Biochemical Pharmacology, Vol. 37, No. 20, pp. 3981–3986, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

COMPARATIVE CELLULAR UPTAKE AND CYTOTOXICITY OF A COMPLEX OF DAUNOMYCIN–LOW DENSITY LIPOPROTEIN IN HUMAN SQUAMOUS LUNG TUMOUR CELL MONOLAYERS

D. J. KERR,*† S. A. HYNDS,‡ J. SHEPHERD,‡ C. J. PACKARD‡ and S. B. KAYE*
*CRC Department of Medical Oncology, 1 Horselethill Road, University of Glasgow G12 9LX, and
‡Department of Clinical Biochemistry, Glasgow Royal Infirmary, Glasgow, U.K.

(*Received* 11 *February* 1988; *accepted* 13 *May* 1988)

Abstract—In an attempt to target cytotoxic drugs to tumour cells daunomycin–low density lipoprotein (LDL) complexes were synthesised. Human squamous lung tumour cells *in vitro* have large numbers of high affinity cell surface LDL receptors (V_{max} = 19 ng LDL/μg cell protein per 24 hr; K_m = 23 μg/ml). Cellular uptake of daunomycin and LDL–daunomycin was rapid and approached equilibrium by approximately 3 hr. Intracellular daunomycin concentrations were similar at each time point regardless of whether free drug or the complex was used. The degree of intracellular drug metabolism differed markedly with significantly higher production of daunomycinol following exposure to free daunomycin for 90 min. Daunomycin and LDL–daunomycin were equally cytotoxic *in vitro* (respective clonogenic ID_{90}s of 1 μg/ml and 0.7 μg/ml). Fluorescence microscopy indicated that both free daunomycin and LDL–daunomycin have a punctate, granular distribution within the cytoplasm.

Low density lipoprotein (LDL) is the major cholesterol-carrying lipoprotein in human plasma. The core of the particle contains a lipid core of approximately 1500 cholesteryl ester molecules surrounded by a polar shell of free cholesterol, phospholipids and protein (apolipoprotein B). After binding to the receptor, an auto-regulated sequence of events occurs which culminates in lysosomal degradation of the lipoprotein and subsequent release of the cholesterol for use in the cell. Recent reports indicate that cells which are undergoing active cell division show a greater LDL receptor activity than non-dividing cells. For example, Gal *et al.* [1] have demonstrated *in vitro* that replicating epidermoid cervical carcinoma (EC_{50}) cells metabolise LDL some 20 times faster than cervical fibroblasts.

Attempts have been made to utilise the LDL-receptor pathway by complexing LDL with cytotoxic drugs. LDL–aclacinomycin A is effective against human glioma cells in culture and an LDL–methotrexate covalent complex has some activity against murine leukemia cells *in vitro* [2]. Iwanik *et al.* [3] have prepared an LDL–daunomycin complex in which the drug appears to be distributed between the hydrophilic surface coat and hydrophobic core of the particle. They demonstrated significantly higher cell associated levels of daunomycin after treatment of P388 leukaemic cell lines with the LDL–daunomycin complex, than after exposure to free daunomycin under similar conditions.

The present study was undertaken to determine the LDL-receptor activity of a human squamous lung tumour cell line and characterise the cellular uptake, metabolism and cytotoxic efficacy of LDL–daunomycin complex in monolayer culture.

† To whom reprints should be addressed.

MATERIALS AND METHODS

Cell culture. The L-DAN cell line was derived from a previously untreated patient with squamous cell lung cancer. The cells were maintained as a monolayer in exponential growth in Ham's F-10/DMEM medium (50:50) with 8 mM sodium bicarbonate supplemented with 10% foetal calf serum. All experiments were carried out in the exponential phase of growth and the cloning efficiency of these cells ranged from 20 to 25%. The doubling time of the cells is approximately 24 hr.

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

During the clonogenic assay for cell survival, the monolayers were exposed to the cytotoxic agents for 1 hr over a wide concentration range (0.5 μg/ml to 10 μg/ml) in growth medium. After drug exposure the cells were trypsinised, centrifuged and washed with ice-cold medium. The cells were then diluted in medium and plated at 200 cells/ml into 5 cm dia. Petri dishes. The plates were incubated for 12 days in a humid 2% CO_2 atmosphere. The colonies were then fixed and stained with a solution of methylene blue (0.1%) in 70% ethanol and colonies of ≥40 cells were counted. Following the usual convention the cloning efficiency of the treated cells was expressed as a percentage of control survival.

Isolation of lipoprotein. This follows the method of Patsch *et al.* [4]. Briefly, human plasma was isolated from venous blood (100 ml) drawn from normolipidaemic adults after overnight fasting. LDL was prepared by rate zonal ultracentrifugation using a linear NaBr gradient constructed in the Ti 14 rotor of a Beckman L5-65B ultracentrifuge. The LDL was then dialysed and its purity checked by immune precipitation. Iodination of the lipoprotein was accomplished by the iodine monochloride method, as modified by Bilheimer *et al.* [5]. Lipoprotein deficient serum was prepared by ultracentrifugation of whole serum for 24 hr at a density of 1.21 kg/l (45,000 rpm, 4°, Beckman T160 rotor). The supernatant was retained, dialysed against 0.15 M NaCl (0.01 M Tris, pH 7.4/0.01% Na_2 EDTA) and used in *in vitro* and incubation studies.

LDL–daunomycin complex formation. The procedure of Iwanik *et al.* [3] was adopted. Daunomycin in methanol was evaporated to dryness in a Buchler Vortex Evaporator and resuspended with LDL (2–5 mg of protein) in 1 ml of LDL buffer. The mixture was stirred with a Teflon-coated stirrer for 2 hr in the dark at room temperature. The mixture was then subjected to gel filtration on G25 Sephadex to separate the free from complex associated daunomycin. The drug–lipoprotein complex was sterilised by passage through a 0.44 μM Millex Millipore filter. Daunomycin concentration was measured by HPLC.

Cellular degradation of ^{125}I–LDL. Isolated L-DAN cells (approximately 2×10^6) were incubated for 24 hr with ^{125}I–LDL in 1 ml of F10DMEM culture medium, at 37° in humidified air containing 5% CO_2. Blank incubations were performed under identical conditions in the absence of cells. The degradation of LDL was determined from the appearance of non-iodide trichloracetic acid (TCA) soluble material in the incubation medium, as described by Goldstein *et al.* [6]. In brief, undegraded high molecular weight ^{125}I–LDL in the medium was precipitated by the addition of an equal volume of 10% TCA. After centrifugation, free iodide was separated from degradation fragments of ^{125}I–LDL in the supernatant by oxidation with H_2O_2 and extraction with chloroform. An aliquot of the aqueous phase was then assayed for radioactivity to determine the degradation of ^{125}I–LDL.

^{125}I–LDL binding to intact cells. 2×10^5 cells were grown in petri dishes. The medium was changed, 24 hr before use, to contain lipoprotein deficient serum which was then replaced with 2 ml of Medium A (Eagle's minimum essential medium containing 20 mM glycine, pH 7.4; and 5 mg of lipoprotein-deficient serum) and the indicated amounts of ^{125}I–LDL protein and native LDL protein. After incubation on a rotary shaker (80 oscillations per min) in air at 37°, the medium was removed and all subsequent operations carried out at 4° in a cold room. Each monolayer was washed 3 times in rapid succession with 3 ml of buffer (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, containing 6 mg of bovine serum albumin) after which a further 3 ml of the same buffer was added and the monolayer incubated for 2 min. This latter step was repeated once, each monolayer was washed finally with 3 ml of albumin-free buffer and the cells were removed from the dish by dissolution in 1 ml of 0.1 M NaOH. Aliquots of 500 μl were removed from each dish for scintillation counting in a gamma counter and 50 μl aliquots were taken for measurement of protein concentration. In all figures, each point represents the value obtained from a single dish and is expressed as the counts per min of ^{125}I–LDL bound per mg of total cell protein. Each dish contained 350–500 μg of total cell protein. Duplicate determinations of ^{125}I–LDL binding varied by less than 10% of the mean values. The specific activity of the ^{125}I–LDL was 20,000 cpm per μg protein.

Cellular accumulation of daunomycin and fluorescent localisation. Monolayers of L-DAN in the exponential phase of growth were treated with complexed or free drug at a concentration of 10 μg/ml for varying times (15 min to 2 hr). The cells were washed twice with ice-cold PBS, harvested by brief trypsinisation (0.25%) counted, and then resuspended in 2 ml of distilled H_2O. In previous experiments we have shown that there is no significant drug loss during this procedure [7].

Parent drug and its metabolites were then extracted and measured by HPLC with fluorescence detection [8]. This method dissociates daunomycin from LDL during extraction, therefore it would measure total intracellular drugs whether bound to LDL or not. Drug levels were expressed as ng/10^5 cells and drug accumulation curves were approximated by non-linear least squares fitting. For fluorescence microscopy studies, the cells were exposed to the drugs at a concentration of 2 μg/ml for 1 hr. The cells were washed and examined under a Polyvar fluorescence microscope (excitation = 460 mm, emission = 550 nm) using oil immersion (×400).

RESULTS

Cellular accumulation and metabolism of daunomycin

LDL–daunomycin was stable in culture medium at 37° for 3 hr with no evidence of complex dissociation. Cellular uptake of daunomycin and LDL–daunomycin proceeded rapidly and approached equilibrium by approximately 2 hr. It is of note that parent drug concentrations on a cellular basis are similar and statistically indistinguishable at each experimental time point (Table 1) regardless of whether free drug or the complex was used. The degree of intracellular drug metabolism, however, differed markedly at the later time points. Daunomycinol which is synthesised by a ubiquitous cytoplasmic NADPH reductase from parent drug was the chief metabolite formed (at the highest drug concentration, small amounts (0.1–0.5 ng/10^5 cells) of the deoxyaglycone of daunorubicinol were measurable). During prolonged incubation with free drug (≥90 min) daunomycinol predominated over the parent drug (Table 1). If the parent drug is expressed as a percentage of total intracellular drug, i.e. daunomycin/daunomycin + daunomycinol × 100%, then it is apparent that relatively less daunomycin is converted to the alcohol after treatment of the cells with the LDL–daunomycin complex at exposures of more than 1 hr.

Table 1. Intracellular levels of daunomycinol and daunomycin (±SD, N = 6) following treatment of L-DAN monolayers with identical concentrations (5 μg/ml) of daunomycin and LDL–daunomycin

Time (min)	Free daunomycin		LDL–daunomycin	
	Intracellular drug ($ng/10^5$ cells)		Intracellular drug ($ng/10^5$ cells)	
	Daunomycin	Daunomycinol	Daunomycin	Daunomycinol
0	0	0	0	0
15	4.8 ± 0.41	6.6 ± 0.5	4.5 ± 0.38	6.7 ± 0.52
30	5.2 ± 0.49	13.1 ± 1.2	7.1 ± 0.62	16.2 ± 1.72
60	10.1 ± 1.2	53.6 ± 4.8	10.4 ± 1.15	41.8 ± 4.3
90	13.2 ± 1.4	82.3 ± 6.7	13.9 ± 1.4	58.5 ± 5.1

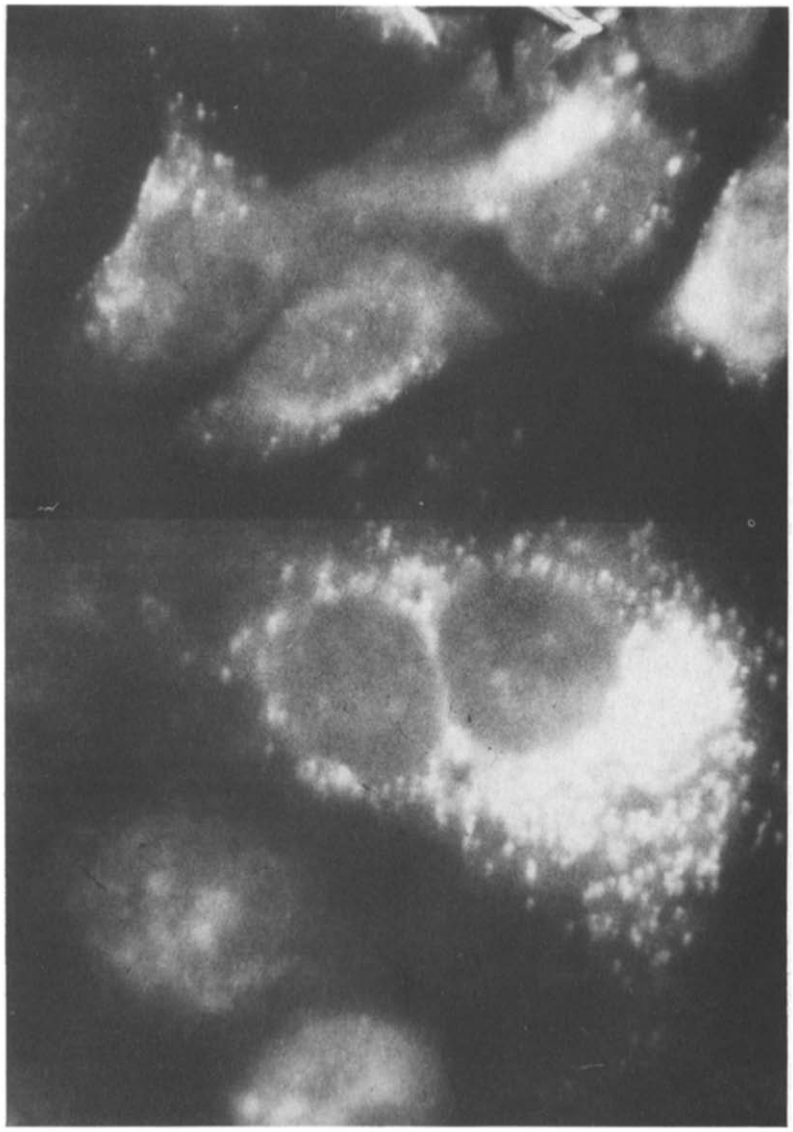

Fig. 1. Fluorescent photomicrograph of L-DAN cells treated with LDL–daunomycin. Granular cytoplasmic fluorescence is also seen following exposure to daunomycin (magnification ×160).

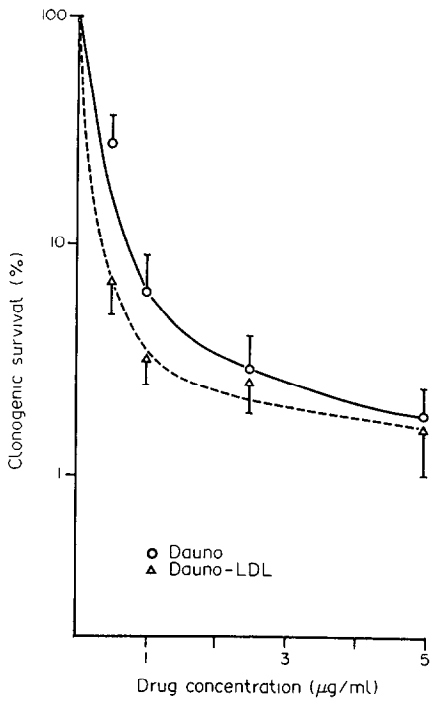

Fig. 2. Monolayer clonogenic survival of L-DAN cells exposed to daunomycin (○) and LDL–daunomycin (△).

The total cellular drug content (daunomycin + daunomycinol) is significantly ($P < 0.05$) higher after exposure of the cells to free daunomycin at the 90 min time point. This is accounted for by the difference in intracellular daunomycinol concentrations ($P < 0.05$).

Intracellular localisation of drug by fluorescence microscopy

The subcellular distribution of daunomycin is apparently independent of its mode of delivery to the cell. Daunomycin is present in a granular distribution within the cytoplasm, the nucleus and the nuclear membrane. This is similar to the fluorescent staining pattern previously reported for 4′-deoxydoxorubicin [6]. The LDL–daunomycin complex does not appear to alter the intracellular disposition of the cytotoxic agent despite the fact that the drug is presented to the cell in a markedly different physico-chemical state, and presumably enters the cells by a different pathway (Fig. 1).

Cytotoxicity to monolayer

The monolayer clonogenic cell survival curves for daunomycin and LDL–daunomycin are shown in Fig. 2. The degree of cell kill produced by both drugs is similar (ID_{90} daunomycin, 1.0 μg/ml; ID_{90} LDL–daunomycin 0.7 μg/ml). The cell survival curves are biphasic with a point of inflection at 5 μg/ml.

^{125}I–LDL degradation and binding to L-DAN cells

The degradation of ^{125}I–LDL by L-DAN cells as a function of the incubation concentration of ^{125}I–LDL is shown in Fig. 3. At concentrations below 40 μg/ml the degradation rate rose sharply with increasing concentration. Further increase in the concentration of ^{125}I–LDL led to a considerably less steep rise in the degradation rate, which approached linearity above 70 μg/ml. In the presence of a 20-fold excess of unlabelled LDL the degradation of radiolabelled LDL was inhibited and degradation increased linearly with the incubation concentration of ^{125}I–LDL. The slope of this curve was similar to the terminal part of the degradation curve obtained in the absence of unlabelled LDL. By subtracting the values for degradation values in its absence, a saturable curve with a maximal velocity of 70 μg/ml results, which probably corresponds to high affinity degradation sites.

High affinity binding sites for ^{125}I–LDL can be demonstrated in a similar manner (Fig. 3). The high affinity binding sites start to saturate above 100 μg/ml of ^{125}I–LDL. Reciprocal Lineweaver–Burke type plots (Fig. 4) of 1/cpm per μg cell protein versus 1/LDL (μg/ml) allow calculation of V_{max} and K_m for

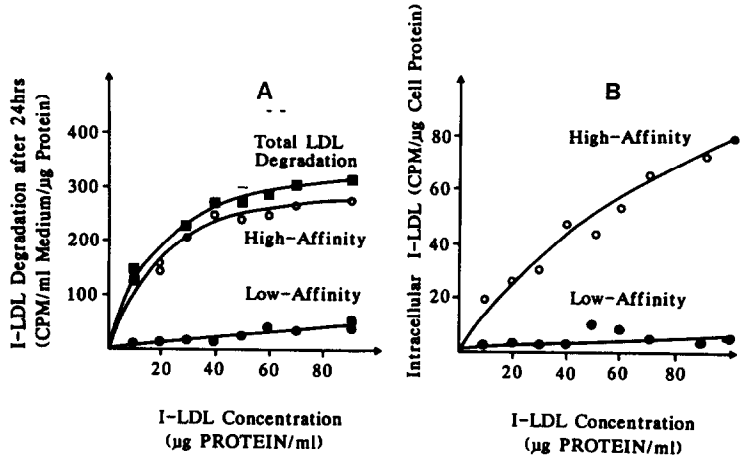

Fig. 3. The relationship between LDL degradation (A) and binding (B) by L-DAN cells to external LDL concentration. High affinity degradation (○) was derived by subtracting the non-specific or low affinity binding (●) from total degradation values (■). High affinity (○) and non-specific binding sites (●) are shown in (B).

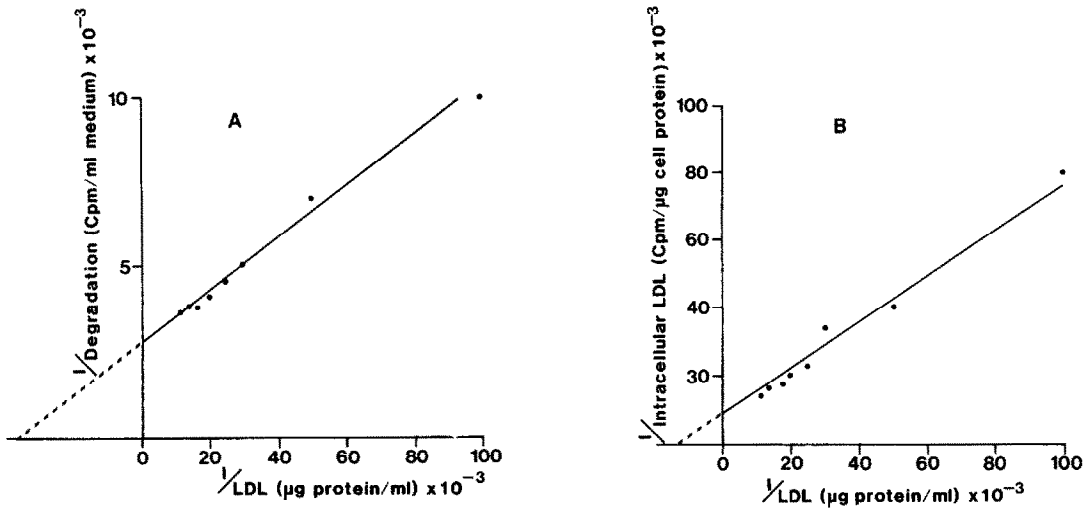

Fig. 4. Lineweaver–Burke plots of degradation (A) and binding data (B), to derive K_m and V_{max}.

degradation (V_{max} = 19 ng/µg cell protein per 24 hr; K_m = 25 µg/ml) and binding (V_{max} = 5.5 ng/µg cell protein per 24 hr; K_m = 71 µg/ml) (Fig. 4).

DISCUSSION

^{125}I-labelled LDL was found to associate with monolayers of cultured human lung tumour cells by two processes, one of high affinity and one of low affinity. The high affinity association appears to represent binding of LDL to specific receptor sites on the cell surface. This binding process exhibited saturation kinetics at low concentrations of the lipoprotein. The other process, designated low affinity uptake, may represent non-specific endocytosis since the uptake was proportional to the lipoprotein concentration in the medium with no apparent saturation.

There is some difficulty in comparing the K_m and V_{max} values for binding and degradation from our study with those of other authors due to variation in the units in which they are expressed, however, the values are similar to those found for fibroblasts [6].

Previous studies have shown that daunomycin is extensively metabolised within the cells to daunomycinol by the cytosolic NADPH-dependent reductase [9]. This occurs to a greater extent after exposure of the cells to free daunomycin, so that after 1.5 hr incubation, intracellular levels of daunomycinol exceed those of the parent drug by approximately 6-fold. There is less metabolism of daunomycin after treatment of the cells with LDL–daunomycin. So, despite the fact that total intracellular drug levels (daunomycin plus daunomycinol) are greater following incubation with free daunomycin, the cellular concentration of unchanged daunomycin is similar at each time point. This implies that the LDL–daunomycin complex inhibits or protects daunomycin from cytoplasmic reduction. There are a number of potential rate limiting steps which could serve to retard uptake of LDL-encapsulated daunomycin, relative to diffusion of free daunomycin through the cell membrane which could account for the lower total intracellular drug levels seen with the LDL–complex.

The monoloayer clonogenic cell survival curves for daunomycin and LDL–daunomycin are similar. Daunomycin has considerably greater cytotoxic efficacy than daunomycinol [9], and one would therefore expect that intracellular daunomycin levels would correlate with clonogenic cell kill. Despite the differing degrees of drug metabolism, daunomycin levels were virtually identical regardless of its mode of presentation to the tumour cells, and this could account for the similar clonogenic cell survival curves.

Although the mechanisms for transmembrane transport were different, the subcellular distribution of daunomycin, demonstrated by fluorescence microscopy was indistinguishable, whether free or carrier bound daunomycin was used. The drug was bound to the nucleus, the nuclear membrane, and was distributed within the cytoplasm in a granular fashion. It is possible that daunomycin diffused rapidly from the LDL complex upon internalisation and distributed in a similar manner to free daunomycin. It has been demonstrated [10] that subcellular organelles cause differential fluorescence quenching of bound daunomycin and therefore fluorescence microscopy remains a useful but rather insensitive method for determining the subcellular distribution of anthracyclines. Iwanik *et al.* [3] examined the subcellular localisation of daunomycin by membrane subfractionation of P388 cells after incubation with ^{125}I–LDL–daunomycin or free daunomycin. They noted that approximately 50% of free daunomycin and about 70% of LDL–daunomycin was associated with the membrane–organelle fraction of the cell, with the remaining percentage being either loosely bound or located within the cytoplasm. The ^{125}I–labelled fragments of LDL became associated in particular with plasma-membranes, microsomal–lysosomal–mitochondrial membranes and the insoluble nuclear pellet. In contrast to daunomycin derived from LDL, free daunomycin showed a strong preference for localisation in the insoluble nuclear pellet.

Further experiments are required *in vivo*, probably in nude mice with human lung tumour xenografts, to determine the affinity of LDL–drug complexes for tumour and normal host tissues, the comparative pharmacokinetics relative to free drug and to explore the possibility of a differential therapeutic effect.

Acknowledgements—The authors thank the Cancer Research Campaign for financial support and Mrs Margaret Jenkins for typing the manuscript.

REFERENCES

1. Gal D, Ohashi M, MacDonald PC, Buchsbaum HJ and Simpson ER, Low-density lipoprotein as a potential vehicle for chemotherapeutic-agents and radionuclides in the management of gynaecologic neoplasms. *Am J Obstet Gynecol* **139**: 877–882, 1981.
2. Halbert GW, Stuart JFB and Florence AT, A low density lipoprotein–methotrexate covalent complex and its activity against L1210 cells *in vitro*. *Cancer Chemother Pharmacol* **15**(3): 223–227, 1985.
3. Iwanik MJ, Shaw KV, Ledwith BJ, Yanovich S and Shaw JM, Preparation and interaction of a low-density lipoprotein:daunomycin complex with P388 leukaemic cells. *Cancer Res* **44**: 1206–1215, 1984.
4. Patsch JR, Sailer S, Kostner G, Sandhofer F, Holosak A and Braunsteiner, Separation of the main lipoprotein density classes from human plasma by rate-zonal ultracentrifugation. *J Lipid Res* **15**: 356–362, 1974.
5. Bilheimer DW, Eisenberg S and Levy RI, The metabolism of very-low-density lipoproteins. 1. Preliminary *in vitro* and *in vivo* observations. *Biochim Biophys Acta* **260**: 212–221, 1972.
6. Goldstein JL and Brown MS, Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolaemia. *J Biol Chem* **249**: 5153–5162, 1974.
7. Kerr DJ, Kerr AM, Freshney RI and Kaye SB, Comparative intracellular uptake of adriamycin and 4'-deoxydoxorubicin by non small cell lung tumour cells in monolayer. *Biochem Pharmacol* **35**: 2817–2823, 1986.
8. Cummings J, Stuart JFB, McArdle CS and Calman KC, Identification of adriamycin and its metabolites in human and animal tissue and blood. *Methodol Surv Biochem Anal* **14**: 245, 1984.
9. Bachur NR, Anthracyline antibiotic pharmacology and metabolism. *Cancer Treatment Rep* **63**: 817–820, 1979.
10. Egorin MJ, Clawson RE, Cohen JL, Ross LA and Bachur NR, Cytofluorescence localisation of anthracycline antibiotics. *Cancer Res* **40**: 4669–4676, 1980.