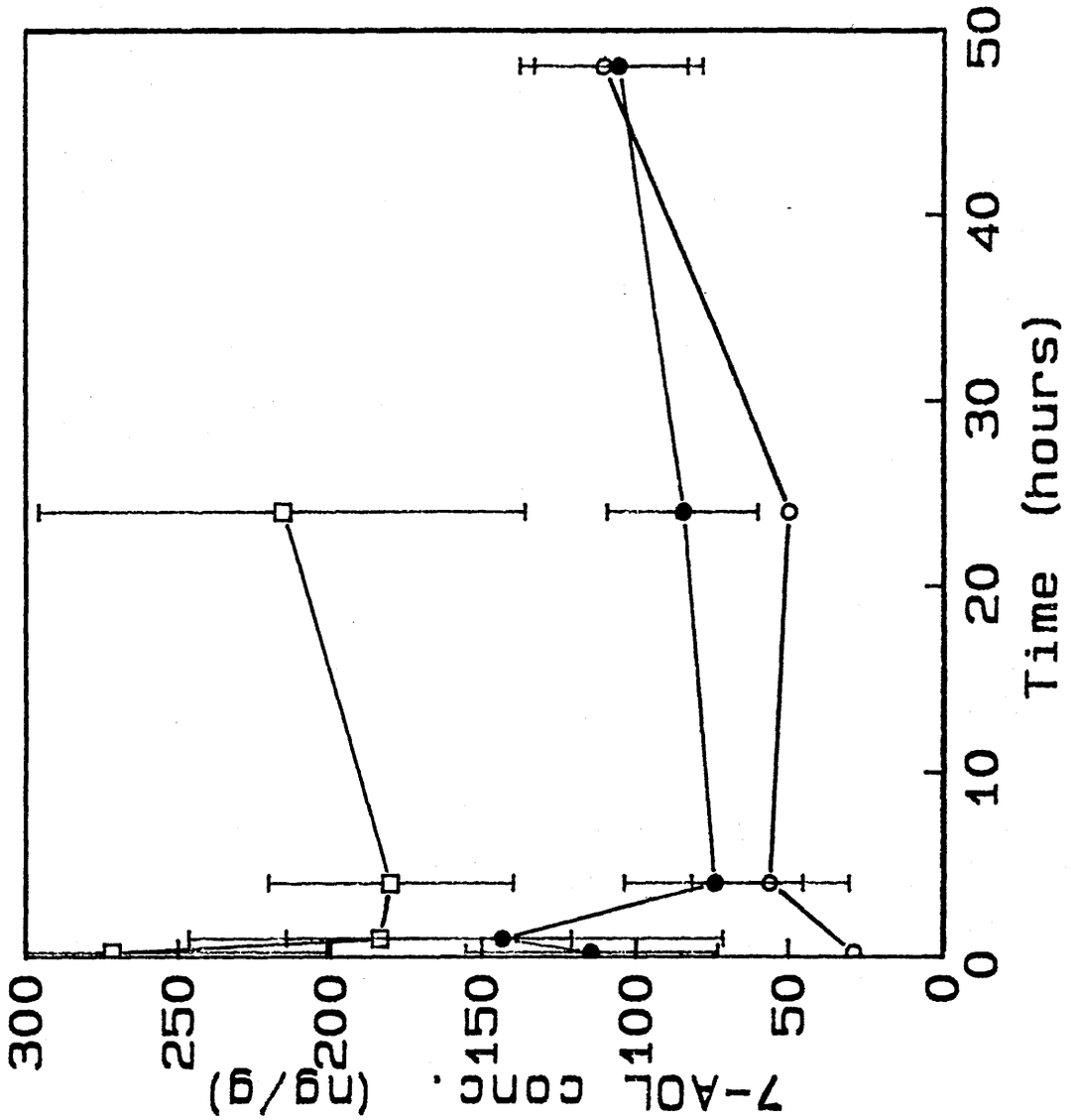


Figure 17: Tumour concentration of adriamycinol 7-deoxy-aglycone versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

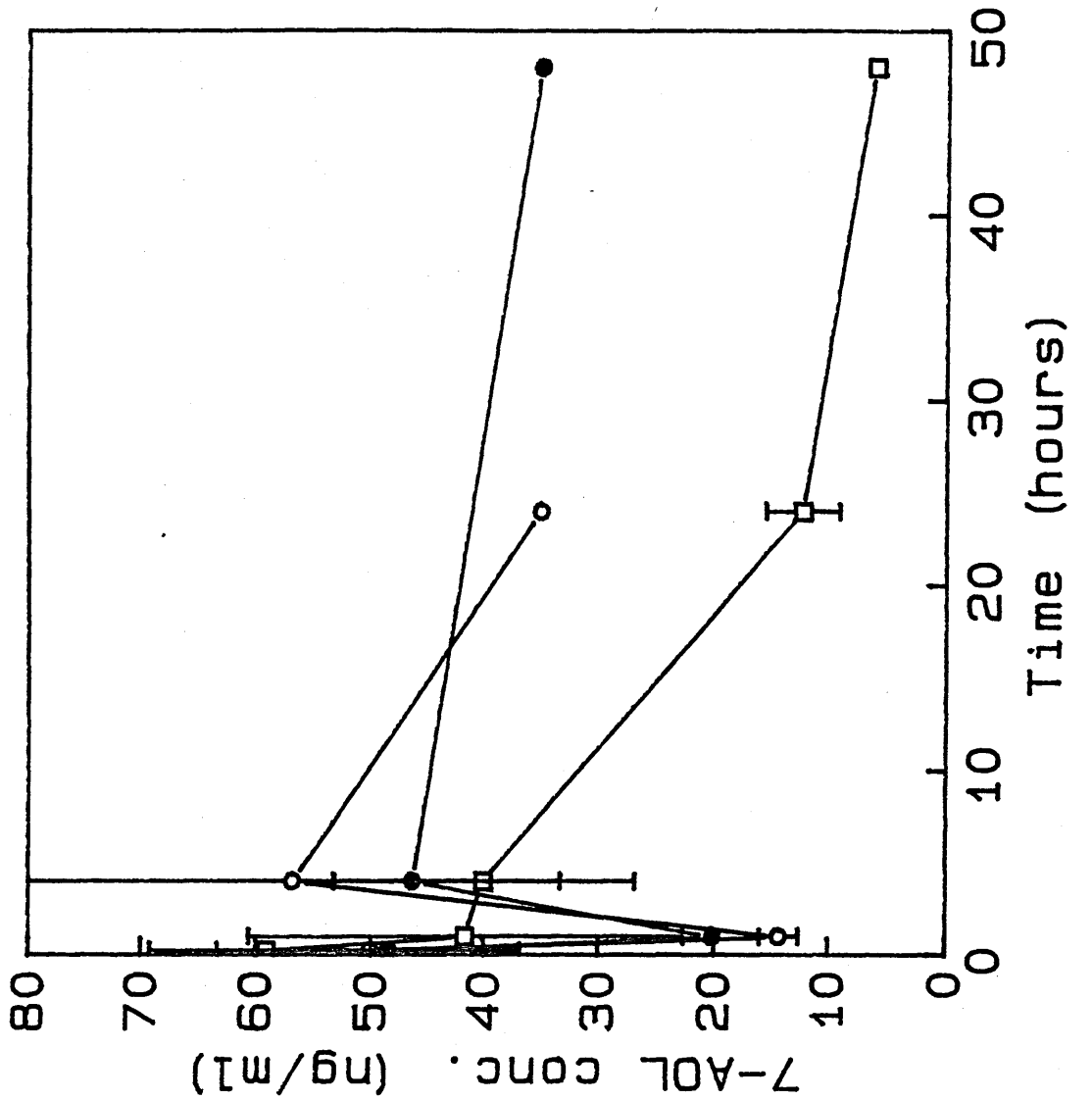


Figure 18: Plasma concentration of adriamycinol 7-deoxy-aglycone versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

levels of the same metabolites and indicate at least comparable metabolite levels following all of the tested formulations. A similar conclusion can be drawn from figure 19 which illustrates the concentration-time profile for AOLONE.

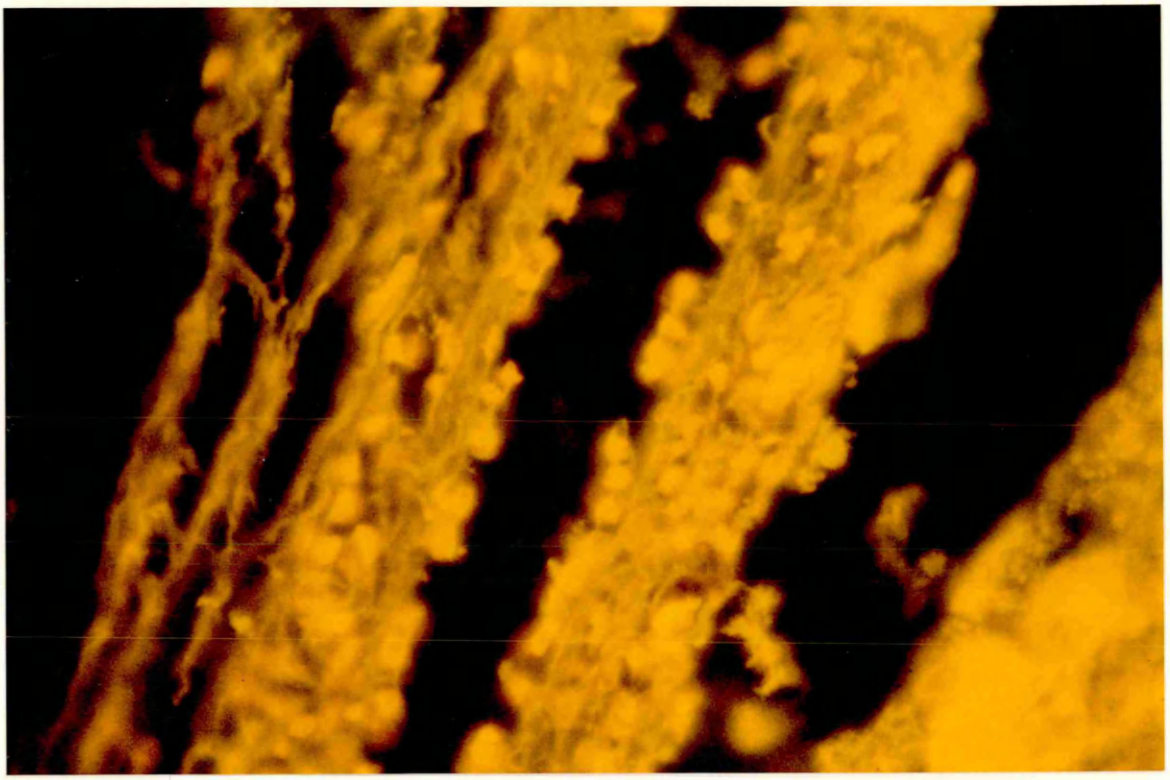
TUMOUR GROWTH RESPONSES (FORMULATION II ONLY)

Figure 20 shows the pattern of growth of ROS tumour in control animals or those given drug treatment as stated. In the treated groups drug was given at day 10 following tumour passage. There is some growth delay in the NIV entrapped adriamycin group but ultimately all of the mice died from tumour burden.

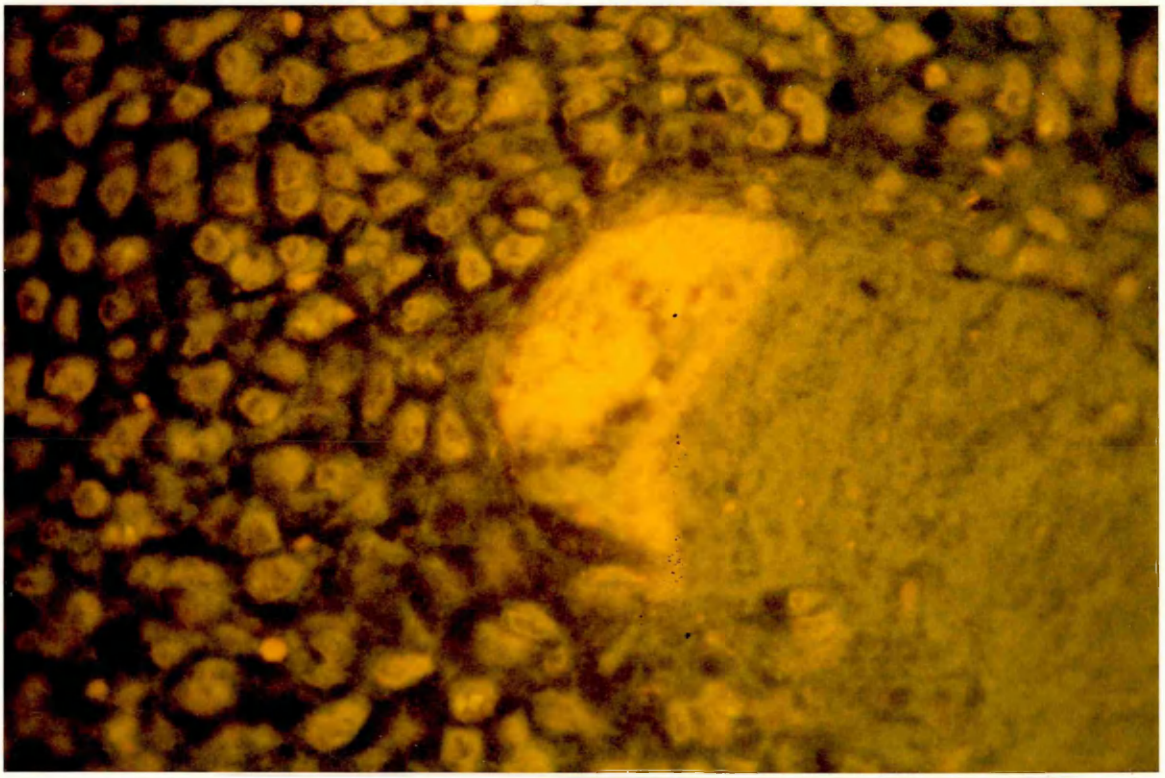
No tumours arose in the mice treated with empty NIV plus adriamycin in solution and only one tumour arose in the four mice given adriamycin in solution confirming the sensitivity of this tumour to anthracyclines.

FLUORESCENT MICROSCOPY (FORMULATION II ONLY)

NIVs containing adriamycin were found to fluoresce with the typical orange-red fluorescence of adriamycin. The intact vesicles could be visualised within the blood vessels of various tissues soon after (5 minutes) intravenous injection of a bolus dose (illustration 1 and 2). At 4 hours after dosing the only tissue in which vesicles could be found was in the tumour



1 Fluorescent photomicrograph (magnification x500) of niosomes loaded with adriamycin lodged in hepatic sinusoids (longitudinal section) five minutes after intravenous bolus injection



2 Fluorescent photomicrograph (magnification x500) of niosomes loaded with adriamycin lodged in tumour vasculature five minutes after intravenous bolus injection

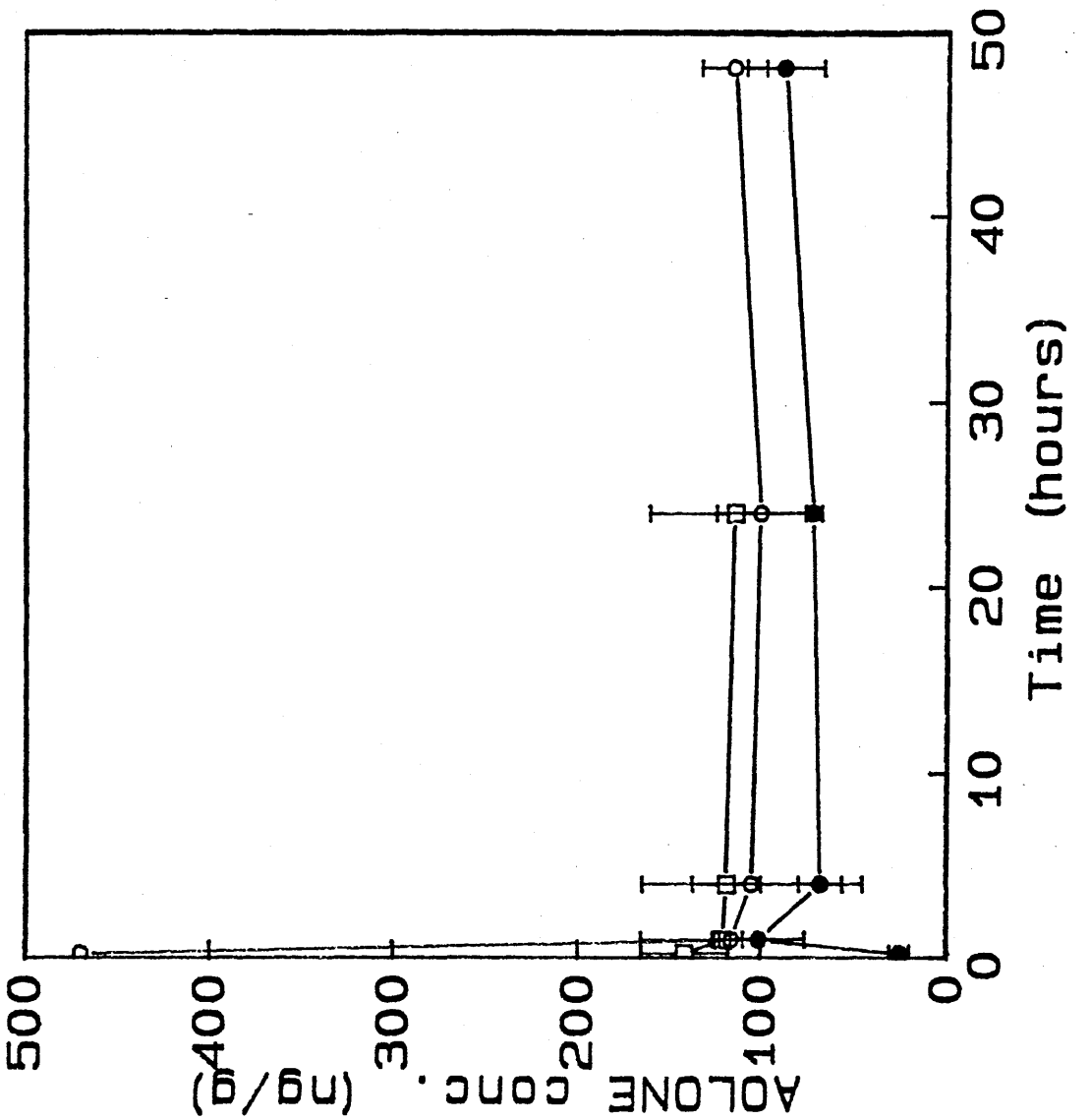


Figure 19: Tumour concentration of adriamycinol aglycone versus time following IV administration of formulation 2 niosomal adriamycin (closed circles), adriamycin in solution (open squares) or a mixture of empty formulation 2 niosomes plus adriamycin in solution (open circles).

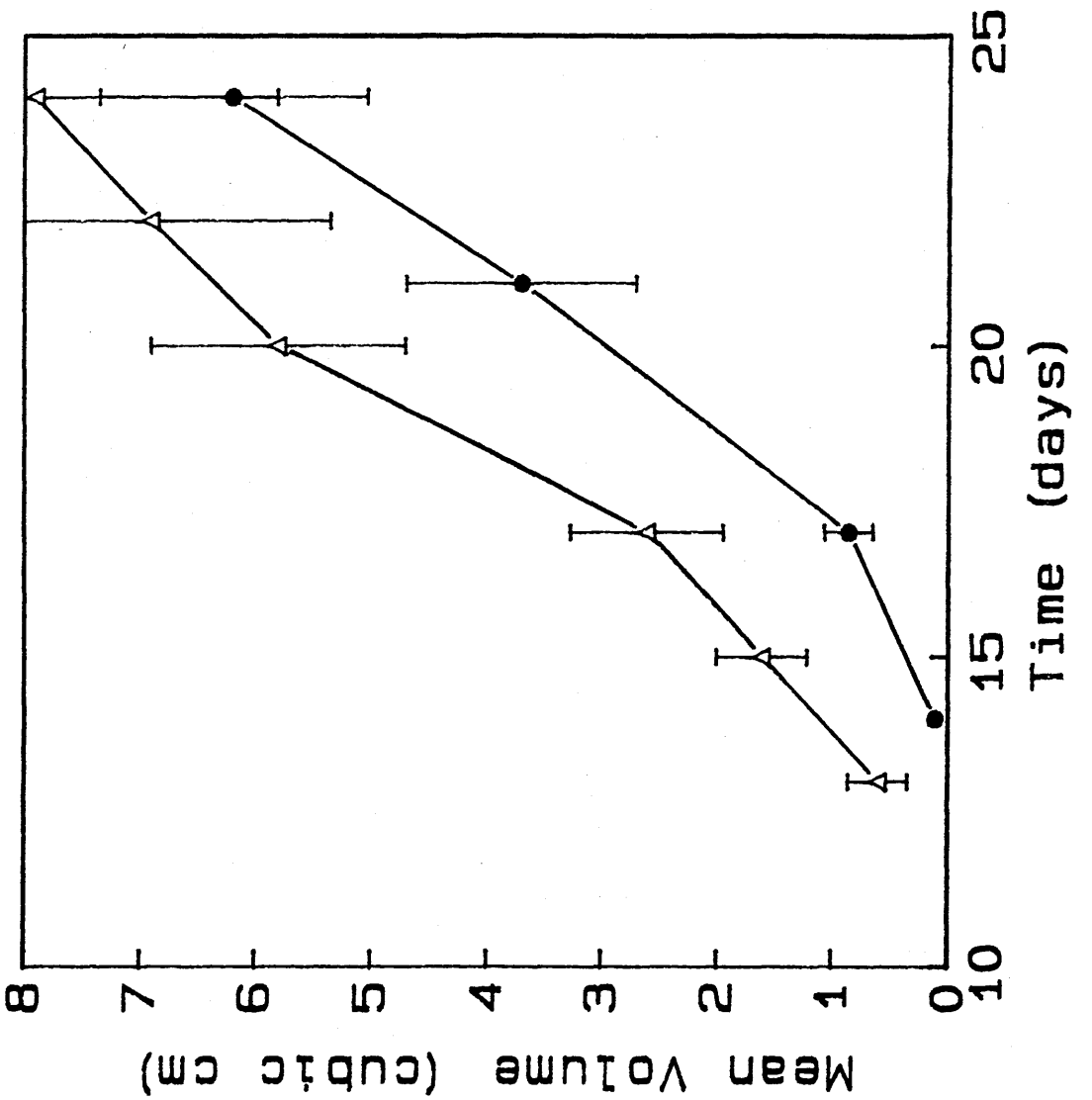
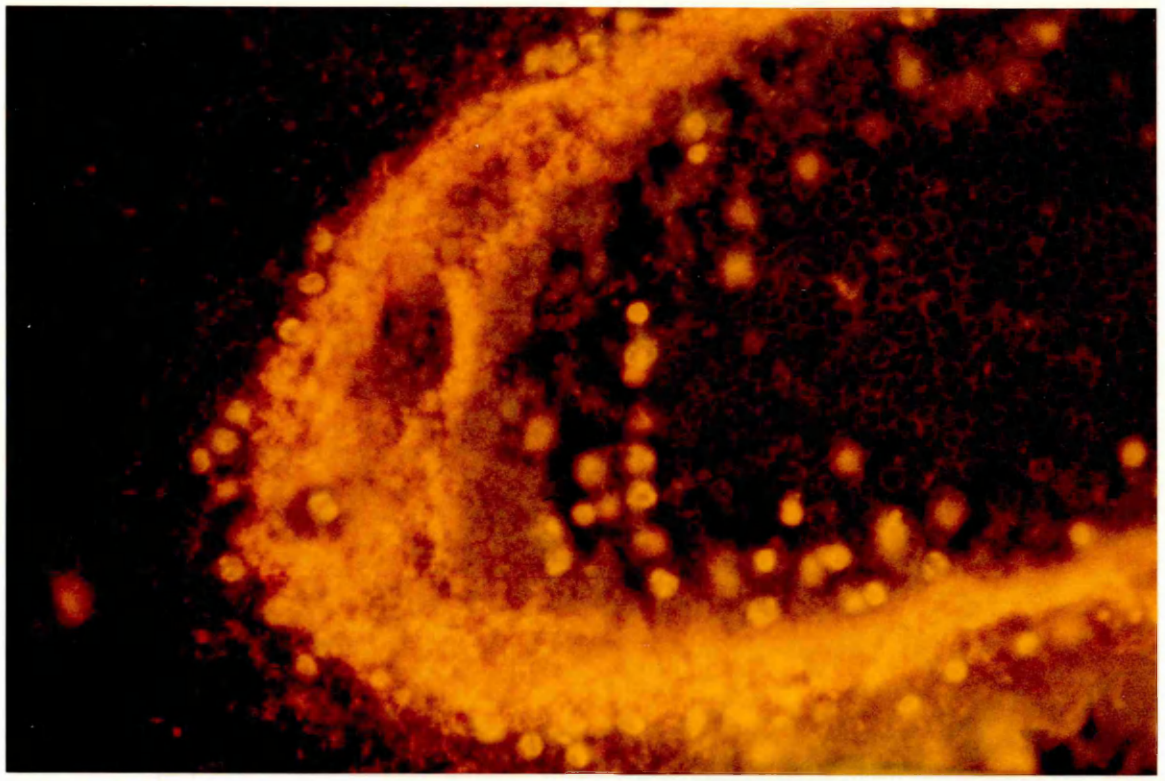
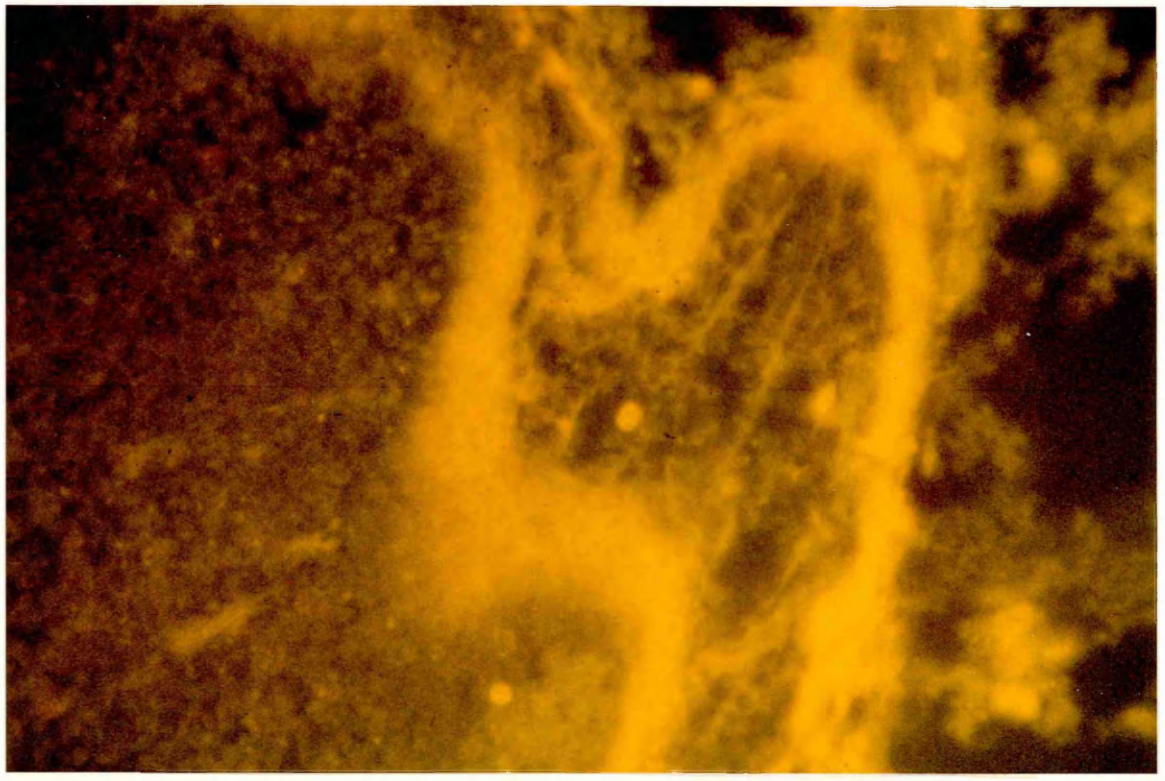


Figure 20: Growth of ROS tumour in untreated (control= open triangles) mice and mice treated with IV formulation 2 niosomal adriamycin at a dose of 10 mg/kg (filled circles).

(illustration 3), and by 24 hours the vesicles appeared to be less numerous (illustration 4) with a more diffuse tissue fluorescence suggesting drug release into the tumour substance. This appearance could not be seen in any other tissue, or after intravenous dosing with adriamycin in solution. In fact after adriamycin in solution no fluorescence could be detected in the tumour at any of the time points.



3 Fluorescent photomicrograph (magnification x500) of niosomes loaded with adriamycin lodged in tumour vasculature four hours after intravenous bolus injection



4 Fluorescent photomicrograph (magnification x500) of niosomes loaded with adriamycin lodged in tumour vasculature 24 hours after intravenous bolus injection

DISCUSSION

The pharmacokinetics of adriamycin in solution are well documented, and the data in this chapter show a general agreement with those in the literature (Cummings, Merry & Willmott, 1986). The NIVs used in these experiments are different from those previously reported (Kerr et al, 1988), in at least three important physico-chemical properties. The NIVs reported here are unilamellar (vs. 6 lamellae), they are sonicated which results in a smaller size with a tighter size distribution (200-400nm vs. around 1000nm) and they are of a different chemical composition. All of these could have profound influences on vesicle stability and any targeting potential. It is therefore not surprising that the data obtained with different formulations differ in several important respects.

Illum et al (1987), have shown that the addition of large molecules with hydrophilic head groups can make particulate carriers avoid reticulo-endothelial trapping. Thus the addition of solulans was made in NIV I in an attempt to mimic this effect. The data presented shows a prolonged maintenance of plasma levels of parent drug, a reduction in hepatic metabolism despite apparent high levels of parent drug in the liver, and a slight reduction in cardiac peak levels and AUC for adriamycin. These changes in pharmacokinetics resemble those achievable by prolonged infusions of the drug in solution. This approach has shown some therapeutic benefit, but is limited by the need for

central venous access and expensive pumps (Legha et al, 1982). The use of NIV entrapped adriamycin may therefore represent a more practical approach to a 'slow-release' type of preparation of adriamycin. The reduction in cardiac concentrations maybe of clinical importance since cardiotoxicity is the ultimate limiting factor to cumulative dosage of adriamycin in humans. The changes in cardiac levels seen with this preparation were much smaller than expected from previous studies (Kerr et al, 1988), and prompted the change to formulation II. Additionally, the supply of surfactant I was discontinued due to changes in the manufacturing process at L'Oreal, France.

The reduced biotransformation to aglycones in the niosomal preparation may indicate an altered metabolic pathway, with implications both for toxicity and efficacy; though this is a disputed point in the literature (Cummings et al, 1986b).

In formulation II experiments there is an apparently low level of parent drug in all of the tissues following the administration of NIV II. This was unexpected and not compatible with the appearance of the fluorescent photomicrographs, which indicate accumulation of drug in the tumour. This suggested some artefactual inability to extract the drug from the tissues. Consequently, attempts were made to make the assay conditions more rigorous. This involved; triplicate extraction for all samples, maintenance of samples in contact with solvent for as long as 3 days, use of extremes of pH 2 - 9.5, use of different solvents, or the use of acid-hydrolysis. None of these proved

able to extract the adriamycin from the samples, but samples passed through bond-elute columns caused a red staining of the column indicating the presence of adriamycin which was presumably firmly bound to some constituent of the NIV or tissue.

One would expect that intratumoural drug content (expressed as AUC) would bear some relationship to the cytotoxic effects of the drug. A gross reduction in tumour AUC is apparent with NIV II, which may account for the relative lack of efficacy in the tumour model system. The presence of an intra-tumoural 'depot' of adriamycin is suggested by the fluorescent photomicrographs, and the low drug levels measured by HPLC are maintained for at least 48 hours. Bearing in mind the therapeutic benefits of prolonged infusions of adriamycin, it should be beneficial to maintain low levels of adriamycin for a prolonged period. The lack of anti-tumour effect for NIV II could indicate that although the material visible by auto-fluorecence is similar to native adriamycin, the drug has been inactivated. The available data using other NIV preparations does not support this view; with only pure drug released from NIVs in dialysis experiments (Rogerson, Cummings & Florence, 1987), and a retention of in-vitro and in-vivo cytotoxicity (Kerr et al, 1988). Another, perhaps more plausible explanation is that the rate of drug release from the NIVs into the tumour is crucial to optimum cytotoxic effect. This hypothesis could be tested by administration of infusions of adriamycin at different rates

into tumour bearing animals, and if proven, the chemical nature of the NIVs could be altered to change permeability in order to optimise drug release rate.

The results of the administration of empty NIVs together withadriamycin in solution are interesting. In formulation II experiments the results are very similar to those for adriamycin alone. Using NIV I the results are intermediate between the drug in solution or entrapped within NIVs. This suggests that there is a loose association between the drug and the outer surface of the vesicles for some, but not all, types of NIVs.

The fluorescent microscopy studies show unexpected and very encouraging results. There appears to be a degree of passive targeting of NIV II to the tumour, through an initial step of vascular accumulation, followed by release of the drug into the tumour tissue. This is in keeping with the initial hypothesis that NIVS would be a way of delivering drug together with a surface active agent which would then enhance penetration of the drug into the tumour substance. The mechanism of this targeting is unclear but may relate to the defective and leaky capillary membranes which have been shown to be present in some tumour types (Warren, 1970), or to the increase in non-specific absorptive endocytosis reported in some tumours (Sehested et al, 1987). It would obviously be important in further studies to try to retain this favourable targeting to the tumour.

The potential for change in the physico-chemical properties of NIVs is almost limitless. In this chapter are described two

formulations; both of which show potential benefits over intravenous bolus administration of adriamycin. Nevertheless, it is clear that neither of our formulations is ideal. Further work is planned which will attempt to retain the targeting potential of NIVs, but have more favourable 'release' characteristics, whilst avoiding the reticulo-endothelial accumulation which is a problem for all vesicular drug carriers.

The limited success achieved with this vesicular carrier turned our attention to other particulate carriers. Lipid based vesicles (liposomes) have been the most intensively investigated over the last 20 years. In the next chapter, data is presented on a liposomal carrier system for anthracyclines.

CHAPTER 5

STUDIES WITH LIPID VESICLES (LIPOSOMES)

Chemotherapy has evolved as a mainly parenteral type of treatment. As a result most cytotoxic drugs are water soluble, and conversely some promising lipophilic drugs were never developed for intravenous use due to solubility problems.

Liposomes are vesicles which are composed of phospholipids and other lipids arranged in concentric bilayers or 'lamellae', thus resembling the 'myelin figures' formed when dried phospholipids are exposed to water (Stoeckenius, 1959). It was not until the observation in 1965 that such multilamellar structures could trap a range of ions and release them at various rates, that the potential for drug delivery of these vesicles was realised (Bangham, Standish & Watkins, 1965). They range in size from 0.05 to 5 um in diameter and can be used as carriers for hydrophilic or lipophilic (Perez-Soler et al, 1988) drugs, which associate with the internal aqueous phase or within the lipid domain respectively (Kaye & Richardson, 1979). Many possible interactions can occur between cells and liposomes, but the major mechanism of internalisation appears to be by endocytosis (Torchillin et al, 1988. Leserman et al, 1980).

Like many other particulate materials, and despite a lipid composition similar to cell membranes liposomes are recognised as foreign and tend to concentrate in organs that are rich

in reticulo-endothelial cells, such as the liver and spleen (Poste et al, 1982. Lopez-Berestein, 1984), with resulting adverse effects on this important host defence mechanism. This is the rationale to the use of liposomes to deliver antibiotics to the hepatic and splenic macrophages which are infected with intracellular organisms in some disease states, e.g. Listeriosis (Bakker-Woudenberg, Roerdink & Scherphof, 1988), and in the use of liposome entrapped muramyl peptides to activate macrophages to a tumouricidal state (Creaven et al, 1989). However, this Kuppfer cell uptake is undesirable in tumour targeting, as a large proportion of the dose is removed. Attempts to inhibit liver uptake by pre-administration of carbon particles, empty liposomes, dextran sulphate or methylpalmitate have been only modestly successful (Proffitt et al, 1983). However, more recently chemical modification of liposomes has been shown to have a significant effect on reticulo-endothelial uptake, and it is now possible to design liposomes which largely avoid this hepato-splenic trap (Allen & Chonn, 1987. Presant et al, 1989)

The ability of liposomes to accumulate in tissues with fenestrated or defective capillaries (Perez-Soler et al, 1985. Palmer et al, 1984) does give some reason for optimism with regard to targeting. Smaller vesicles can pass through the 100 nm pores in the hepatic sinusoids to enter the space of Disse and interact directly with hepatocytes (Rahman et al, 1982). Certain tumour types are known to have defective capillaries (Warren, 1970), and the available data in the literature

suggests that tumour cells show increased endocytic activity when compared with normal cells (Sehested et al, 1987. Trouet, Deprez de Campeneere & DeDuve, 1972). If this is true one would expect selective delivery to such tumours. The contribution of endocytic activity is not totally clear since liver endothelial cells are known to have a high endocytic rate but take up few liposomes in at least two independant studies (Rahman et al, 1982).

Work done in several laboratories (Gabizon & Papahadjopoulos, 1988) suggests that liposome entrapment of anthracyclines results in prolonged maintenance of high plasma levels of parent drug, similar to that seen with prolonged continuous infusion of the drug (Legha et al, 1982), together with a reduction in uptake by other tissues such as the bone marrow, small intestine, kidney and heart (Kaye, Boden & Ryman, 1981. Rahman et al, 1985. Rahman et al, 1986a). The therapeutic implications of this redistribution will depend on the tumour cell uptake in vivo, since this would have to be at least maintained for an improvement in therapeutic index to be apparent. A reduction in toxicity (particularly cardiotoxicity) has been demonstrated in several studies of liposomal adriamycin (Forssen & Tokes, 1983 a and b. Rahman et al, 1986b. Gabizon, Meshorer & Barenholz, 1986). In most studies this is associated with a maintenance of anti-tumour activity (Olson et al, 1982. Van Hoesel et al, 1984), though in some there is enhancement of activity (Forssen & Tokes, 1983a. Presant et al, 1989. Dusre,

Forst & Rahman, 1989. Gabizon et al, 1985). Interestingly, in the latter study this superior activity was limited to intrahepatic tumour, and the formulation was inactive against intramuscular deposits of the same tumour.

For over ten years investigations have been carried out to determine the therapeutic potential of liposomes as a drug carrier system. Early studies were mainly based on the 'passive' targeting aspect with drugs chosen which have therapeutic effects on, or mediated through the macrophages. Since the properties required to avoid reticulo-endothelial uptake have become clear, the clinical studies have taken on new impetus. Several groups have demonstrated tumour localisation for diagnostic purposes and therapeutic purposes (Presant et al, 1989. Gabizon & Papahadjopoulos, 1988), and human phase I - II studies of liposomal-anthracycline preparations are underway, with some encouraging preliminary results (Sulkes, Barenholz & Gabizon, 1989. Treat et al, 1989. Cowens et al, 1989). In addition, recent preclinical studies suggest that liposomal encapsulation may be a way of circumventing adriamycin resistance in-vitro (Fan et al, 1989), which if confirmed in-vivo would be a major step forward in cancer chemotherapy.

More detailed reviews of the history and development of liposomes have been published in articles and book form (Kaye, 1981. Perez-Soler, 1989. Gregoriadis, 1988).

The work detailed in this chapter uses liposomes under commercial development by Vestar Inc. (San Dimas, California,

USA). Consequently, certain details of formulation and composition cannot yet be published in full. In short, these liposomes are made from ultra-pure ingredients, resulting in an extremely stable final product, which can be lyophilised and therefore has a much longer shelf life. This is obviously advantageous for formulation and clinical applications. The liposomes discussed in this chapter were already loaded with daunorubicin on delivery, and were used immediately to minimise any potential drug leakage without any further preparative work.

MATERIALS AND METHODS

PREPARATION OF LIPOSOMES

Daunorubicin loaded liposomes were a gift from Vestar Inc. (650 Cliffside Dr, San Dimas, CA, USA) and used as supplied. Vesicle formulations containing daunorubicin consisted of distearoyl phosphatidylcholine and cholesterol (1:10:5 mole ratio). Vesicle composition and preparative procedures were designed to enable the usual step of removal of unincorporated drug from the vesicles to be eliminated (Presant et al, 1989). The integrity of entrapped drug was verified by HPLC and thin layer chromatography, the final concentration of entrapped drug was 1.05 mg/ml. The vesicles were unilamellar, and ranged in size from 40 to 60 nm in diameter. They were sterilised by Millipore filtration at 0.22um. Physical and chemical analyses indicated that they are stable for at least two months at room temperature.

Daunorubicin was purchased from May and Baker, Dagenham, England; as the clinically used formulation. It was dissolved in 0.9% Sodium chloride solution immediately prior to use.

ANIMALS AND TUMOUR MODEL

Six week old female Wistar rats were purchased from Harlan

Olac (Shaws Farm, Bicester, UK). They were kept in a 12 hour alternating light/dark cycle and allowed food and water ad libitum. Within one week of purchase (when mean weight = 200 grams) a 1 cubic mm fragment of Walker 256 tumour was implanted into a surgically created subcutaneous pouch on the right flank of lightly ether anaesthetised animals, and the wound closed by surgical clips. Approximately 24 hours later, three groups of 10 animals each were given tail-vein injections of 5mg/kg of daunorubicin in solution, 1 ml of sterile normal saline as a negative control or 5mg/kg of daunorubicin entrapped in liposomes. The animals were then observed and resultant tumours measured at least twice weekly in two perpendicular diameters using calipers, and tumour volume calculated as in the previous chapter. The life-span of control animals is around 13 to 15 days. In our experience with this tumour the percentage failure rate for tumour 'takes' is less than 10%.

PHARMACOKINETIC STUDIES

About 5 days after tumour implantation tail-vein injections (as detailed above) of each formulation were administered to groups of 3 rats each, which were subsequently sacrificed by cervical dislocation at 5, 30 and 60 minutes, 3, 5, 12 and 24 hours post-dose. The blood of each animal was immediately collected from the inferior vena cava. Samples were collected in Lithium Heparin tubes (Sherwood medical industries, County Oak

Way, Crawley, UK) and centrifuged at 100g for 2 minutes and the plasma removed and rapidly frozen, then held at -20^{0}C until the day of assay. The heart, liver and tumour were dissected out of each animal, blotted dry and frozen in liquid nitrogen to await analysis.

DRUG ANALYSIS

This is described in detail in the previous chapter. The only difference being that in this case adriamycin was used as the internal standard.

MATHEMATICAL ANALYSIS

The area under the concentration time curve (AUC) was measured by the log trapezoidal rule from time zero to the last measured time point. Statistical comparisons were made by two way analysis of variance or Student's T-test with Bonnferroni correction where appropriate. As it was impossible to take multiple plasma samples from the same rats, it is impossible to statistically compare AUCs as this parameter was derived from single samples from multiple animals.

The growth curves for treated and untreated animals are given only out to day 15 following passage, as at this time several animals from each group have died from their tumour burden,

thereby biasing the results towards those with smallest tumours. A more realistic idea of outcome is given in the 'survival' graph. Mann-Whitney U-test was applied in the statistical comparison of the growth curves.

RESULTS

PHARMACOKINETIC STUDIES

Figures 1 to 4 show the daunomycin concentration-time profiles for plasma, liver, heart and tumour respectively. Each point is the mean with associated standard errors (n=3 for liposomes, n=8 for controls). In all cases the concentrations for liposome entrapped drug are higher over the major part of the time course of the experiments. Table 13 details the derived values of AUC. In each case the daunomycin concentration for the liposome group appears to fall rapidly at around 30 minutes, with recovery at 3 hours and thence a more orderly form of exponential decay is exhibited in all the tissues examined. Even allowing for this unusual feature the tissue AUCs are larger for the liposomal form of the drug (see Table 13).

TUMOUR RESPONSE

Figure 5 shows the mean tumour volume (+/- SE) against time for each of the three treatment groups (n=10 each). The control curve is similar to that in other experiments using the same model (see next chapter). The free daunomycin curve shows a small growth delay, but this fails to reach statistical significance, and as shown in the survival data (figure 6) all tumour bearing animals in this group have died by day 17. (Two 'no takes' occurred in this group). The curve for liposomal

treatment does show a statistically significant difference from the control curve. In this group there were no animals in which the tumour failed to 'take' and the downward slope after day 11 therefore represents true tumour regressions, rather than the deaths of those animals with the largest tumours. In fact, this is the only group in which animals with palpable tumours showed regression and long term survival. Figure 6 details the survival pattern for all 3 groups until 18 days post-passage. Those surviving until then are still alive and tumour free at approximately 6 months post-passage to date.

TABLE 13

AUC measurements for free or liposomal daunomycin

Sample		Free	Liposomal	x Factor
Plasma	(ng/ml).h	411	692	1.7
Heart	(ug/g).h	31	92	2.9
Tumour	(ug/g).h	22	124	5.8
Liver	(ug/g).h	25	89	3.6

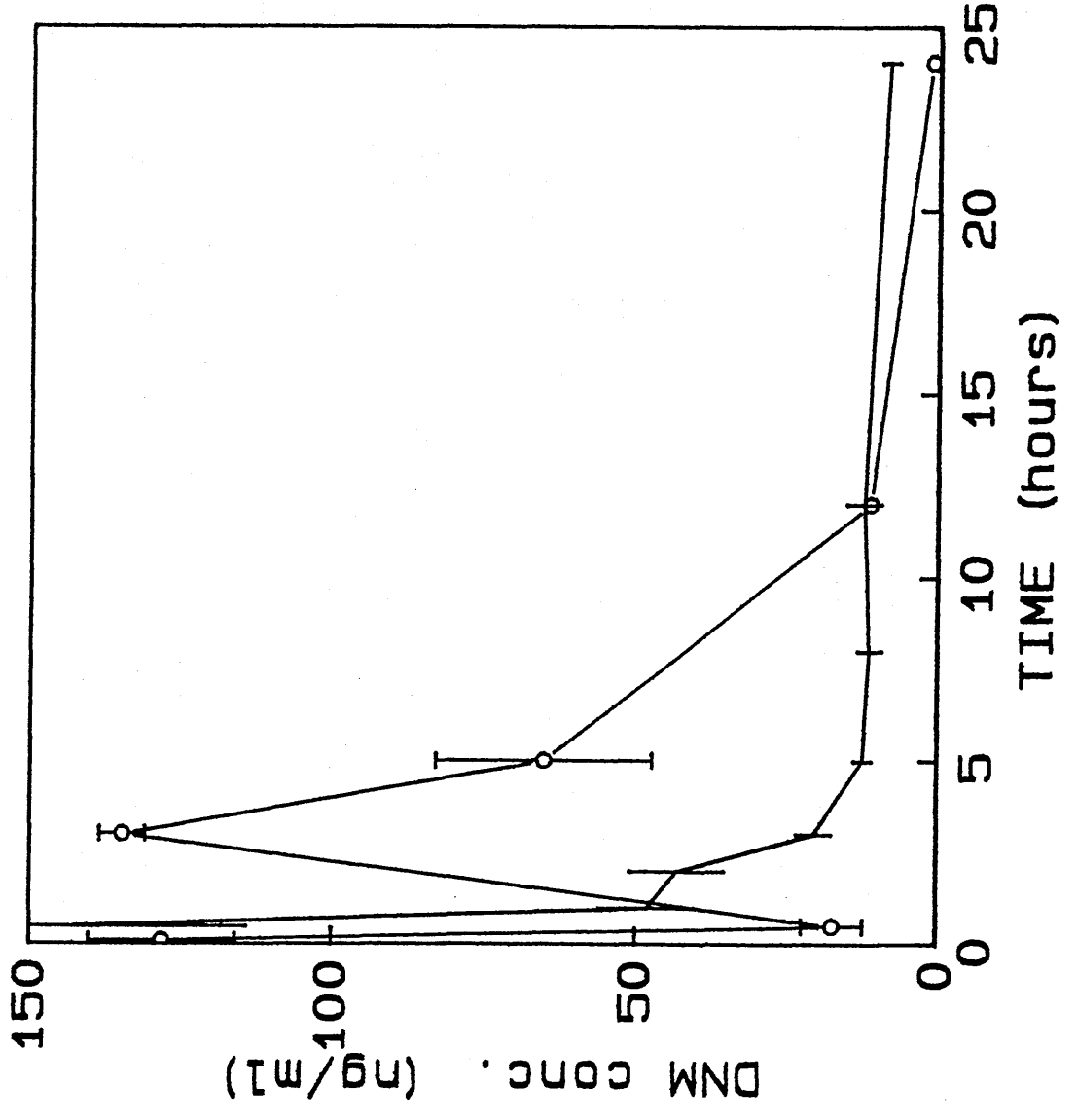


Figure 1: Plasma daunomycin concentrations versus time following IV administration of daunomycin in solution (dots) or liposomal daunomycin (open circles).

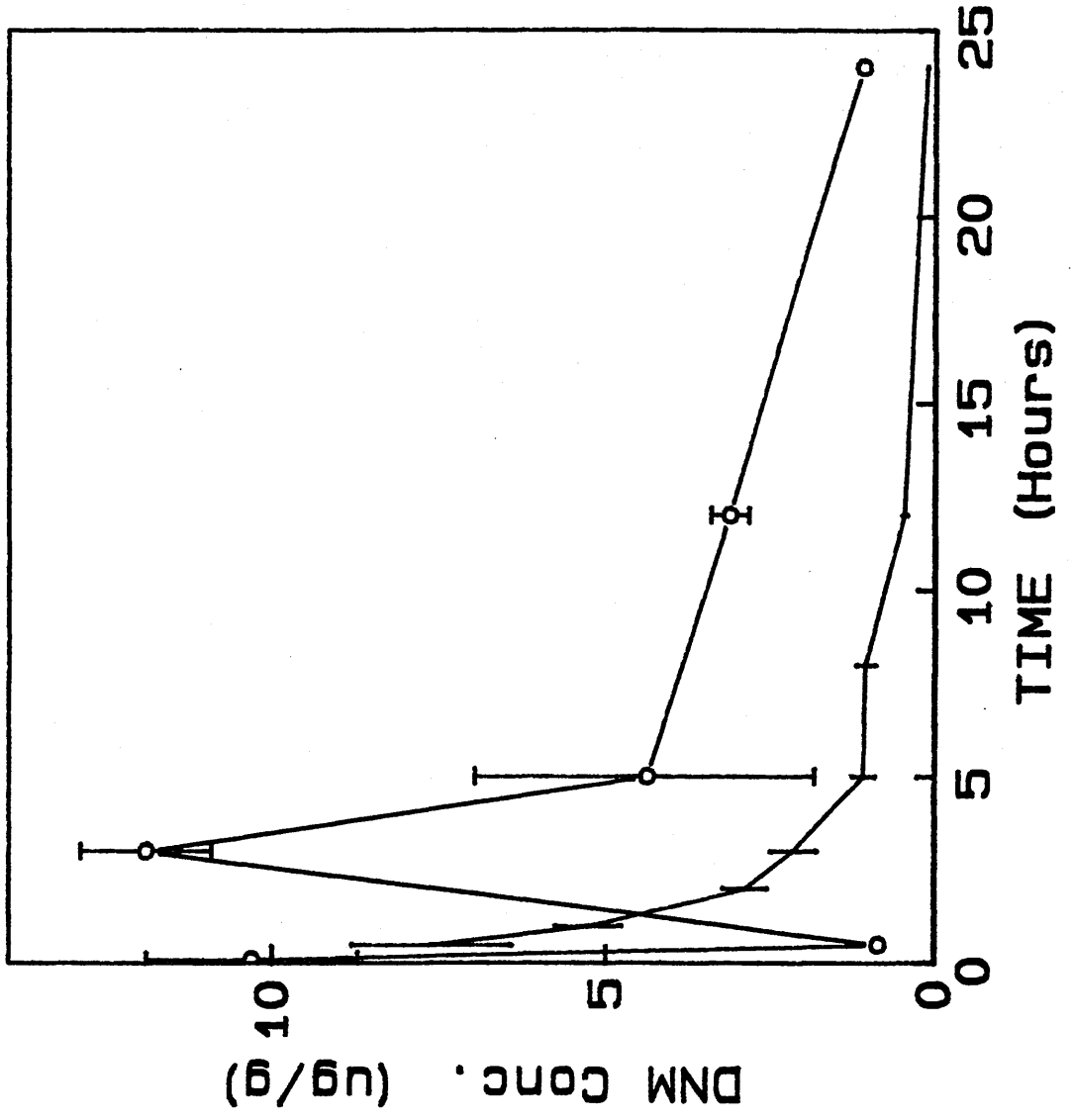


Figure 2: Liver daunomycin concentrations versus time following IV administration of daunomycin in solution (dots) or liposomal daunomycin (open circles).

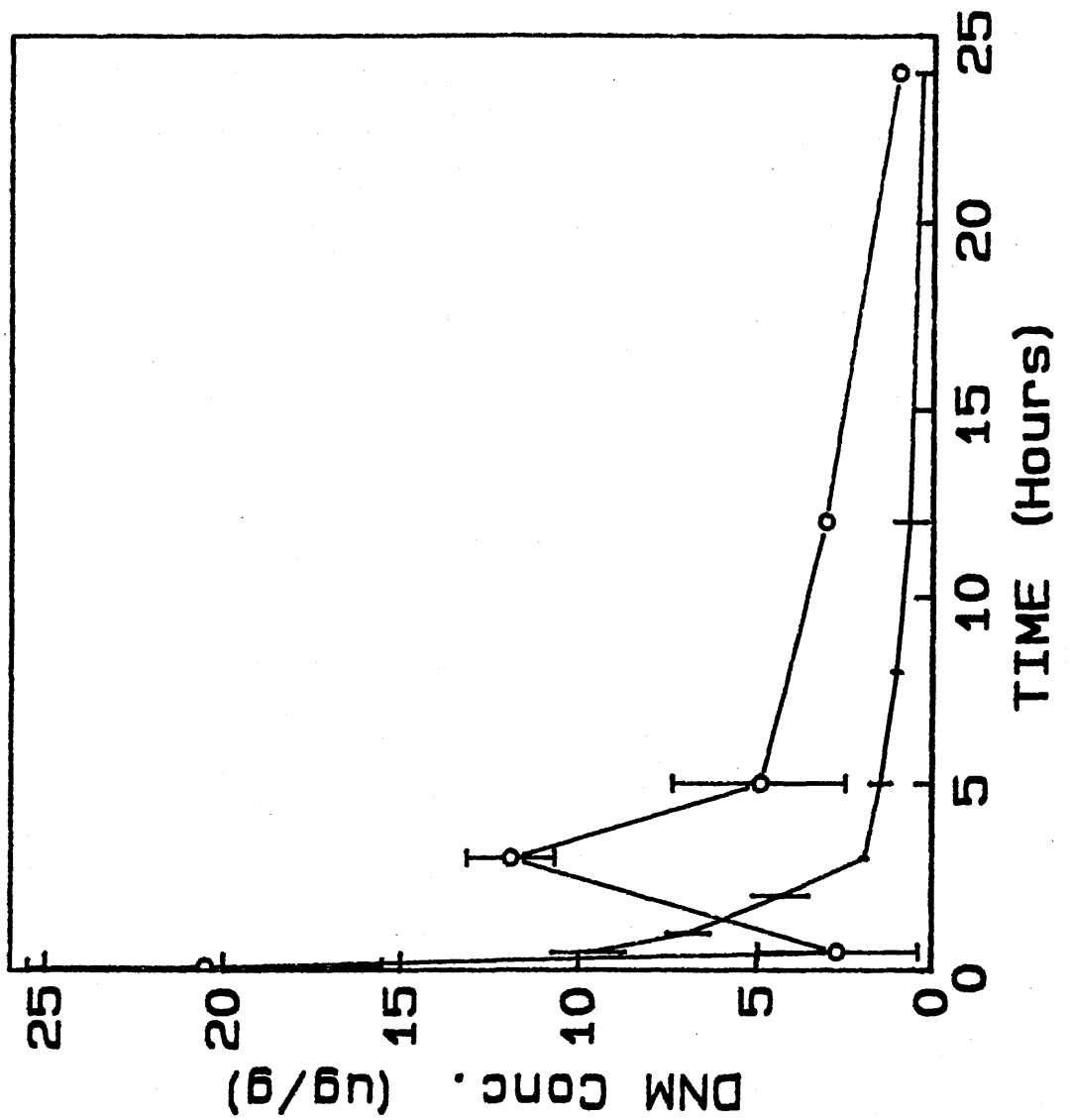


Figure 3: Heart daunomycin concentrations versus time following IV administration of daunomycin in solution (dots) or liposomal daunomycin (open circles).

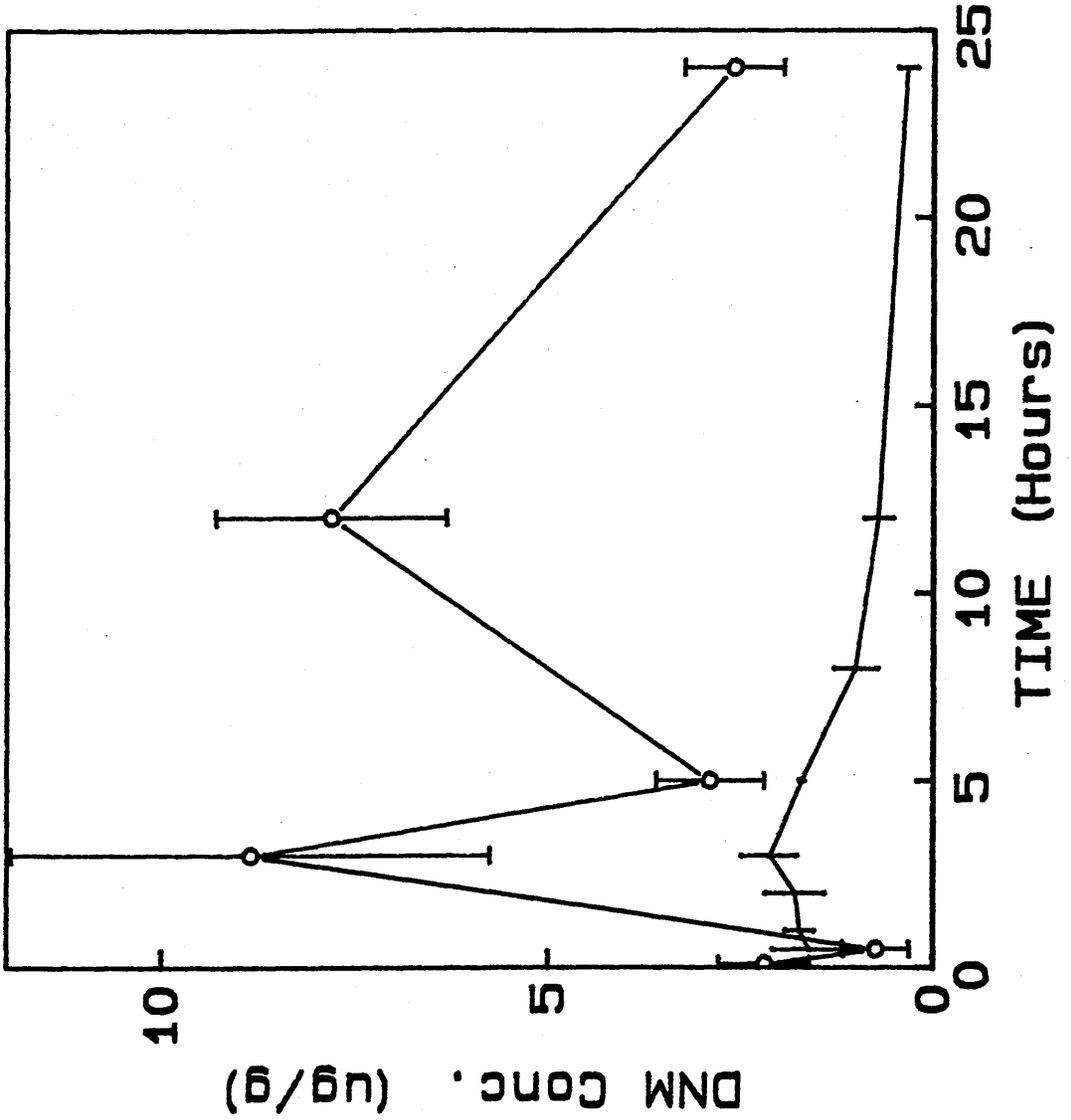


Figure 4: Tumour daunomycin concentrations versus time following IV administration of daunomycin in solution (dots) or liposomal daunomycin (open circles).

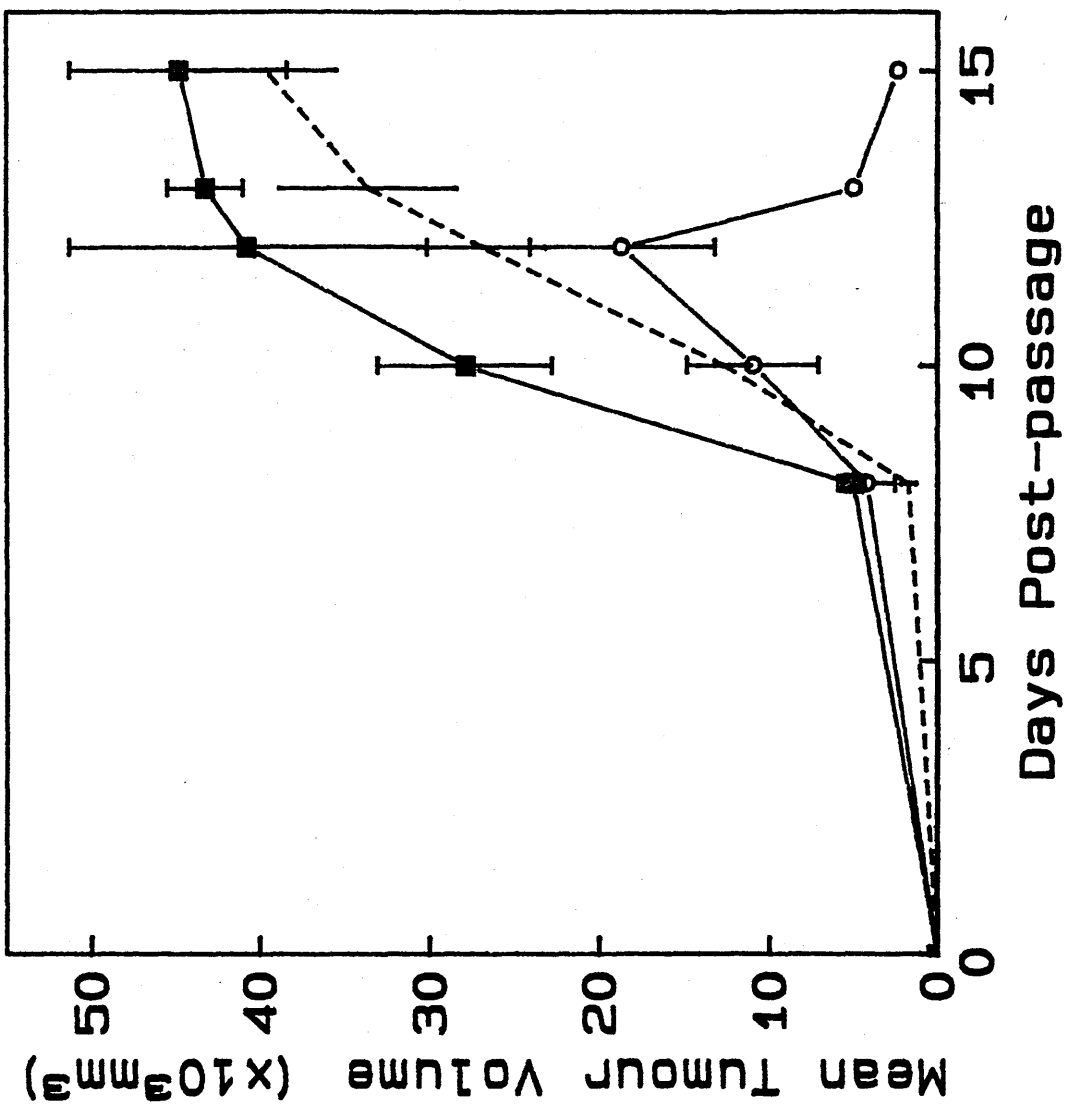


Figure 5: Growth of subcutaneously implanted Walker 256 carcinoma in untreated (control= filled squares) rats, or following IV administration of daunomycin in solution (dashed line) or liposomal daunomycin (open circles).

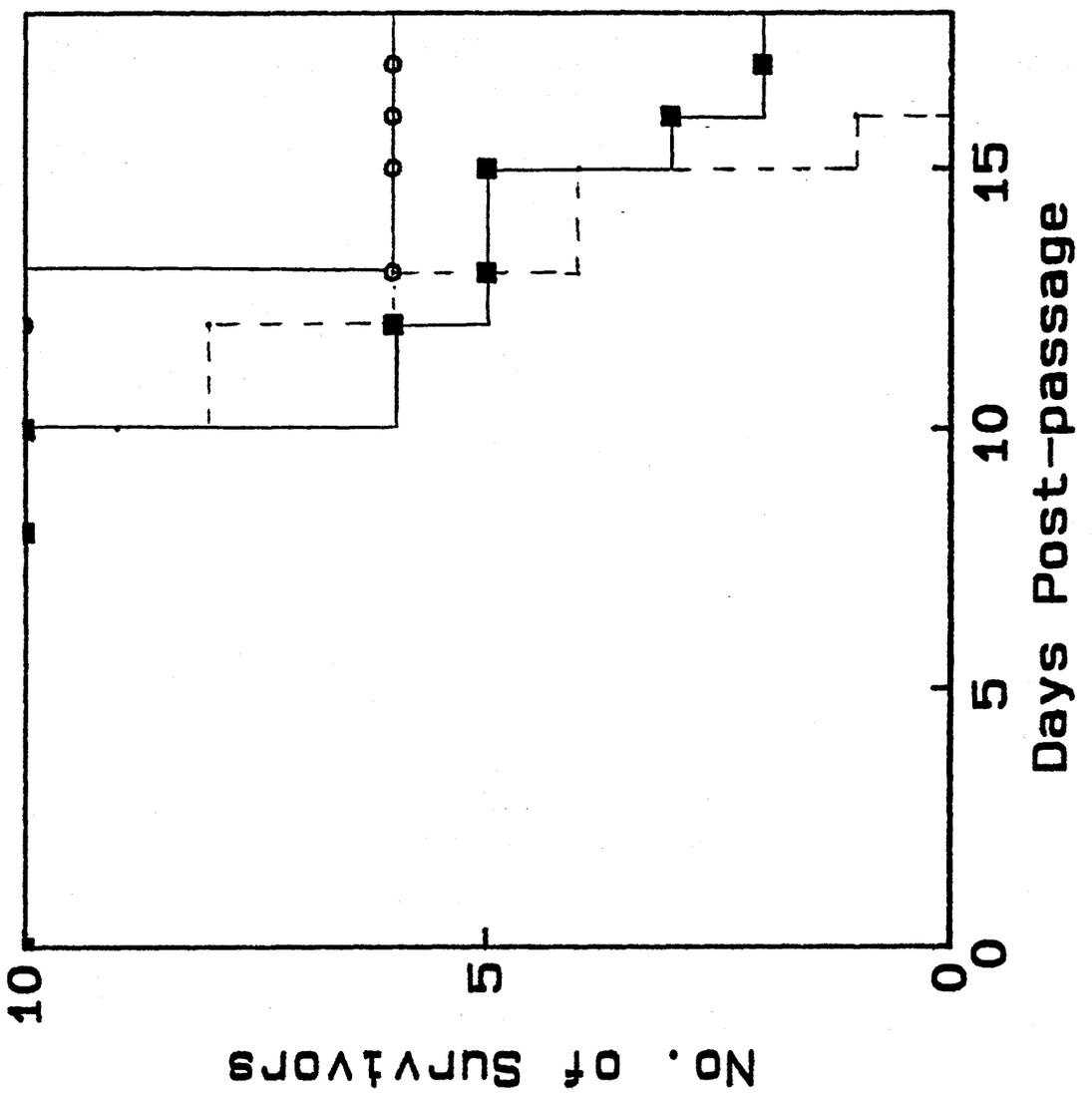


Figure 6: Survival of rats bearing subcutaneously growing Walker 256 carcinoma without treatment (control= filled squares) or following IV administration of daunomycin in solution (dots) or liposomal daunomycin (open circles).

DISCUSSION

Interestingly, in all of the examined tissues the major portion of the liposomal concentration-time curve is above that for free drug. This difference is most obvious in the tissues rather than the plasma, as shown by the factorial changes in AUC detailed in table 1. No correction for enclosed blood volume has been made as some would suggest is necessary for this type of data. Great care was taken to exclude gross contamination of tissues with blood, and one would expect that for this source of experimental error to be foremost, that the plasma concentration profiles would show the most extreme differences.

The curious form of the concentration time profiles for liposomal-daunomycin is difficult to explain: it does not seem to have been a problem with tail-vein injection technique, nor with the analytical methods used. It is perhaps possible that this represents some form of redistribution from an initial (but not instantaneous) depot within a particular organ, such as the lung. This observation has not been made in any other animal test system (personal communication, E.A. Forssen) using this particular liposomal formulation. Further work would be required to elucidate this point.

The observed pharmacokinetic alterations are accompanied by a significant increase in therapeutic activity of the liposomal

preparation against this particular tumour model. The experimental protocol for this set of experiments involves treatment only 24 hours after tumour implantation, this is necessary because later treatment with anthracyclines (even at doses around the LD_{50} level) causes no tumour growth delay at all (data not shown). Even at this early stage histological studies show a developing blood supply in these small tumour masses, but it is possible that liposomes are being delivered in a non-specific way to an area with damaged vasculature following an operative procedure. However, all of the long term survivors in the liposome treatment group developed tumours at around day 8-10, with subsequent regression of tumour from day 11 onwards.

In this model system the use of this liposome formulation has resulted in considerable changes in the pharmacokinetics of daunomycin. More drug appears to be delivered to the tumour, and this results in improved therapeutic responses. However it does appear that more drug finds its way to certain target organs for anthracycline toxicity (such as the heart). Two animals in the group scheduled for sacrifice at one hour post-treatment died immediately following tail-vein injection; in the control group no deaths were experienced. It is possible that even within the limited time course of these experiments overt cardiotoxicity was demonstrated. Thus, it remains to be demonstrated that a worthwhile change in therapeutic ratio has been acheived by liposomal entrappment of daunomycin. Unfortunately, at the time

of writing the limited availablity of this formulation has prevented further therapeutic toxicity studies. Nevertheless, the results presented are encouraging enough to warrant further investigation of this type of carrier in-vivo, and further studies are envisaged.

CHAPTER 6

STUDIES WITH A MACROMOLECULAR DRUG DELIVERY SYSTEM

INTRODUCTION

A different approach to improvement in therapeutic ratio of a chemotherapeutic drug might be to employ a high molecular weight carrier. Such macromolecularised drug derivatives are expected to increase the efficacy of the drug through more effective biodistribution, retardation of metabolic degradation and maintenance of prolonged non-toxic levels of the drug in the circulation due to slow release from the carrier.

Various chemotherapeutic drugs have been shown to be more effective when used in association with macromolecular carriers such as DNA (Cornu et al, 1974. Marks & Venditti, 1976. Trouet & Jolles, 1984); dextran (Bernstein et al, 1978. Levi-Schaffer et al, 1982. Kojima et al, 1980); proteins (Posnansky et al, 1982. Trouet et al, 1982) or polypeptides (Kato et al, 1984. Zunino et al, 1984. Schechter, Wilchek & Arnon, 1987). In certain cases these drug carriers have also been claimed to circumvent drug resistance (Ryser & Shen, 1980). Such carriers are non-specific in their actions, but more recently various groups have denmonstrated the efficacy of carrier systems based on tumour specific antibody-drug complexes (Schechter et al, 1987. Dillman et al, 1986).

N-(2-hydroxypropyl)methacrylamide (HPMA) homopolymers were originally developed as plasma expanders, for use in emergency volume replacement if blood or plasma protein solutions were unavailable. The homopolymer is chemically inert, and exhibits little immunogenicity even when complexed to anthracyclines (Rihova et al, 1985. Seymour et al, 1987), which can be a problem with other macromolecular carriers (such as DNA). Side-chains are readily introduced into HPMA copolymers and they can be used to carry a variety of prosthetic groups, such as drugs or targeting moieties (Duncan et al, 1987 and 1988). Drugs containing an aliphatic amine group such as the anthracyclines are particularly suitable for conjugation with HPMA copolymers. These copolymers can be tailor made to include oligopeptide drug-polymer linkages that are stable in the bloodstream (Rejmanova et al, 1985. Seymour et al, 1987), but can be cleaved inside the cell by lysosomal cysteine proteinases (Duncan et al, 1982. Rejmanova et al, 1983), thus releasing the drug into the lysosomes. The possibility of targeting these macromolecules has been investigated using a wide range of side-chain substitutions; from the simple addition of galactosamine residues (Duncan et al, 1983) to the more complex introduction of tumour specific monoclonal antibodies (Dr. L. Seymour, personal communication).

HPMA copolymer anthracycline conjugates are too large and too hydrophilic to enter cells by passage across the cell membrane; the only possible mode of entry is by endocytosis. The drug

conjugate in the endosome is then cleaved by lysosomal enzymes and the released drug (like physiological degradation products, such as amino acids and simple lipids) is free to make its way through the lysosomal membrane and to other parts of the cell to exert its cytotoxic effect. In the case of anthracyclines the most likely intracellular target is DNA; but as discussed in a previous chapter this may not be the only site of action for this class of drugs.

The binding of daunomycin to HPMA copolymers could theoretically change drug effect in two fundamental ways :-

1) Modification of pharmacokinetics

2) Delivery to specific cell populations i.e. targeting to tumours, with enhanced uptake through increased endocytic activity (Sehested et al, 1987).

The data presented in this chapter supports both these mechanisms using HPMA copolymers as a delivery system in a rat tumour model. The data has been published (Cassidy et al, 1989) and a reprint is bound into the back cover of this thesis.

MATERIALS AND METHODS

SYNTHESIS OF HPMA-DAUNOMYCIN COPOLYMERS

The chemical synthesis and drug-loading of HPMA copolymers was performed by Dr. Ruth Duncan and co-workers at CRC Polymer-controlled Drug Delivery Research Group, Keele University, Staffordshire, UK. The synthesis involves a two step procedure, described in detail elsewhere (Duncan et al, 1988 and 1989). Briefly, in the first step a reactive copolymer of HPMA with methacryloylated oligopeptide p-nitrophenyl ester was synthesised (polymer precursor); the second step involves drug binding by consecutive aminolysis (Kopecek et al, 1985) to yield the basic structure shown in Figure 1.

In the experiments described in this chapter, daunomycin was bound to HPMA copolymer via a biodegradable spacer (Gly-Phe-Leu-Gly, POLYMER 1), or the same dose of daunomycin bound via a non-biodegradable linkage (Gly-Gly, POLYMER 2).

ANIMALS AND TUMOUR MODEL

Six to eight week old Wistar rats were purchased from Harlan Olac (Shaws Farm, Bicester, UK). They were kept in a 12 hour alternating light/dark cycle and allowed food and water ad libitum. When the mean weight was 250 grams, a 1 cubic mm

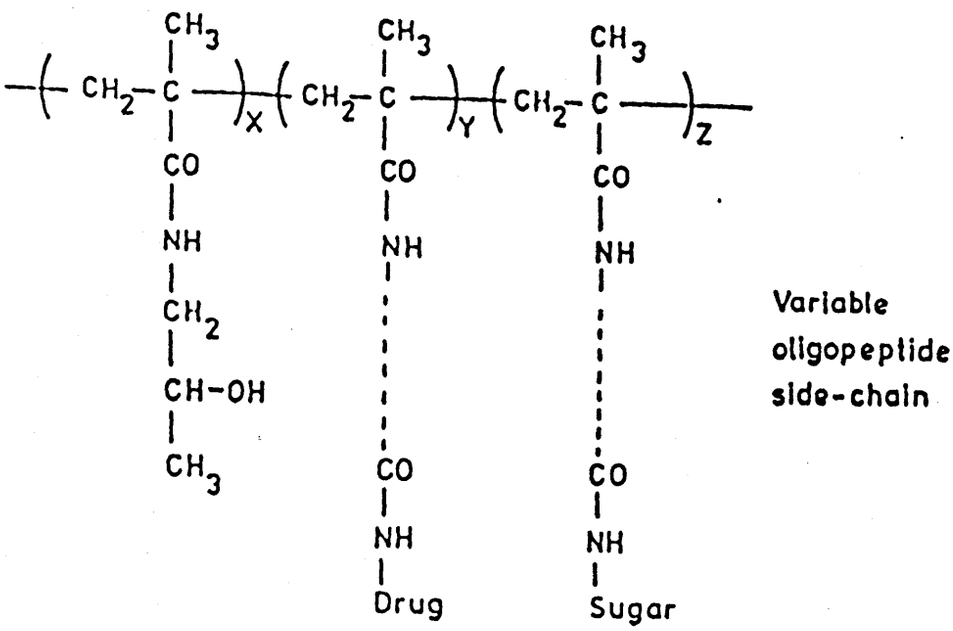


Figure 1: Oligopolymeric structure of N-(2-hydroxypropyl) methacrylamide copolymers

fragment of fresh Walker 256 tumour was implanted into a surgically created subcutaneous pouch on the right flank of lightly ether anaesthetised animals, and the wound closed by surgical clips. The animals were then observed and resultant tumours measured by the same observer at least twice weekly. Two perpendicular diameters were taken using calipers, and the tumour volume calculated using the formula given in chapter 4. The life-span of control animals is around 13 to 15 days. In our experience with this tumour the percentage failure rate for tumour 'takes' is less than 10%.

PHARMACOKINETIC STUDIES

Five mg/kg of either free daunomycin or daunomycin bound to HPMA copolymer (POLYMER 1 only) was administered by tail vein injection to Wistar rats bearing subcutaneous implants of Walker 256 tumour. The total volume of intravenous injection was approximately 0.5 ml. At various times up to 24 hours thereafter animals were sacrificed by cervical dislocation. Blood was removed from the inferior vena cava, and plasma separated by centrifugation. Tissues of interest were dissected out immediately, washed twice in ice cold saline, blotted dry and frozen in liquid nitrogen to await analysis. The experiments were carried out on two separate occasions using 2-4 animals per time point on each occasion.

DRUG ANALYSIS

The determination of free daunomycin and its metabolites was performed using an established HPLC method (see chapter 4 for details).

The hydrophilicity of HPMA copolymer-daunomycin prevents its extraction into the organic phase. In addition, polymer bound daunomycin is only weakly fluorescent. Hence, to permit accurate quantitation of polymer-bound daunomycin the free drug must first be released from the conjugate. The glycosidic bond between the daunosamine ring and the aglycone moiety is relatively easily acid hydrolysed, releasing the free aglycone. A suitable hydrolysis protocol for quantitation of polymer-daunomycin was designed and validated by Dr. Len Seymour (CRC Polymer-controlled drug delivery group; Personal communication). This involves exposure of samples to HCl (final concentration 1M) at 85^{0}C for 20 minutes, an equal volume of tris-phosphate buffer (pH 9.5) was then added, followed by NaOH, volume adjusted to neutralise the acid. This was performed on all tumour samples obtained and results in release of approximately 100% of bound daunomycin. Subsequent extraction and processing was the same as for the free drug.

TUMOUR RESPONSE

Five animals in each of four groups were given (by tail vein

injection); saline as control, 5mg/kg of daunomycin, the same dose of daunomycin bound to HPMA copolymer by a biodegradable spacer (POLYMER 1) or the same dose of daunomycin bound to HPMA copolymer via a non-biodegradable linkage (POLYMER 2). The injections were performed on the same day as subcutaneous implantation of 0.5 cm^3 cubes of Walker tumour. Development of tumours was measured as above.

RESULTS

PHARMACOKINETIC STUDIES

The results of HPLC analyses are shown for plasma (figure 2), liver (figure 3), tumour (figure 4) and heart (figure 5). The results are shown as mean values +/- standard errors from two separate experiments. In general the levels of daunomycin metabolites parallel those of the parent drug (data not shown).

The results show a significant change in the pharmacokinetics of the HPMA preparation. The polymer bound drug was found in the tumour at greater concentrations than the free drug at all time points*. Tumour AUC (calculated by the log trapezoidal rule) was increased approximately 4-fold at 24 hours. There was also a reduction in the cardiac concentrations with the HPMA preparation suggesting the possibility of an improved therapeutic index in this model system.

Figure 6 details the results of tumour analyses both before and after processing by the acid hydrolysis method to release all the daunomycin which initially was polymer bound. The form of the curves implies the formation of a depot of HPMA copolymer-daunomycin within the tumour, followed by a slow release of free daunomycin into the tumour substance.

* more than three hours post injection.

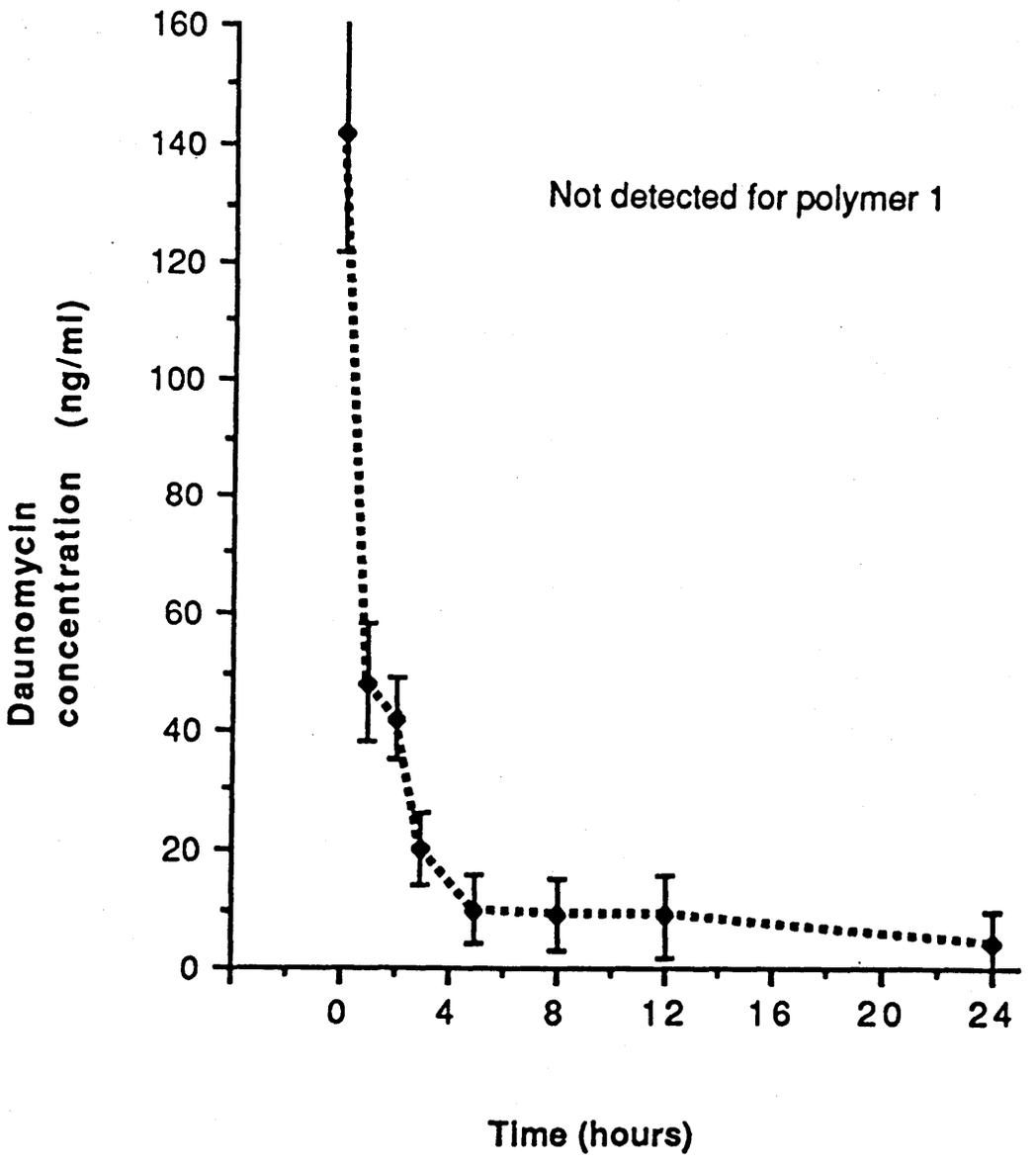


Figure 2: Plasma daunomycin concentration versus time following IV administration of daunomycin in solution or HPMA copolymer bound daunomycin

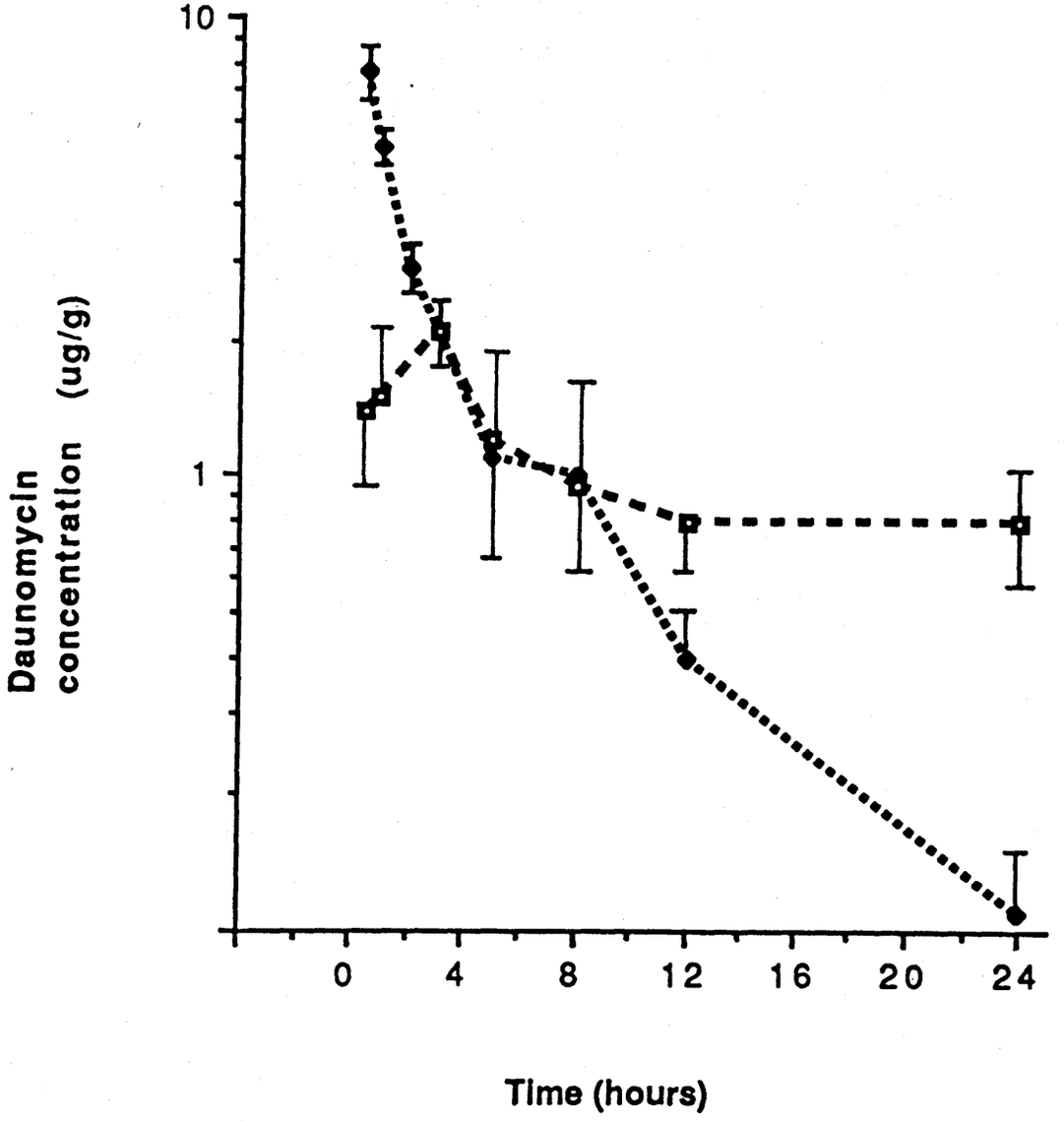


Figure 3: Liver daunomycin concentration versus time following IV administration of daunomycin in solution (filled diamonds) or HPMA copolymer bound daunomycin (open squares).

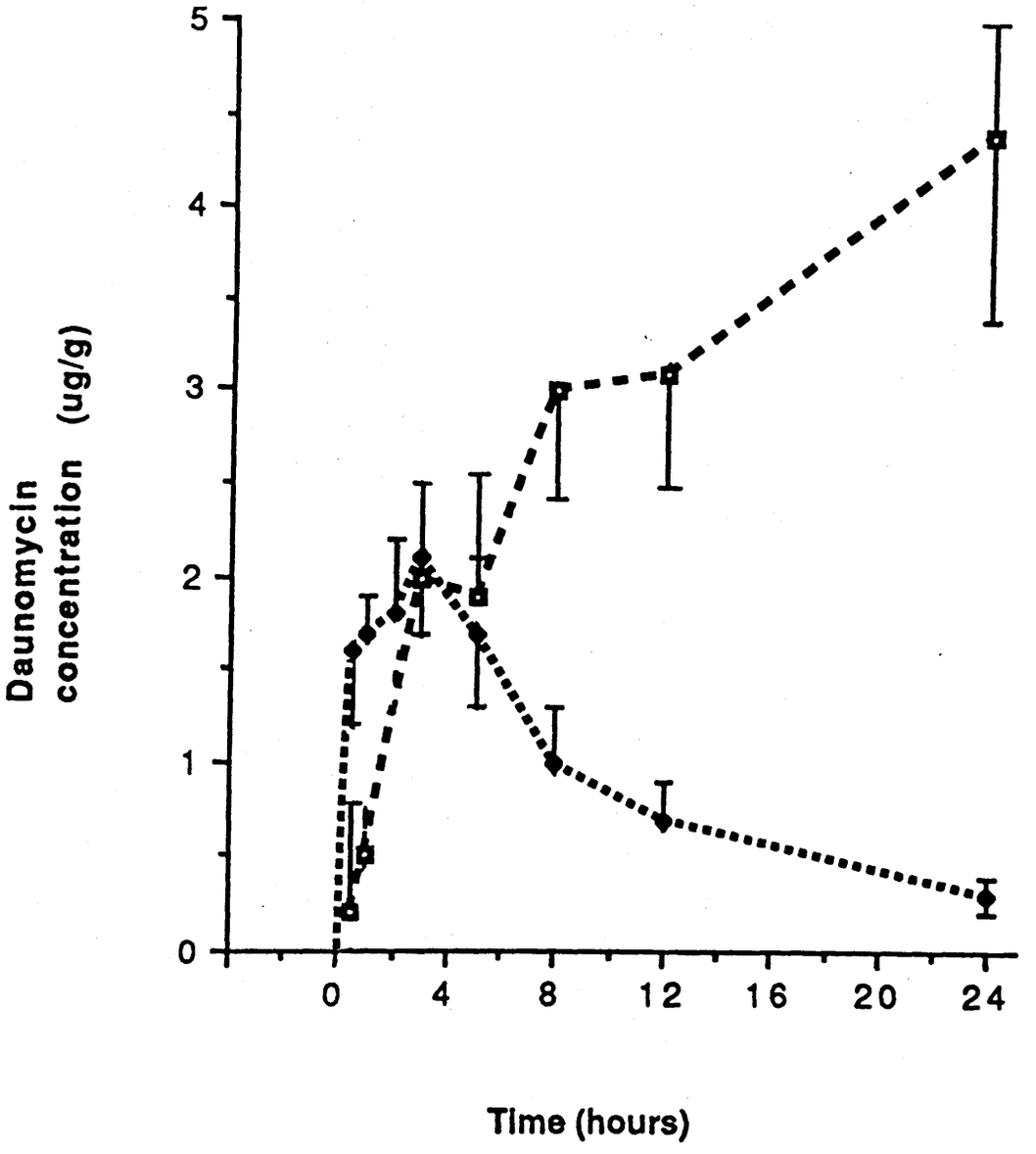


Figure 4: Tumour daunomycin concentration versus time following IV administration of daunomycin in solution (filled diamonds) or HPMA copolymer bound daunomycin (open squares).

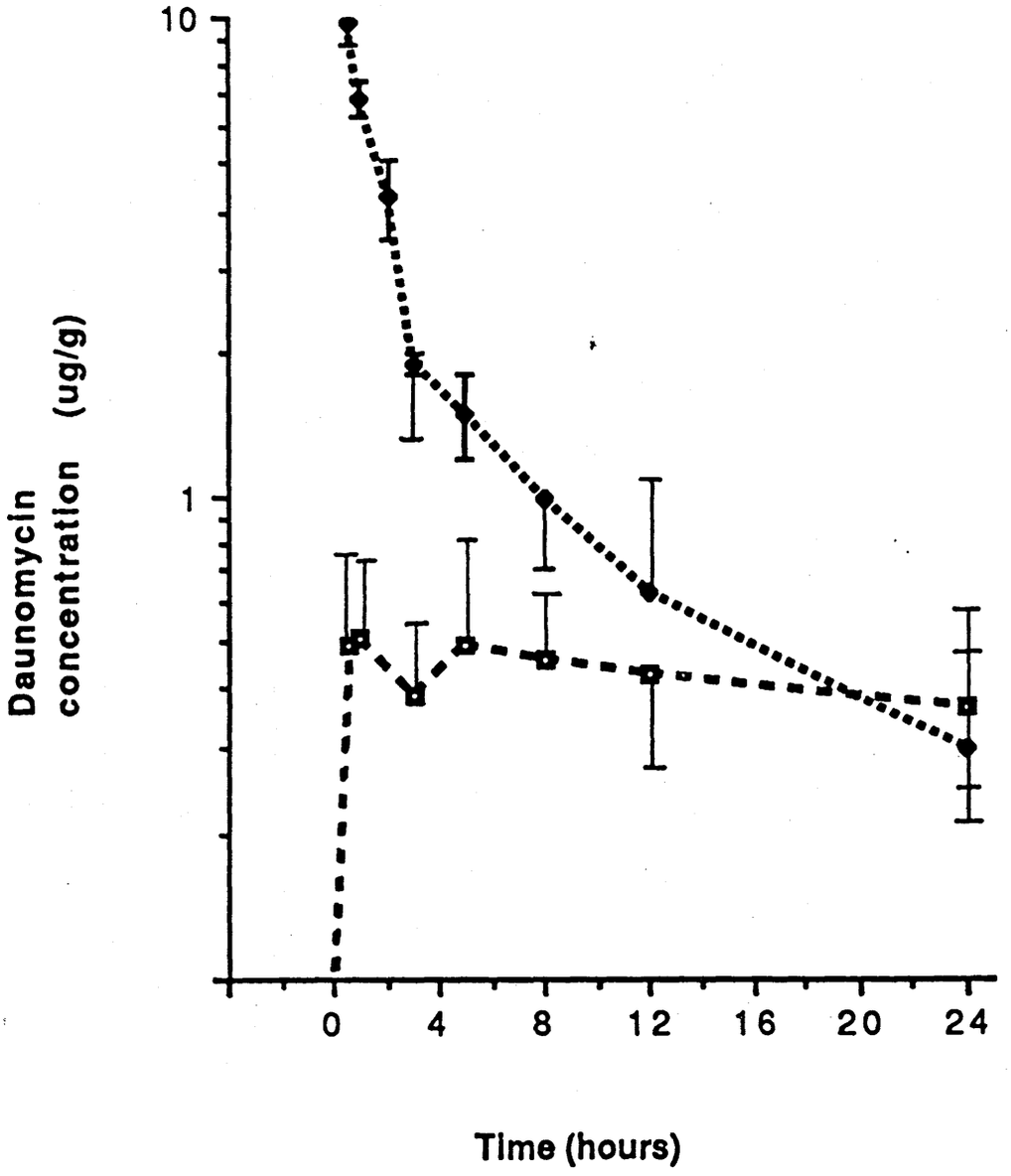


Figure 5: Heart daunomycin concentration versus time following IV administration of daunomycin in solution (filled diamonds) or HPMA copolymer bound daunomycin (open squares).

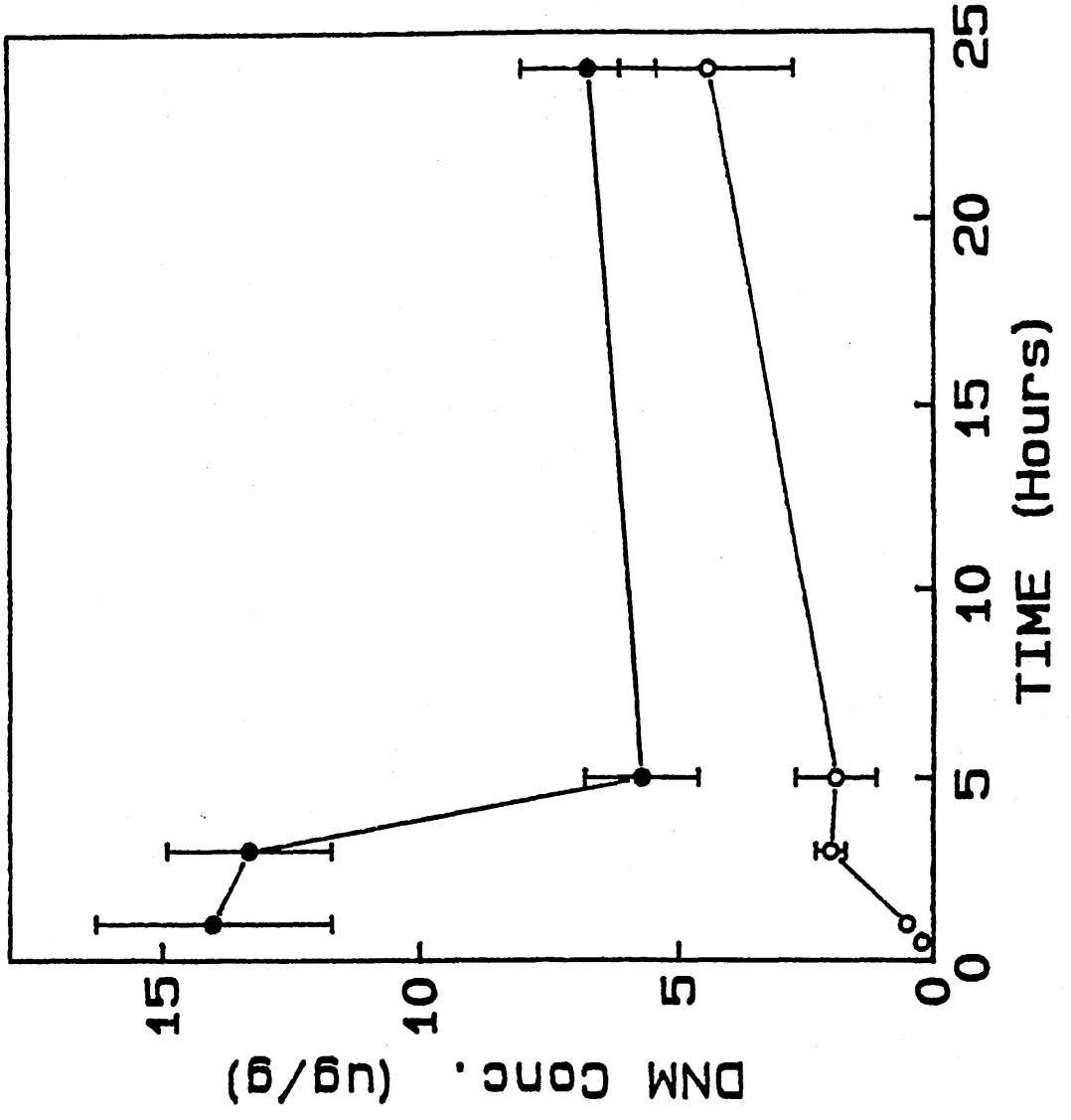


Figure 6: Tumour daunomycin concentration versus time following IV administration of HPMA copolymer bound daunomycin (with effect of addition of an acid hydrolysis step to the analytical methodology). KEY: Open circles= before hydrolysis, filled circles= after hydrolysis.

TUMOUR RESPONSE

Figure 7 shows the pattern of tumour growth in the four groups shown as the mean volumes +/- standard errors against time from passage. Animals were sacrificed before their tumour burdens became intolerable (>6 cm in diameter) which occurred from day 14 onwards. Thus in figure 8 the data are converted into a survival curve.

The only group showing a statistically significant growth delay (Mann-Whitney U test) was the group given the biodegradable polymer 1. In fact, 4/5 in this group were long term survivors (> 1 year so far), with no evidence of tumour. Only one other animal from any group survived and this was from the daunomycin group.

Although a control group using HPMA copolymer on its own has not been included in this series of experiments, no anti-tumour activity has been noted in any other system for HPMA alone.

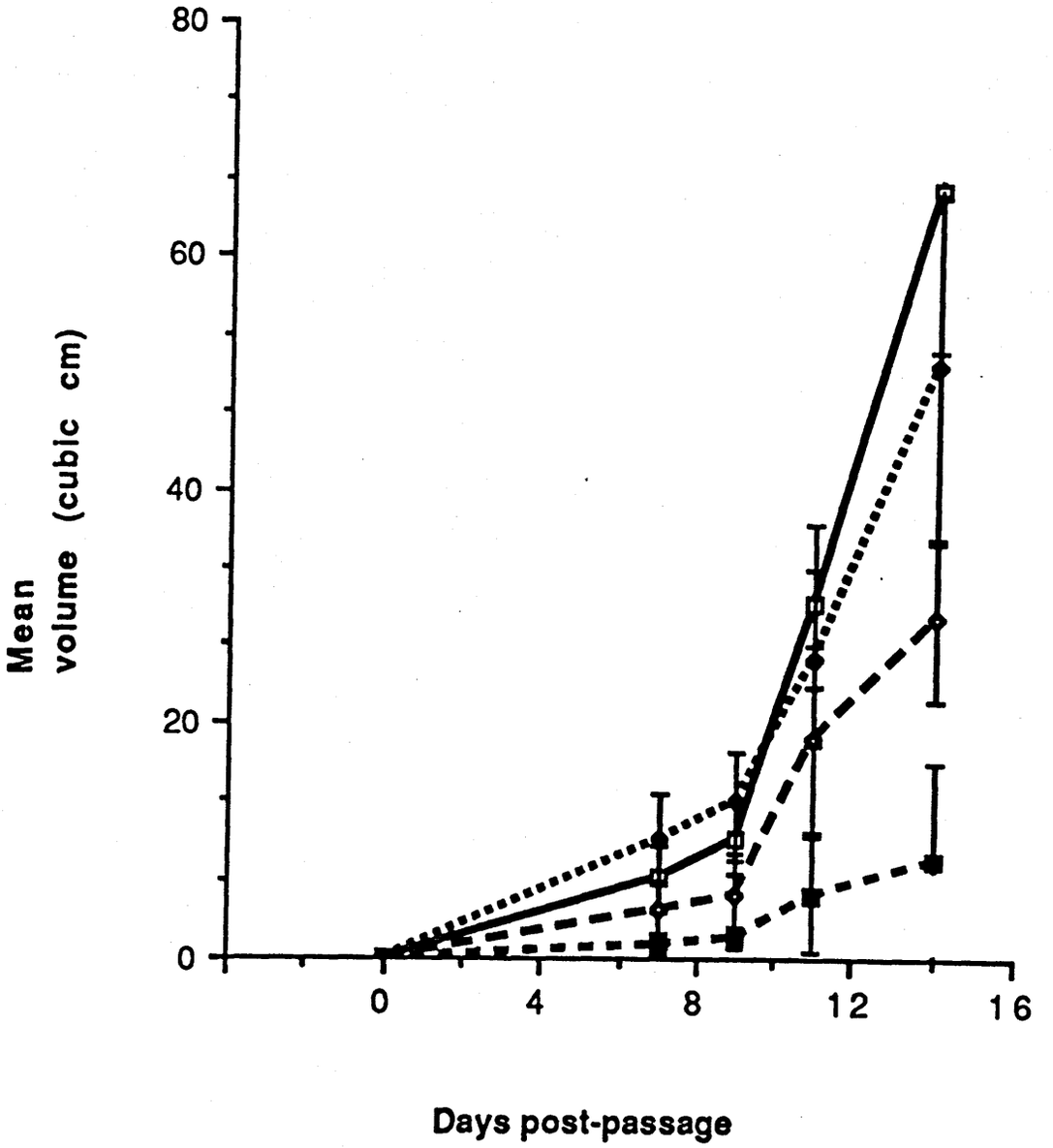


Figure 7: Growth of subcutaneously implanted Walker 256 carcinoma in untreated (control= open squares) rats, or following IV administration of daunomycin in solution (filled diamonds) HPMA copolymer 1 bound daunomycin (filled squares) or HPMA copolymer 2 bound daunomycin (open diamonds).

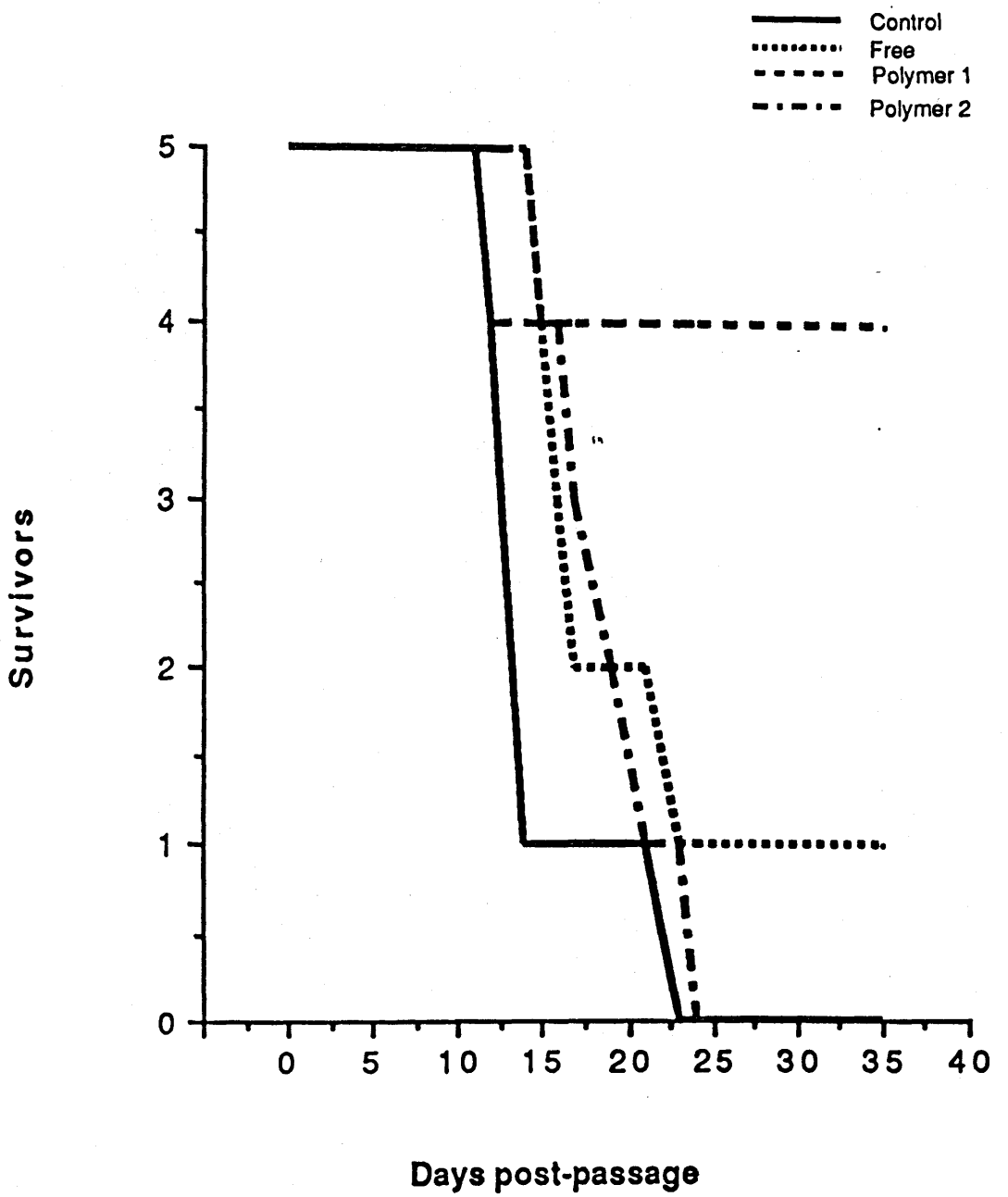


Figure 8: Survival of rats bearing subcutaneously grown Walker 256 carcinoma without treatment, or following IV administration of daunomycin in solution, HPMA copolymer (1) bound daunomycin or HPMA copolymer (2) bound daunomycin.

DISCUSSION

In this model system the use of HPMA copolymers to deliver daunomycin has resulted in a favourable modification of the pharmacokinetic behaviour of daunomycin. More drug reached the 'target' tumour tissue, and less reached the myocardium, suggesting that cardiotoxicity of the anthracycline maybe reduced. This has now been confirmed independently by Yeung et al (1989), who found the HPMA copolymer to be about four-fold less toxic than the native drug. The other major site of toxicity for the anthracyclines is the bone marrow, causing dose limiting myelosuppression at around 10 days following administration. We did not measure marrow uptake or monitor the haematology of the treated animals. However, in the tumour response study, the animals all died of tumour burden rather than drug toxicity, and in the long term survivors from the biodegradable polymer group there was no sign of haematological toxicity which one would expect if the bone marrow 'dosage' had been substantially increased in this group.

It is reasonable to suppose that tumour drug exposure as reflected by AUC should correlate with response to the drug. The pharmacokinetic changes observed in this study could thus explain the increase in therapeutic effect of the HPMA copolymer daunomycin conjugates. However, in addition to the quantitative changes in drug delivery, the time course of tumour drug exposure has been changed, with concentrations rising throughout

the 24 hour period in the HPMA group (see figure 4), and a slow release of drug from a depot of intact HPMA copolymer within the tumour (see figure 6). These qualitative changes in drug delivery may also be important for tumour response. The following chapter presents further data on this subject.

The effects of polymer 2 on tumour growth are worthy of comment. Statistically the growth rate for polymer 2 treated animals is not different from control. However, the impression is of a growth pattern which is intermediate between control and the active (biodegradable) polymer 1. This is difficult to explain as the drug theoretically should still be bound to HPMA within the lysosomes of non-biodegradable polymer 2 treated tumour cells. One possiblity is that even in this bound state the anthracycline is still able to exert a cytotoxic effect, perhaps by disrupting membranes as proposed by Tritton & Yee (1982).

The biological reason for selective delivery to tumour with this preparation is unclear. No specific targeting moiety was included in this particular formulation. The macromolecular nature of the HPMA copolymers means that cellular uptake must be through an endocytic mechanism. It is possible that at least some tumour types have an increased endocytic activity, resulting in a non-specific enhancement of uptake of macromolecules (Sehested et al, 1987), though the literature is unclear on this subject because of major methodological problems in measurement of endocytosis. It has been suggested that Walker

256 tumour contains fenestrated capillaries (Warren, 1970), which may allow preferential 'leakage' of the polymer into the tumour vascular bed. The experimental protocol used in the tumour response study may also account for some of the 'targeting', since the vascular damage caused by the surgical implantation of the tumour may have resulted in preferential deposition of polymer in that region. However, the pharmacokinetic experiments were performed in animals with established tumours, and similar anti-tumour responses have been independantly observed by other groups using established tumour masses (Duncan et al, 1989).

These encouraging preliminary results have been reproduced in other model systems. Clinical trials using a form of HPMA copolymer targeted to hepatocellular carcinoma and other forms of currently unresponsive tumours are planned for the near future under the auspices of the CRC Phase 1 trials committee.

CHAPTER 7

STUDIES ON POSSIBLE MECHANISMS OF ACTION OF HPMA COPOLYMER-ANTHRACYCLINE CONJUGATES AND RELEVANCE TO MULTI-DRUG RESISTANCE

INTRODUCTION

As stated in the previous chapter, the use of HPMA copolymer carriers for daunomycin results in substantial changes in pharmacokinetics and an enhanced therapeutic effect when compared with the free drug.

These two observations could be causally related; or the pharmacokinetic changes may be coincidental to a more fundamental change in cellular drug handling resulting in improved effect. It is possible that the conjugation itself enhances the cytotoxic effect of the daunomycin by changing the chemical reactivity of the anthracycline. Alternatively, the conjugate may in some way have the effect of increasing the tumour penetration of daunomycin (as discussed for surfactants in chapters 3 and 4). Data presented in this chapter attempts to explore these possibilities.

In clinical oncology a major problem is the development of resistance to chemotherapy and subsequent relapse. Ultimately patients may fail to respond to any therapy, with the appearance of a form of multiple drug resistance. The molecular biochemistry and pharmacology underlying this development is

a major area of research effort. In contrast to the clinical situation much of the molecular background to the development of in-vitro multi-drug resistance (MDR) is already well known. In this instance MDR is used to imply a broad pattern of cross-resistance to 'natural product' cytotoxics, such as the vinca alkaloids and the anthracyclines. MDR is usually a phenomenon induced in immortal cell-lines by serial passage in ever increasing concentrations of the cytotoxic agent. One of the major correlates with this type of resistance is the overexpression of a membrane component known as P-glycoprotein. The current state of knowledge is that this molecule functions as a membrane pump, causing efflux of drug, and hence protection from the cytotoxic effect (Gottesman & Pastan, 1988). The gene reponsible for the production of this protein has been identified and found in samples from some, but not all, resistant tumour types in humans (Fojo et al, 1987). If one accepts this hypothesis one way of circumventing MDR may be to present the drug in a different form (as a macromolecular conjugate) or by a different route (through the endosome-lysosome system). In collaboration with Mr. S. Wedge from the CRC Polymer Controlled Drug Delivery Group at Keele University, we therefore looked at a sensitive and MDR expressing resistant cell-line to test these possibilities.

Finally; if the enhanced therapeutic effect of HPMA copolymer daunomycin conjugates is solely due to the observed changes in pharmacokinetics, it should be possible to reproduce the

observed intra-tumour pharmacokinetic profile by different methodology and expect the same enhancement of therapeutic effect. This hypothesis has been tested by the crude methods of either increasing the intravenous dosage or using timed direct intra-tumoural injections of the native drug, and the results are further discussed in this chapter.

MATERIALS AND METHODS

IN-VITRO CYTOTOXICITY OF HPMA COPOLYMER DAUNOMYCIN CONJUGATES AGAINST MONOLAYER CULTURES OF L-DAN CELLS

L-Dan cells were grown in monolayers then exposed to graduated concentrations of daunomycin or HPMA daunomycin conjugates for 1 or 24 hours in two independent sets of experiments. A clonogenic assay was then performed on single cell suspensions prepared from the cultures. Each assay was performed in triplicate. The details of cell growth and the assay procedure are given in chapter 2.

IN-VITRO CYTOTOXICITY OF HPMA COPOLYMER ADRIAMYCIN CONJUGATES AGAINST L-DAN MULTICELLULAR TUMOUR SPHEROIDS

The methods of spheroid culture and details of the cytotoxicity assay employed are given in chapter 2. One series of experiments used a one hour drug exposure time. As this may be too short a time for penetration of the spheroid followed by endocytic uptake of the conjugates, a further series of experiments were performed with a drug exposure time of 72 hours. In this set of experiments the drug containing medium was replenished every 24 hours. Each assay was performed in triplicate with 16-20 spheroids in each treatment group.

IN-VITRO CYTOTOXICITY OF HPMA COPOLYMER DAUNOMYCIN CONJUGATES AGAINST SENSITIVE AND 'MDR' RESISTANT HUMAN OVARIAN CANCER CELL LINES

The cell-lines used in these experiments were the kind gift of Dr. R.F. Ozols (National Cancer Institute, Washington, USA). The parental 'sensitive' cell line was derived from an untreated patient with ovarian carcinoma. The 'resistant' line was derived from the parental line by serial passage in adriamycin; it exhibits a classical MDR phenotype and has been shown to express elevated levels of P-glycoprotein (Hamilton, Young & Ozols, 1984).

The cells were maintained in RPMI supplemented with bovine foetal calf serum 10 % and insulin 6 U/ml. The resistant line was routinely maintained in adriamycin but this was removed and cells allowed two passages prior to testing. Previous experiments (data not shown) confirm that this does not alter the pattern or degree of resistance of these cells. The 'MTT assay' as described by Carmichael et al (1979), and subsequently modified in our own laboratory was used (Plumb et al, 1989) for this set of experiments, because the assay is faster than the standard 'clonogenic' types. Cells are plated out at 1000 cells/well in flat bottomed, 96 well microtitre plates (Flow, UK), incubated at 37^{0}C for 48 hours and then a series of drug concentrations added in fresh culture medium. Fresh drug containing medium was added again after 24 hours incubation, to give a continuous drug exposure of 48 hours. Plates were then

fed with fresh medium for each of the following five days. On the eighth day cells are fed with medium plus 10 mM Hepes buffer (which minimises differences in absorbance) and then 50 ul of a 5mg/ml solution in PBS of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] is added to each well. The plates are then incubated for 4 hours at 37^{0}C whilst protected from light by an aluminium foil wrapping. The medium is then removed and the insoluble formazan crystals (formed by the reduction of MTT by living but not dead tumour cells) dissolved in 200 ul of DMSO (Sigma Chemicals) plus Sorensen's glycine buffer (20 ul of a 0.1 M glycine/0.1 M NaCl solution, equilibrated to pH 10.5 with 0.1 M NaOH). Finally the absorbance of light at 570nm was measured using a microtitre plate reader linked to a dedicated micro-computer system (Bio-rad, UK) The assay was performed in duplicate for daunomycin or in triplicate for HPMA-daunomycin conjugates.

PHARMACOKINETICS AND THERAPEUTIC EFFECT OF DIRECT INTRA-TUMOURAL INJECTIONS OF DAUNOMYCIN

Walker 256 tumour was subcutaneously implanted into Wistar rats, as described in the previous chapter. Daunomycin (May and Baker, Dagenham, England) was dissolved in 0.9% Sodium chloride immediately prior to use. Whilst restraining the animals (without anaesthetic) an appropriate volume of drug solution was injected using a hypodermic syringe and 18g needle into the

centre of the tumour mass. Using data derived from control experiments on the intra-tumoural half-life of daunomycin it was calculated that three intratumoural doses at 4 hour intervals would closely simulate the kinetic profile using intravenous HPMA copolymer daunomycin conjugates (see figure 4 in the previous chapter). Groups of three animals each were sacrificed by cervical dislocation at 1, 4, 8, 12 and 24 hours following the first dose. The tumours were rapidly dissected out, washed twice in ice cold saline, and frozen in liquid nitrogen for subsequent analysis.

The anti-tumour effect of this regimen was assessed by serial tumour volume measurements and overall survival in 6 rats given three intratumoural doses of 1.67 mg/kg each, compared with 6 rats given 5 mg/kg as an IV bolus.

THERAPEUTIC EFFECT OF HIGHER DOSE INTRAVENOUS DAUNOMYCIN

The improvement in tumour AUC was of the order of four-fold when HPMA copolymer conjugates were used in comparison to daunomycin alone. Therefore a group of 10 Walker tumour bearing rats were given 20 mg/kg daunomycin (i.e. 4 times the previously used dosage) as an intravenous bolus, in an attempt to improve the anti-tumour efficacy. This dose is above the LD_{10}, and as expected some animals died almost immediately post injection. In order to maintain a reasonable size group for tumour response estimations it was necessary to use 16 animals in total, but the

analysis only includes those animals that survived for at least 24 hours following dosing.

RESULTS

IN-VITRO CYTOTOXICITY AGAINST MONOLAYER CELLS

Figure 1 shows the clonogenic survival versus daunomycin dosage, for a one hour drug exposure to daunomycin or HPMA copolymer daunomycin conjugates. The conjugated drug appears to have little or no cytotoxicity over the tested concentration range. A similar result was obtained following 24 hour drug exposures (data not shown).

IN-VITRO CYTOTOXICITY AGAINST MULTICELLULAR TUMOUR SPHEROIDS

The results of 1 hour exposure of these spheroids to adriamycin at various concentrations are given in chapter 2 (figure 14), from which the growth delay data shown in figure 2 are derived. Following one hour exposure to HPMA copolymer adriamycin conjugates some growth delay was evident, but only at substantially higher concentrations of adriamycin.

Exposure of spheroids for 72 hours failed to produce reliable data, due to technical problems. The cultures invariably became infected; presumably due to the repeated changes of medium and the necessity for an additional transfer of intact spheroids following the three day drug exposure. As a consequence the spheroids failed to return to a regrowth pattern parallel to control, and failed to reach 10 times their original volume,

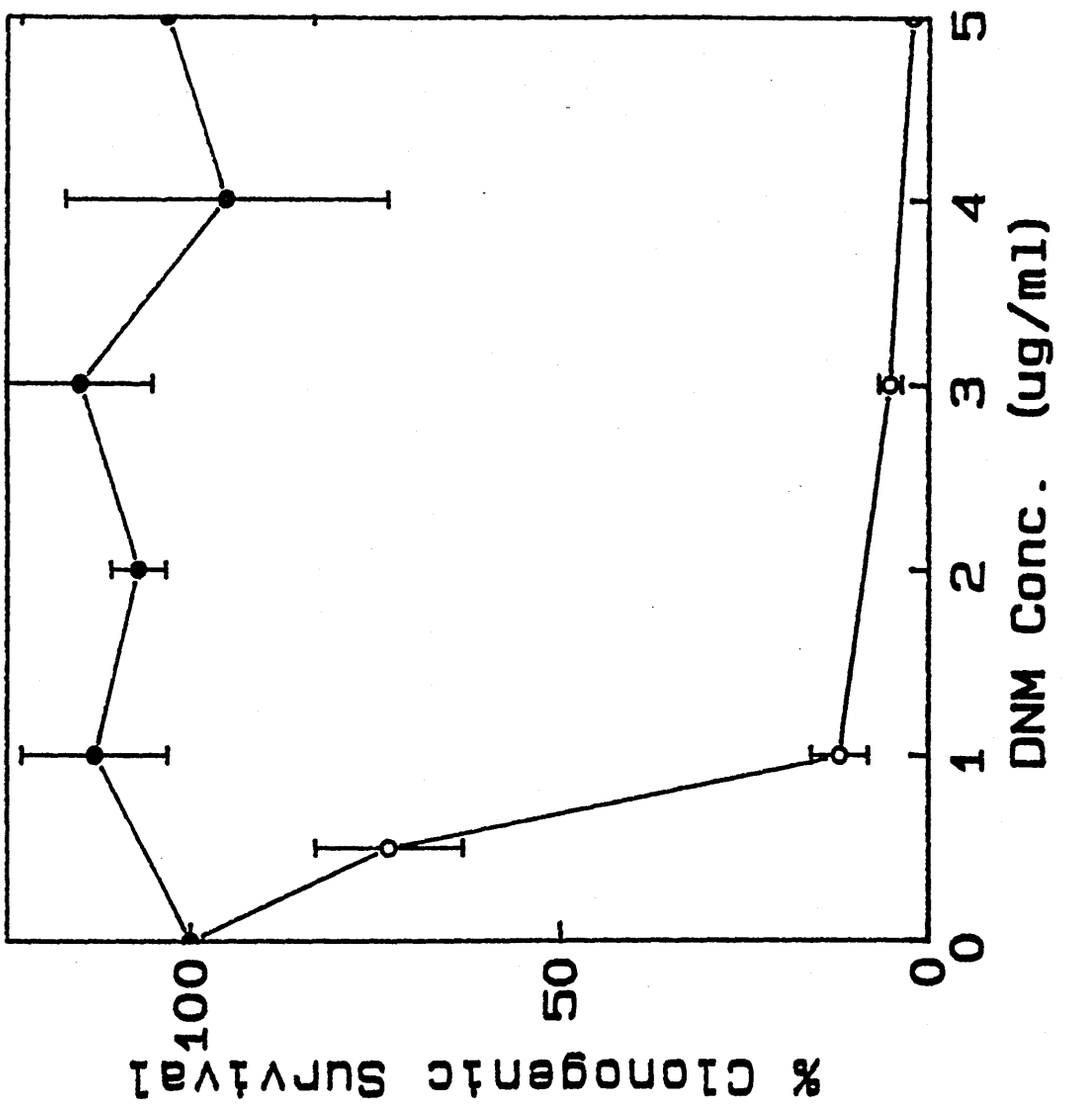


Figure 1: Dose survival curve for L-Dan cells exposed to free daunomycin (open circles) or HPMA-copolymer bound daunomycin (filled circles) in monolayer cultures

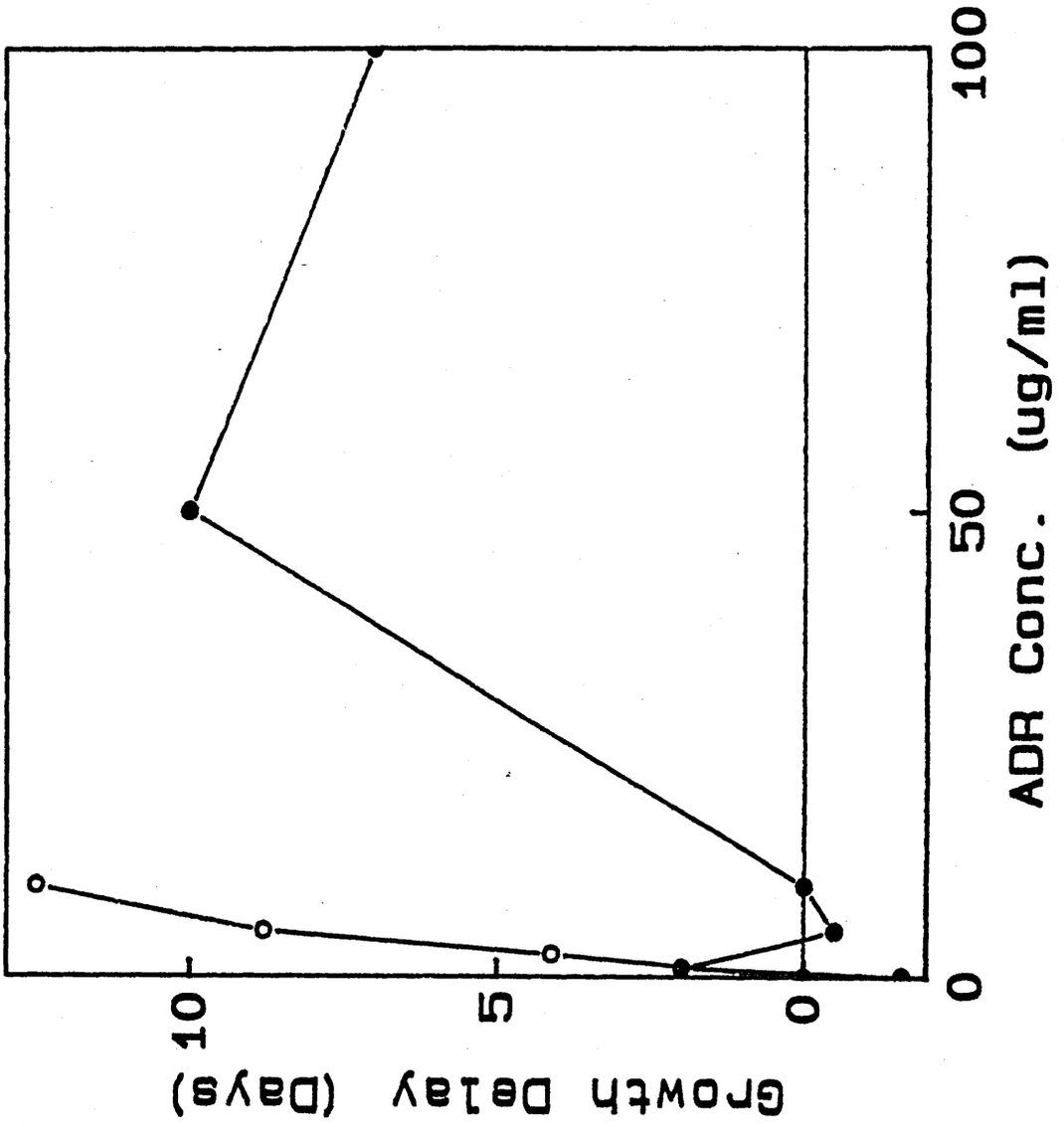


Figure 2: Derived growth delay for L-Dan spheroids exposed to free adriamycin (open circles) or HPMA-copolymer bound adriamycin (filled circles).

thereby invalidating two of the assumptions implicit in the mathematical handling of these data. Nevertheless, it was clear that the conjugated form was less active than the drug alone.

IN-VITRO CYTOTOXICITY AGAINST SENSITIVE/RESISTANT CELLS

Figure 3 shows the mean optical density (directly proportional to viable cell number) +/- the standard error versus the log of daunomycin dosage for both cell lines. Both curves show the expected sigmoid pattern. Values of ID50 are around 0.4 ng/ml for A2780 and around 500 ng/ml for 2780AD; giving a relative resistance factor of (500/0.4) about 1250.

FIgure 4 shows the same data for the cell lines when exposed to HPMA copolymer daunomycin conjugates. It is obvious that both curves are shifted to the right (confirming the rather poor in-vitro results observed with L-Dan), but that the relative separation of the curves remains unchanged. Values of ID50 in this case are approximately 200 ng/ml for A2780 and around 200,000 ng/ml for 2780AD; giving a resistance factor of about 1000.

If the HPMA copolymer conjugates were able to circumvent the resistance of 2780AD, one would expect the two curves to become closer (with a left hand shift in the curve for 2780AD). Therefore it appears that there was no modification of resistance in this model.

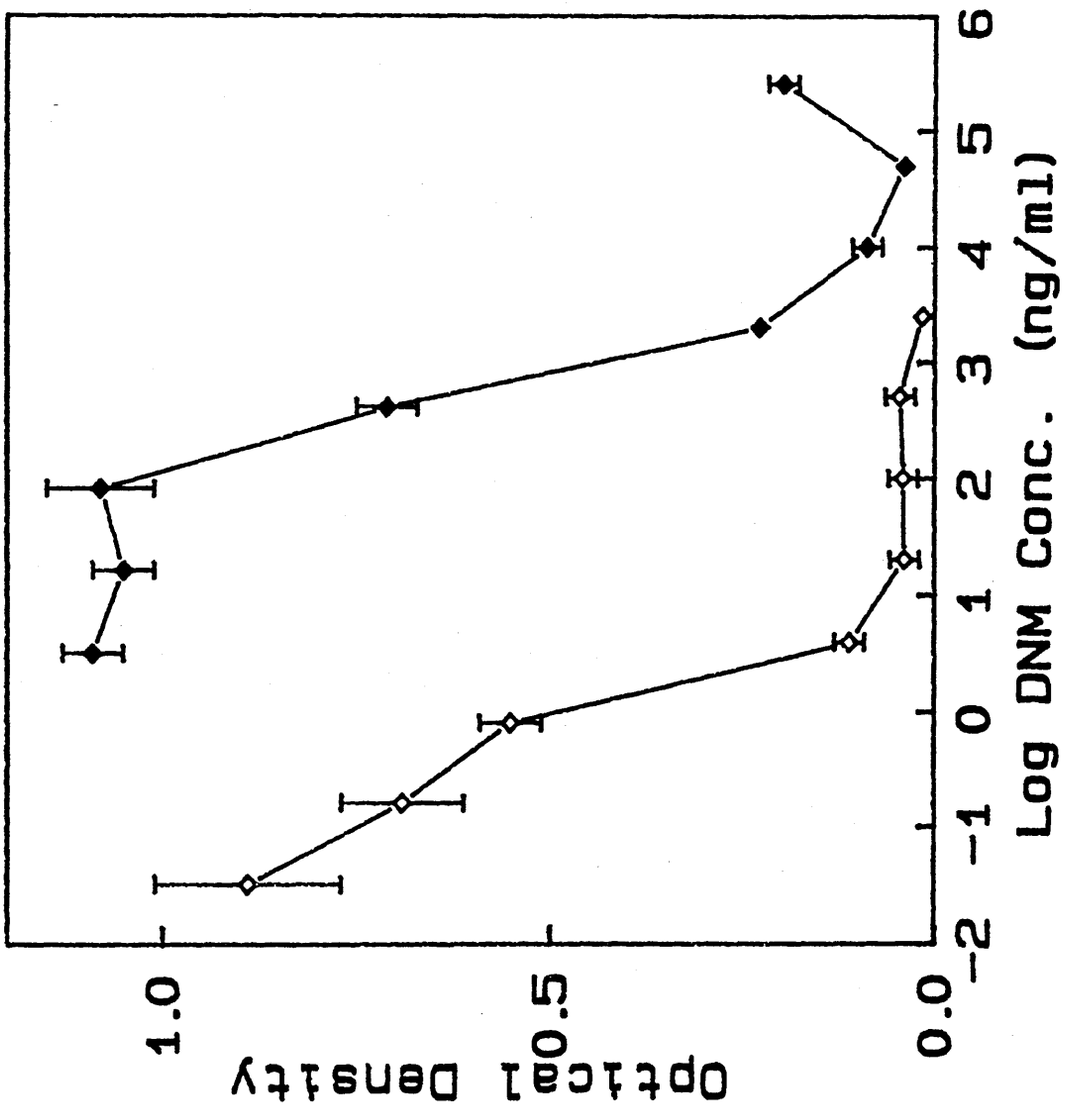


Figure 3: Optical density versus log daunomycin concentration for sensitive A2780 ovarian cancer cells (open diamonds) and for resistant 2780 AD cells (filled diamonds) exposed to free daunomycin in monolayer cultures.

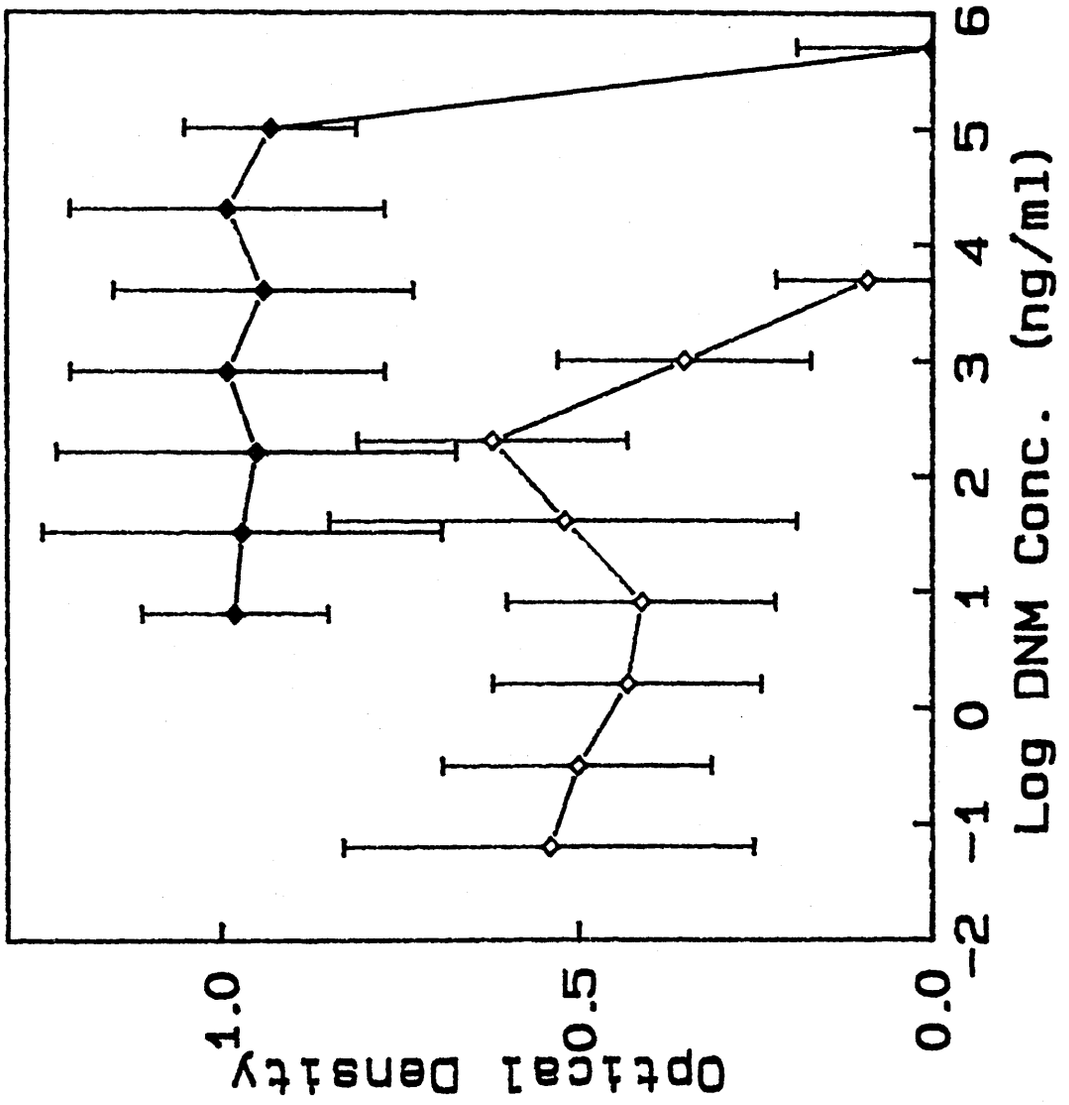


Figure 4: Optical density versus log daunomycin concentration for sensitive A2780 ovarian cancer cells (open diamonds) and for resistant 2780 AD cells (filled diamonds) exposed to HPMA-copolymer bound daunomycin in monolayer cultures.

INTRA-TUMOURAL INJECTIONS OF DAUNOMYCIN

The results of tumour daunomycin concentrations versus time are given in figure 5. Each point is the mean +/- standard error from three estimations. The line is a computer generated best fit to the data, using an in-house fitting program based on the least squares method (r=0.98). Although the form of the curve is similar to that with HPMA copolymer daunomycin, the absolute values are approximately 10 fold higher using direct intra-tumour injections. This is reflected in a change in AUC (measured by the log trapezoidal rule) from 71 ug/g.h for the polymer to 1027 ug/g.h for the intra-tumoural daunomycin. Despite comfortably exceeding the tumour drug levels acheivable by the HPMA-daunomycin, this route failed to result in enhancement of anti-tumour effect as shown in figure 6.

HIGH DOSE DAUNOMYCIN

Assuming linearity of pharmacokinetics a four-fold increase in drug dosage should result in a four-fold increase in AUC. However, as shown in figure 7 this increase in dosage to 20 mg/kg also failed to enhance anti-tumour efficacy in this model system.

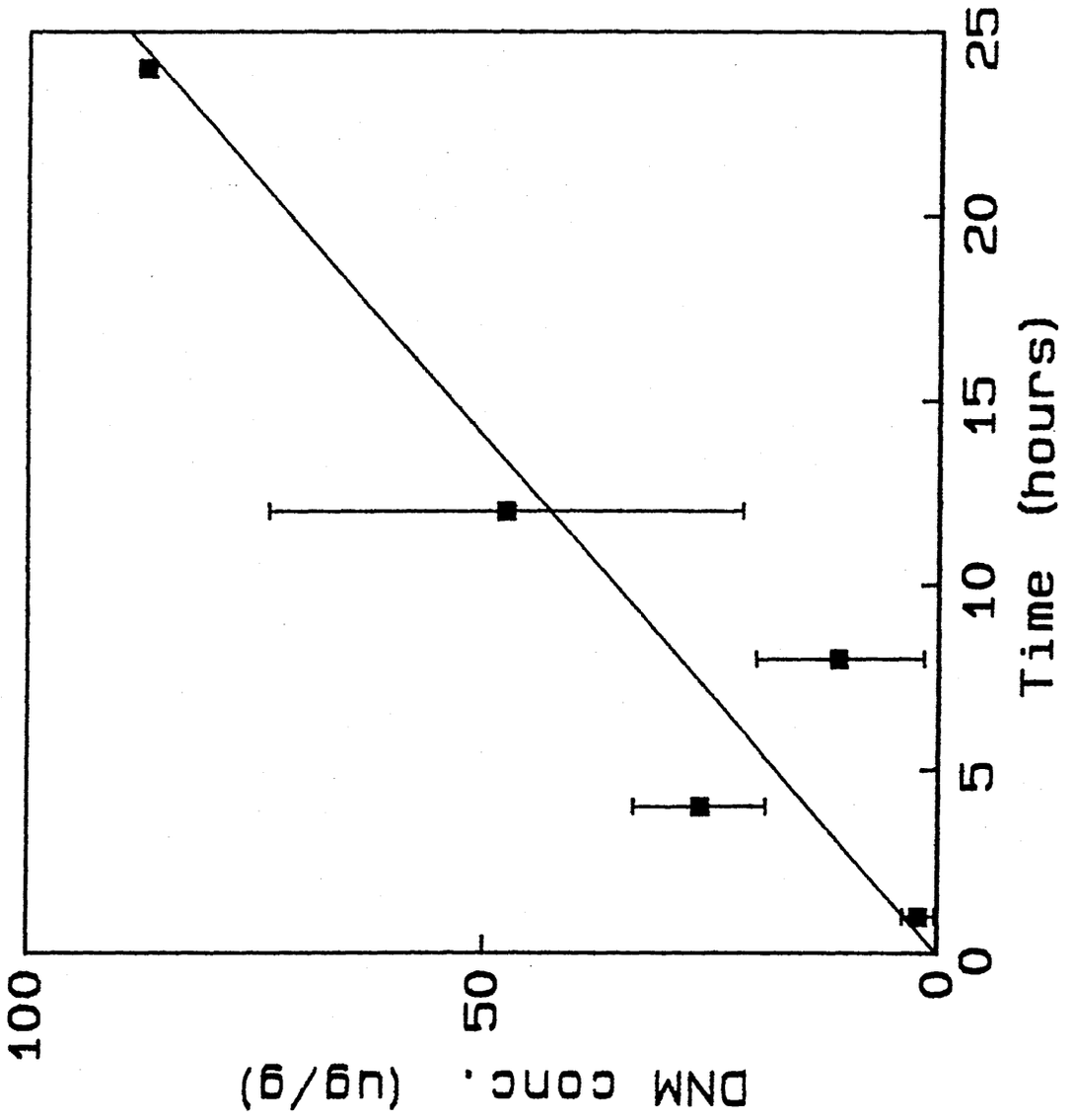


Figure 5: Tumour daunomycin concentration versus time following three intra-tumoural injections of 1.67 mg/kg of free daunomycin at 4-hourly intervals.

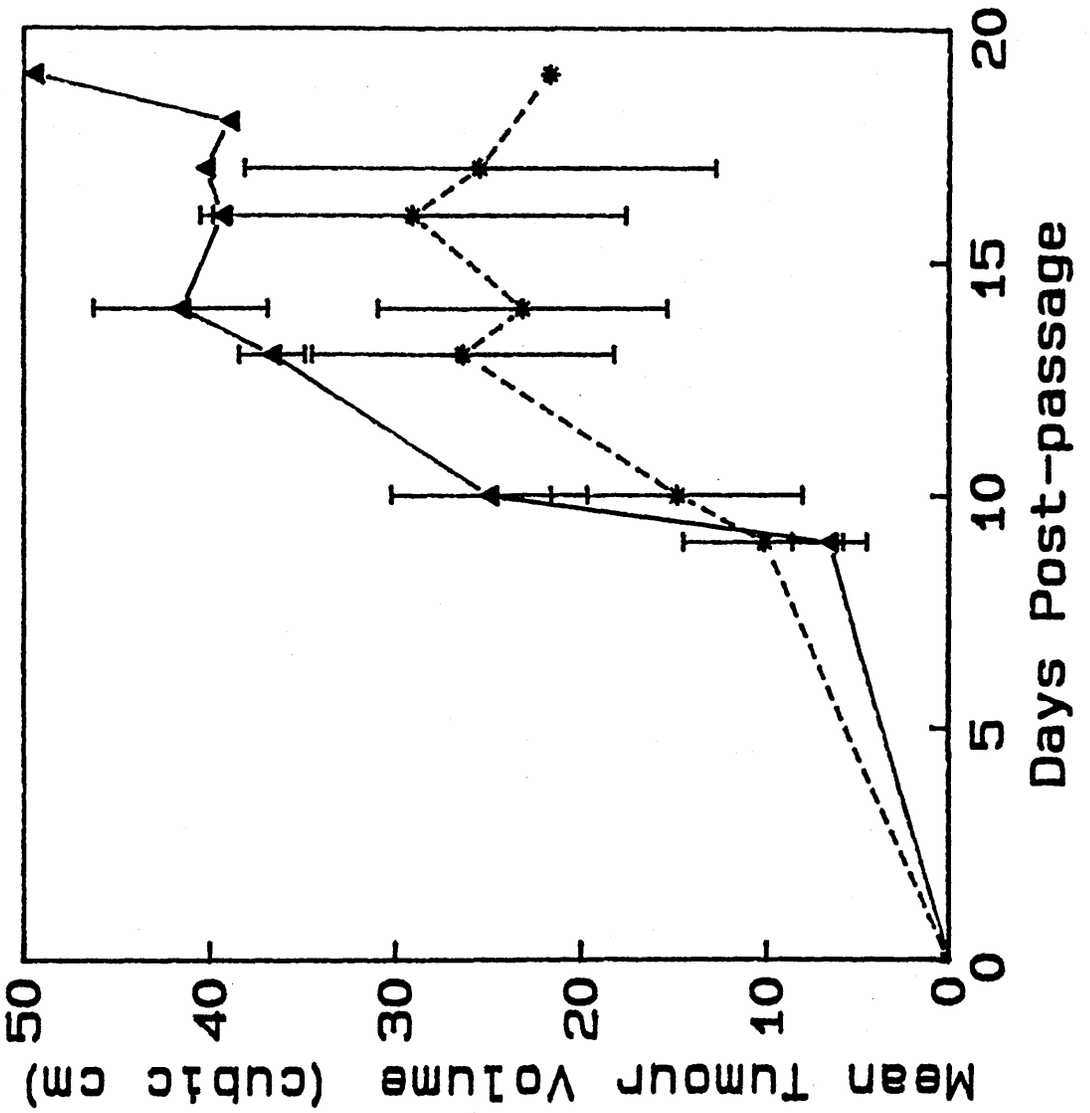


Figure 6: Growth of subcutaneously implanted Walker 256 carcinoma in rats given a single 5 mg/kg intravenous bolus injection of daunomycin (stars) or three intra-tumoural injections of 1.67 mg/kg of free daunomycin at 4-hourly intervals (filled triangles).

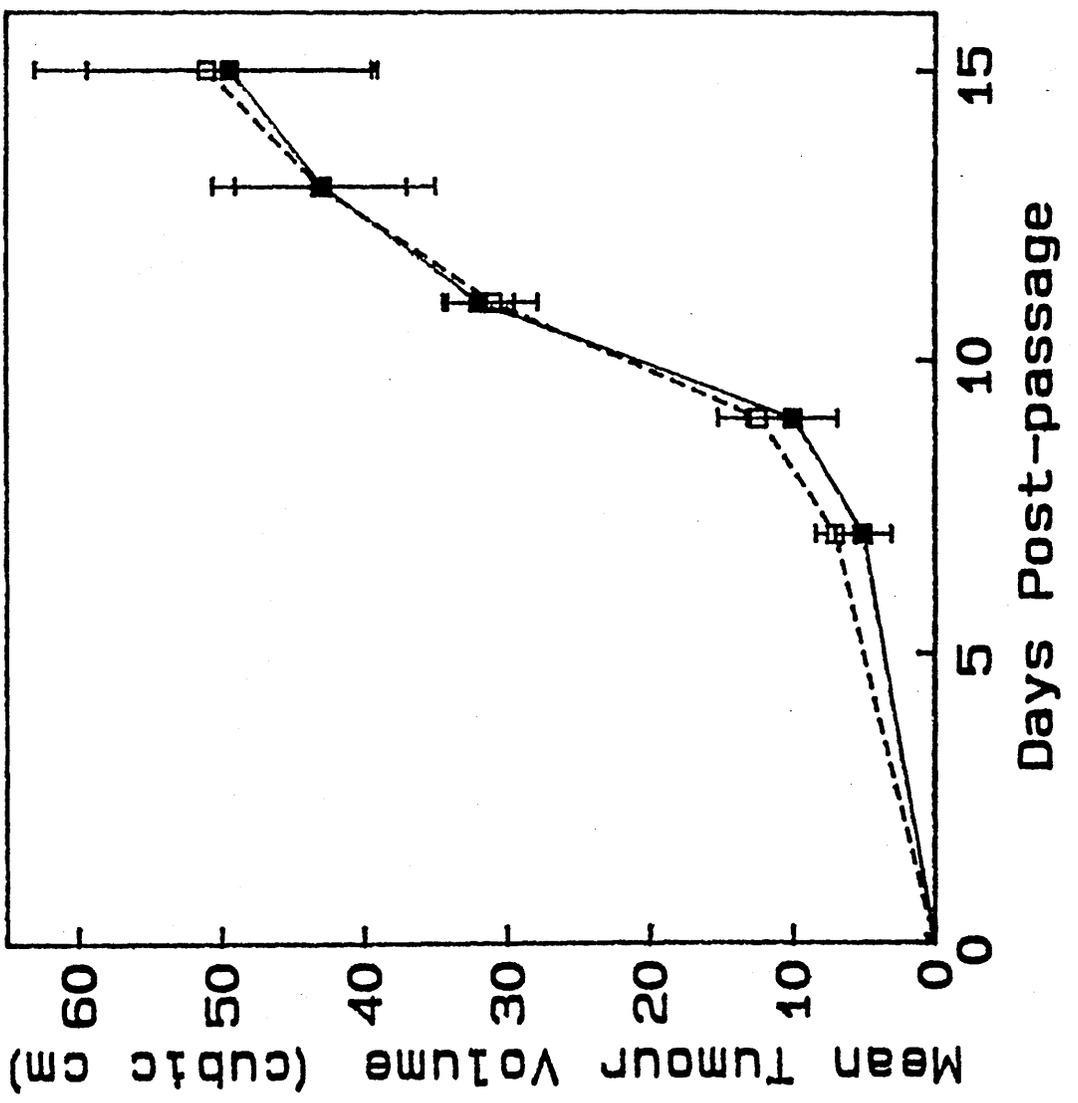


Figure 7: Growth of subcutaneously implanted Walker 256 carcinoma in rats given 5 mg/kg (open squares) or 20 mg/kg (filled squares) of free daunomycin given as a single IV injection.

DISCUSSION

The relative lack of in-vitro activity for HPMA copolymer daunomycin conjugates was not unexpected, and similar results have been obtained with other macromolecular carriers. It is clear from the spheroid experiments that cytotoxicity is present in-vitro if sufficient dosages are employed.

The bond between the polymer backbone and the daunomycin can only be cleaved by lysosomal enzymes, which requires that the conjugates are first internalised by endocytosis. The diffusion of these large molecules into a multicellular spheroid would be limited, and slow. It is not surprising that no growth delay was observed after a short drug exposure. In the previous chapter, the slight anti-tumour effect of a non-biodegradable polymer might have been a result of membrane activity of bound daunomycin (Tritton & Yee, 1982). The data in this chapter would seem to negate this hypothesis.

Other drug carriers have been shown to overcome drug resistance to methotrexate when the resistance was due to a transport defect (Ryser & Shen, 1980). It appears that the MDR phenotype expressed by 2780AD cells cannot be altered by the use of HPMA copolymer anthracycline conjugates. However, it should be noted that the degree of resistance exhibited by these cell-lines is much greater than can be demonstrated in patient derived material (where a resistance factor of less than 10 is

the norm). This extreme degree of resistance may have overwhelmed any more subtle modifications which may have been made by this form of lysosomotropic treatment. Therefore, it is intended to repeat this series of experiments using cell lines with less extreme resistance characteristics.

It would be possible to explain the increase in therapeutic efficacy of HPMA-daunomycin purely in terms of beneficial changes in pharmacokinetic handling, as demonstrated in chapter 6. However, despite reproducing the form, and exceeding the quantitative changes, by direct intra-tumoural injection of drug, we were unable to reproduce the therapeutic effect. The measurement of tumour drug levels is a crude estimation of drug delivery, as it does not take account of the cellular distribution of the drug. It is possible that the specific delivery of drug into the lysosomes is the important factor, and this is unlikely to be the normal route of intracellular entry of native anthracyclines.

In conclusion, in vitro studies to date indicate that changes in whole tissue penetration are not relevant, but the effect of HPMA copolymer presumably is explained by enhanced cellular uptake via the lysosomal pathway. Further studies using other tumour models, i.e. human tumour xenografts, are necessary, prior to clinical trials of this very promising avenue for drug delivery.

CHAPTER 8

CONCLUDING REMARKS AND FUTURE PROSPECTS

It is clear that the efficacy, and at least by implication the therapeutic index, of existing anthracyclines can be improved by the use of various synthetic drug carrier systems.

The simplistic approach of co-administration of a penetration enhancing agent such as a surfactant was found to be unhelpful, but perhaps this may be more logically applied to the setting of regional chemotherapy where high local concentrations can be achieved with simultaneous high drug concentrations in the vicinity of the tumour. Penetration enhancers may yet find a role in intraperitoneal therapy or in isolated limb perfusion techniques.

These remarks may also apply to those carriers which aim to improve whole tumour penetration such as non-ionic surfactant vesicles which could, for example, be administered intraperitoneally to form a 'depot' of drug. The demonstration of a degree of tumour targeting using NIVs was a promising, if somewhat unexpected observation. Perhaps the physico-chemical properties of the NIVs are more important for tumour targeting than the constituent molecules i.e. the nature of the vesicle forming lipid may not be critical. Further work is necessary to define the optimal chemical and physical properties of NIVs, and

in the light of the problems encountered with inactivation of entrapped drug, this should be a particular focus.

Other systemic carriers of more potential are those which may improve therapeutic index by reducing uptake in normal tissue and / or improving uptake in tumour. This seems to be the case for both liposomes and HPMA copolymer conjugates. One could hypothesise that a common mechanism involving increased tumour endocytosis may be responsible for the results obtained in both cases. It is possible that this increase in endocytosis is a peculiarity of certain animal tumour models, so it would be important in future studies to include human tumour xenografts to exclude this possibility and confirm the results found here. Possibly the constituents or some physico-chemical property of the carrier actually causes the increase in endocytosis, such as when macrophages encounter particulate matter. In addition, there appears to be a change in the pharmacokinetics of the anthracyclines using these carriers to a more 'slow release' preparation. This may also be involved in the improved efficacy as can be demonstrated by prolonged intravenous infusion of anthracyclines, so even if human tumours do not exhibit increased endocytosis, all is not lost. However, the studies on the mechanism of action of HPMA copolymer conjugates suggest that this is not a complete explanation of the benefits of at least that carrier system.

The diverse biochemical and biophysical properties of drug carriers means that the possible changes in size, chemistry,

charge, etc. are almost endless. Future development work particularly with an emphasis on improved tumour targeting is justified. The history of liposome development illustrates the likey path for drug carriers; initial optimism, followed by scepticism and a realisation of the potential problems, and finally a concerted research effort to define a clinical role.

The liposomes used in this work are currently in early clinical trials with interesting effects, such as a diminution in alopecia and bone marrow toxicity; the results in terms of therapeutic efficacy are eagerly awaited. HPMA copolymers are at an earlier stage of development but plans exist to perform clinical trials, probably involving patients with Hepatoma because some formulations show extremely efficient targeting to hepatocytes.

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ACTIVITY OF N-(2-HYDROXYPROPYL)METHACRYLAMIDE COPOLYMERS CONTAINING DAUNOMYCIN AGAINST A RAT TUMOUR MODEL

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INTRODUCTION:

Many drug delivery or 'targeting' systems have been developed with differing degrees of success in improving therapeutic efficacy. Polymeric drug carriers based on water soluble non-immunogenic copolymers of N-(2-hydroxypropyl)methacrylamide [HPMA] have been developed in recent years by Duncan et al [1,2]. This group have shown that it is possible to covalently bind drugs to these carriers so that the polymer-drug linkage is stable in the bloodstream [3,4,5], but can be cleaved inside the cell by lysosomal cysteine proteinases [6,7]. Theoretically this could change drug effect in two fundamental ways:-

1) Modification of pharmacokinetics.

2) Delivery to specific cell populations i.e. 'targeting' to tumours (with enhanced uptake through increased endocytic activity [8]).

We present data to support both of these mechanisms using HPMA copolymers as a delivery system (for daunomycin) in a rat tumour model.

MATERIALS AND METHODS:

SYNTHESIS:

The HPMA copolymer-daunomycin conjugates were synthesised using a two step procedure [1,2]. In the first step a reactive copolymer of HPMA with methacryloylated oligopeptide p-nitrophenyl ester was synthesised (polymer precursor). To the latter, daunomycin was bound by aminolysis.

PHARMACOKINETICS,

Five mg/kg of either free daunomycin or daunomycin bound to HPMA coplymer (polymer 1 only) was administered by tail vein injection to Wistar rats bearing subcutaneous implants of Walker 256 tumour. At various times up to 24 hours thereafter animals were sacrificed by cervical dislocation. Blood was removed and plasma separated by centrifugation. Tissues of interest were dissected out immediately, blotted dry and frozen in liquid nitrogen to await analysis. The determination of free daunomycin (and its metabolites) was performed using an established HPLC method [9]. The experiment was carried out on two separate occasions using 2-4 animals per time point on each occasion.

Footnote; Abbreviations used Gly = glycine
Phe = phenylalanine
Leu = leucine
AUC = area under concentration-time curve
HPMA = N-(2-hydroxpropyl)methacrylamide

TUMOUR RESPONSE,

Five animals in each of four groups were given (by tail vein injection); saline as control, 5mg/kg of free daunomycin, the same dose of daunomycin bound to HPMA copolymer by a biodegradable spacer (Gly-Phe-Leu-Gly,POLYMER 1) or the same dose of daunomycin bound to HPMA copolymer via a non-biodegradable linkage (Gly-Gly,POLYMER 2). The injections were performed on the same day as subcutaneous implantation of 0.5 cm^3 cubes of Walker tumour. Subsequent tumour growth was measured bi-dimensionally by calipers every 2-3 days and the measurements converted to a volume assuming spherical geometry.

RESULTS:

PHARMACOKINETICS,

The results of HPLC analyses are shown for plasma (FIG. 1a), liver (FIG. 1b), tumour (FIG. 1c) and heart (FIG. 1d). The results are shown as mean values +/- standard errors from two separate experiments. In general levels of daunomycin metabolites parallel those of the parent drug (data not shown).

The results show a significant change in the pharmacokinetics of the HPMA preparation. The polymer-bound drug was found in the tumour at greater concentration than the free drug at all time points. Tumour AUC was increased approximately 4-fold at 24 hours. There was also a reduction in the cardiac concentrations with the HPMA preparation suggesting the possibility of an improved therapeutic index in this model system.

TUMOUR RESPONSE,

FIG. 2a shows the pattern of tumour growth in the four groups shown as the mean volumes +/- standard errors against time from passage. Animals were sacrificed before their tumour burdens became intolerable (>6 cm in diameter) which occurred from day 14 onwards. Thus in FIG. 2b the data are shown converted into a 'survival curve'.

The only group showing a statistically significant growth delay (Mann-Whitney U test) was the group given the biodegradable polymer 1. In fact 4/5 animals in this group were long term survivors (>120 days so far), with no evidence of tumour. Only one other animal from any group survived and this was in the free daunomycin group.

DISCUSSION:

In this model system the use of HPMA copolymers to deliver daunomycin has resulted in a favourable modification of the pharmacokinetic behaviour of daunomycin. More drug reached the 'target' tumour tissue, and less reached the myocardium, suggesting that cardiotoxicity of the anthracycline may be reduced. The other major site of toxicity for the anthracyclines is the bone marrow, causing dose limiting myelosuppresion [4]. We did not measure marrow uptake or monitor the haematology of the treated animals. However, in the response study,the control animals all died of tumour burden rather than drug toxicity, and in the long-term survivors from the biodegradeable polymer group there was no sign of haematological toxicity which one would expect if the bone marrow 'dosage' had been substantially increased. These pharmacokinetic changes have been shown to result in enhanced anti-tumour activity in this model system. It is interesting that the non-biodegradable polymer group also seemed to show some growth delay though this did not reach statistical significance.

The biological reason for selective delivery to tumour with this preparation is unclear. The macromolecular nature of the HPMA copolymers means that cellular uptake must be through an endocytic mechanism. It is possible that tumour cells have increased endocytic activity resulting in enhanced uptake of macromolecules [8]. It has been suggested that Walker 256 tumour contains fenestrated capillaries [10] which may allow preferential 'leakage' of the polymer in the tumour vascular bed. These preliminary

results are encouraging, but need to be reproduced in other model systems. Clinical trials using a form of HPMA copolymer targeted to hepatocellular carcinoma are planned for the near future.

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Fig. 1a

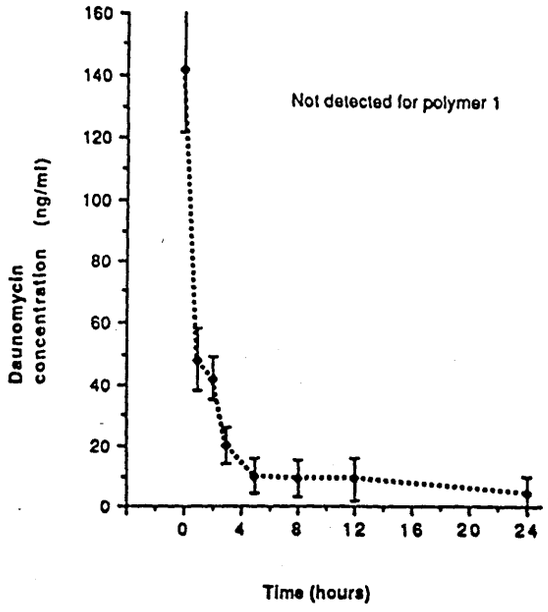

Fig. 1b

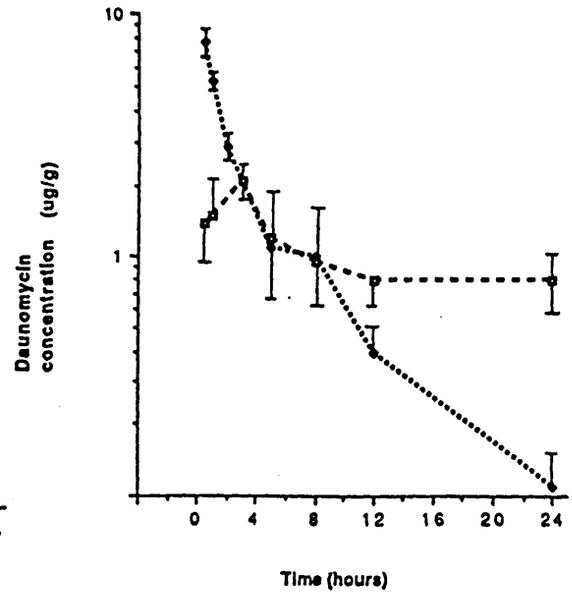

Fig. 1c

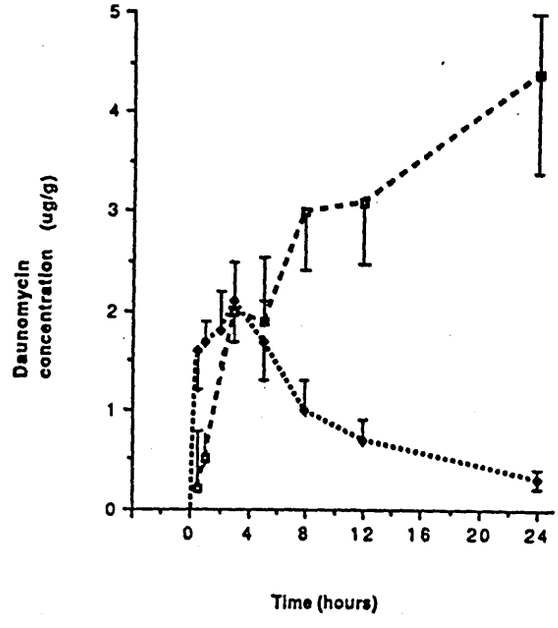

Fig. 1d

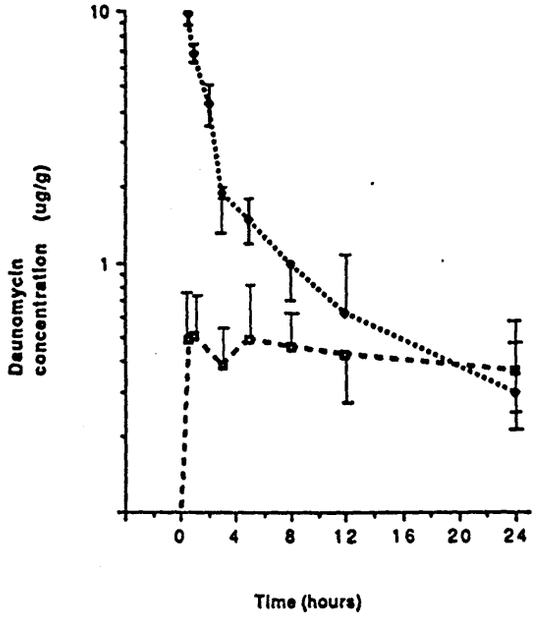

Figure 1. Free daunomycin levels by HPLC analysis in samples from (a) plasma, (b) liver, (c) tumour, (d) heart. ····· Free ----- Polymer 1

Fig. 2a

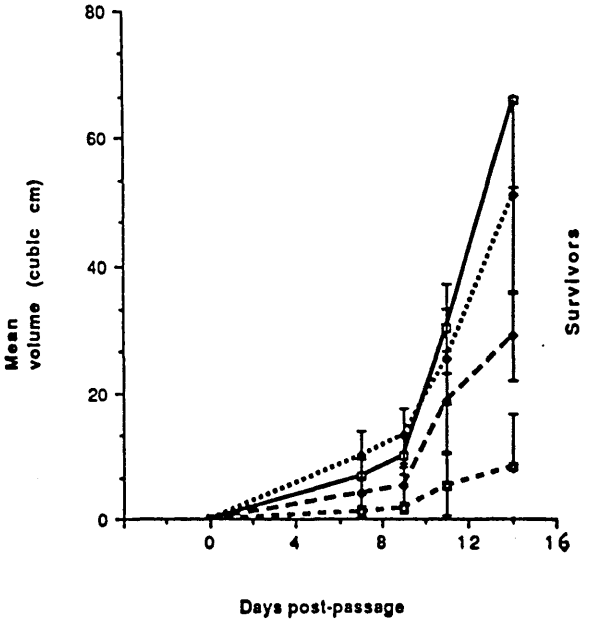

Fig. 2b

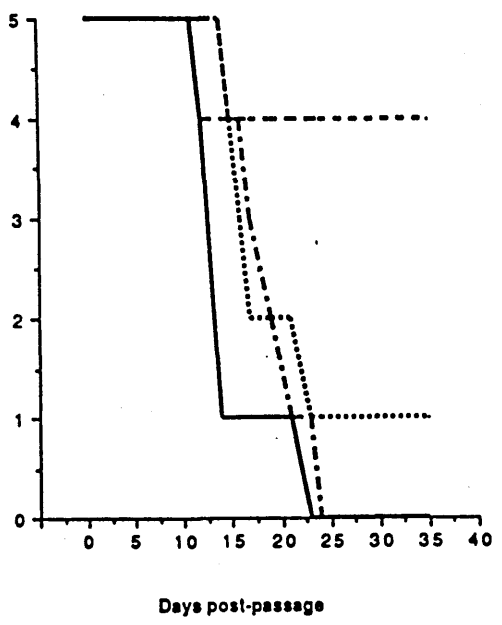

Figure 2a. Walker tumour growth in four groups of rats given saline (—□—), free daunomycin (·····◆·····), polymer 1 (- - ■ - -), polymer 2 (— · ◆ · —).

Figure 2b. Survival of rats in groups given saline (———), free daunomycin (·····), polymer 1 (- - - - -), polymer 2 (— · — · —), following subcutaneous passage of Walker tumour.