UNIVERSITY OF SOUTHAMPTON

# Fluorescence based assay for the identification of multidrug resistance in transitional cell bladder carcinoma

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## Abstract

The cytotoxic response of cancer cells to chemotherapeutic drugs may be influenced by the development of multidrug resistance (MDR) which phenomenon affords cross-resistance to a variety of structurally unrelated drugs, including the anthracyclines and possibly mitomycin C. The reduction in cytotoxic efficacy seen in MDR cancer cells is associated with the interaction of a number of cellular protein systems including *transmembrane glycoproteins*, *intracellular organelles*, *topoisomerases* and *glutathione transferases*.

Superficial bladder cancer, although primarily treated by surgical resection, can be adjuvantly treated by means of intravesical chemotherapy, most commonly employing the anthracycline *epirubicin*, or *mitomycin C*. However, the recurrence rate of these tumours can approach 60% despite such treatment. It is suggested that this represents the development of MDR in many instances. There is currently no reliable means of identifying the presence of MDR in patient tumours, and it is therefore impossible to predict an individual's likely response to intravesical chemotherapy.

The work which follows initially describes the current philosophy of intravesical chemotherapy in the management of superficial bladder cancer, the mechanisms of MDR and the means by which it might be mediated. Thereafter is described the development of a novel functional assay for the identification of MDR within bladder cancer biopsy explants in culture. In addition is given a description of the place of mitomycin C in the MDR family. Finally some mechanistic observations are described on the subcellular handling of the anthracyclines, and of the membrane characteristics of MDR.

## Declaration and Acknowledgements

This work is original, has not been submitted to any other institution, and all experiments were performed by the author except where stated below.

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My wife Anne, and daughters Eleanor and Harriet, have patiently tolerated my musings and have been a constant source of delightful distractions from this work!

## Abbreviations

| | |
|---|---|
| AA | Antibiotic antimycotic solution |
| ABC | ATP binding cassette |
| APS | ammonium persulphate |
| BCG | Bacille Calmette Guerin |
| bp | base pairs |
| CIB | chromosome isolation buffer |
| cis | carcinoma in situ |
| CMV | cisplatin, vinblastine and methotrexate |
| cDNA | complementary DNA |
| dATP | deoxyadenine triphosphate |
| dCTP | deoxycytosine triphosphate |
| DEPC | diethyl pyrocarbonate |
| dGTP | deoxyguanine triphosphate |
| DMEM | Dulbecco's modification of Eagle's medium |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| dTTP | deoxythymine triphosphate |
| DXT | deep X-ray therapy - syn. radiotherapy |
| Epi | epirubicin |
| Epo | epodyl |
| EDTA | ethylene diamine tetracetic acid |

| | |
|---|---|
| EGTA | ethylene glycol-bis(β-aminoethyl ether) N,N,N'N', tetraacetic acid |
| EORTC | European Organisation for Research and Treatment of Cancer |
| FBS/FCS | fetal bovine/calf serum |
| FITC | fluoroscein isothiocyanate |
| GST | glutathione-S-transferase |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid |
| IC50 | inhibitory concentration (50%) |
| IgG | immunoglobulin G |
| K/S-D | D value in Kolmogorov-Smirnoff two sample test |
| kDa | kiloDalton |
| LB | Lennox broth |
| LRP | lung resistance protein |
| MAb | monoclonal antibody |
| MDR | multidrug resistance |
| MMC | mitomycin C |
| MRC | Medical Research Council |
| mRNA | messenger RNA |
| MRP | multidrug resistance-related protein |
| MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide |
| MVP | major vault protein |
| MVR | minisatellite variant repeat |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NPC | nuclear pore complex |
| oligo dt | (deoxythymine triphosphate)12-18 oligo primer |

| | |
|---|---|
| PBS | phosphate buffered saline |
| PBSI | PBS containing protease and phosphatase inhibitors |
| PCR | polymerase chain reaction |
| P-gp | P-glycoprotein |
| PMSF | phenylmethylsulphonyl fluoride |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RT | reverse transcriptase |
| SEM | scanning electron microscopy |
| TAE | tris-acetic acid EDTA |
| TBE | tris-boric acid EDTA |
| TCC(B) | transitional cell carcinoma (bladder) |
| TE | tris-EDTA |
| TEM | transmission electron microscopy |
| TEMED | N,N,N'N' - tetramethylethylenediamine |
| TNM | tumour nodes metastases (classification) |
| Topo II | topoisomerase II |
| TRIS | tris(hydroxymethyl)aminomethane |
| TURBT | transurethral resection of bladder tumour |
| X-Gal | 5-bromo-4-chloro-3-indolyl B-D-galactopyranoside |

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# 1. INTRODUCTION

## 1.1 Transitional Cell Carcinoma of the Bladder

The twentieth century world epidemic of cigarette smoking has resulted in a parallel rise in the incidence of many cancers. This is exemplified by the annually increasing number of patients who present with transitional cell carcinoma of the bladder (TCCB), induced primarily now in Westernised societies by nitrosamines (primarily 2-naphthylamine and 4-aminobiphenyl) in tobacco (Morrison et al 1984). Although the incidence in males remains around three-fold greater than that in females, the increasing numbers of women who smoke tobacco has been reflected in a narrowing of this 'incidence gap'.

Recent figures from the Wessex Cancer Intelligence Unit (kindly supplied by Mr. J Woodall) reveal that bladder cancer (greater than 90% of which is TCCB) is now the third most common cancer among men and the tenth most common cancer among women within Wessex region. Bladder cancer contributed 8.8% of new cancer registrations to the WCIU, and 4.8% of deaths among men during 1990, as opposed to figures of 3% and 2.4% for women respectively in the same year.

In 1990 there were 801 cases of bladder cancer in Wessex with crude regional incidence rates of 41.4 and 13.4 per 100,000 for men and women respectively. This compares with (lower) national crude incidence rates of 32 and 12 per 100,000 respectively for the same year. This implies that there are an estimated 2900 people in Wessex living with bladder cancer at present.

Historically, in the United Kingdom, industry has also contributed significantly to bladder cancer causation. Aniline dye workers were identified as being at increased

risk of developing bladder cancer in 1895 (Rehn 1895) although the carcinogen was not identified as 2-naphthylamine until 1938 (Hueper et al 1938). The usage of benzidine in the rubber industry was implicated in 1954 (Case et al 1954), since when a host of other industrial carcinogens have been identified. However industry-related bladder cancer has become much less common than smoking-related disease in recent years since the successful identification of such carcinogens, and limitation of human exposure to them.

Recently, more interest has focused on the molecular aetiology of bladder cancer. Some oncogenes, including p21 *ras* (Meyers et al 1989) *c-myc* (Del Senno et al 1989) and *c-jun* (Lamph et al 1988) have been associated with TCCB carcinogenesis, and the expression of epidermal growth factor (EGF) has consistently been demonstrated to have a poor prognostic implication (Mellon et al 1995).

In addition deletions of four tumour suppressor genes are closely associated with bladder cancer - *p53* (Wright et al 1991), the retinoblastoma (*Rb*) gene on chromosome 13q (Logothetis et al 1992), and *p15 and p16* on chromosome 9 (Orlow et al 1994). To date chromosomes 1, 5, 9, 11, and 17 have all shown non-random changes in bladder cancer, and allelic deletions of chromosomes 9 and 17 occur in greater than 60% of bladder tumours (Olumni et al 1990) - chromosome 9 aberrations are rarely reported in other solid tumours.

In the United Kingdom the most common presenting clinical feature (80%) of bladder cancer is macro- or microscopic haematuria, the latter frequently detected by dipstick urinalysis. Presentation with recurrent symptomatic urinary tract infections account for the majority of the remainder, with a minority presenting with sterile pyuria. Rarely (5%) patients may attend with symptomatic anaemia, uraemia or

metastatic disease. Few clinical signs are apparent unless the patient has advanced malignancy, and as a result clinicians depend heavily on diagnostic investigations (including urine cytology, cystoscopy, intravenous urography, ultrasound examination and computed tomography) to establish a diagnosis and treatment plan. The mainstay of treatment remains transurethral diathermy ablation or resection of bladder tumour (Herr 1991) or open surgery, but other treatment modalities are employed. There is increasing evidence that the intravesical instillation of chemotherapeutic agents has an adjuvant role to play in the treatment of 'superficial bladder cancer' (see below).

Pathology, Stage and Grade

There is no doubt that accurate prognosis, and therefore treatment plan design, for patients with transitional cell bladder cancer is heavily dependent on histopathological assessment of tumour biopsy/resection material. In 1992 the Union Internationale Contre le Cancer (UICC) facilitated this by the introduction of a comprehensive tumour staging system based on the T (tumour) N (node) M (metastasis) classification, the most recent modification of which is shown below. The following stages are based on a combination of the histopathological assessment of surgically obtained tumour material and clinical examination/investigation findings:

**Ta :** papillary non-invasive (no invasion of lamina propria)

**Tis:** carcinoma in situ (flat, preinvasive)

**T1a:** invasion up to muscularis mucosae

**T1b:** invasion into muscularis mucosae

**T1c:** invasion beyond muscularis mucosae

**T2a:** invasion of inner half of detrusor muscle

**T2b:** invasion of outer half of detrusor muscle

**T3a:** microscopically beyond detrusor muscle

**T3b**: extravesical mass

**T4a:** invasion of prostate/uterus, vagina.

**T4b:** invasion of pelvic or abdominal wall.

In accordance with this classification, tumours staged as **Ta and T1** are currently designated as **'superficial bladder cancer'** although there is some debate in the literature about the inclusion of T1 tumours in this category in view of their dissimilar recurrence/progression behaviour - for example the chance of a tumour recurring and then becoming invasive has been estimated at 2 to 4% for Ta lesions, as opposed to approximately 30% for T1 lesions (Cutler et al 1982).

The phenomenon of **Tis** also remains controversial and there is now much evidence to suggest that patients who have developed carcinoma in situ are at high risk of developing aggressive invasive cancers in due course (Soloway 1987). Indeed between 40% and 83% of patients with carcinoma in situ will progress to muscle-invasive disease if treated by endoscopic resection alone (Althausen et al 1976). For this reason many consider Tis to be a separate entity from Ta/T1 disease and consequently treat it in a more aggressive manner (Jakse et al 1992).

In terms of cytological grade, tumours of any stage may be classified as **grade 1 (G1), grade 2 (G2) and grade 3 (G3).** These grades represent well-differentiated, moderately-differentiated and poorly-differentiated tumours respectively.

Tumour grade has a profound influence on recurrence rates. It has been suggested that 25% of G1 tumours will recur at one year, compared with 45% of G3 tumours (Cutler et al 1982). If the initial lesion is T1, a G3 tumour will have a 45% progression-to-invasion risk, as opposed to a G1 lesion which will have a 15% risk

(Anderstrom et al 1980). For this reason T1G3 tumours are independently classified by many urologists, and treated much more aggressively. One fifth to one half progress to muscle invasion, and almost one third metastasise within two to five years (Abel et al 1988, Birch & Harland 1989, Mulders et al 1994). There is currently no consensus of opinion regarding optimal treatment for such patients, with some urologists advocating early cystectomy and others preferring a bladder-conserving approach with the use of adjuvant intravesical chemo/immunotherapy or radiotherapy.

The internationally accepted tumour grading classification is dependent on fairly subjective assessments of cell numbers and size, and nuclear features:

| | *N$^{0's}$ epithelial cell layers* | *Superficial cells* | *Nuclear enlargement* | *Nuclear hyperchromasia* |
|---|---|---|---|---|
| **G0 papilloma** | ≤ 7 | Present, small | Insignificant | Absent |
| **G1 carcinoma** | ≥ 7 | Usually present | Slight/moderate | Slight in occasional cells |
| **G2 carcinoma** | ≥ 7 usually more | Variable | Moderate/marked | Slight/moderate in 25-50% cells |
| **G3 carcinoma** | ≥ 7 often more | Usually absent | Marked and very variable | Marked in ≥50% cells |

## 1.2 Intravesical Chemotherapy - historical development, agents and current philosophy

Historical development of intravesical chemotherapy

In September 1961 Jones and Swinney, working in Newcastle, published a landmark paper in the Lancet in which they described their experience of the intravesical administration of ***thiotepa*** (triethylene thiophosphoramide - an alkylating agent) in the treatment of bladder tumours. They treated sixteen patients with four doses (on alternate days) of between 30mg and 120mg thiotepa in sterile water, having made an attempt to assess the effect of transvesical systemic absorption by means of measuring blood leucocyte counts. Of the patient group, 13 had well-differentiated papillary tumours and of these the tumour 'had almost completely disappeared' in eight patients at 6- and 12-week cystoscopies. Three patients complained of frequency and dysuria but all had confirmed urinary tract infections. Two patients developed haematological sequelae, with one mild leucopenia and one severe thrombocytopenia requiring treatment.

In 1960 Yeates had demonstrated, in a personal communication to Jones and Swinney, that transvesical molecular absorption was related to molecular size, and that the critical molecular weight in this regard was around 200. These haematological complications were therefore attributed to the low molecular weight (189) of thiotepa.

So began the search for the optimal intravesical chemotherapeutic agent, a drug which ideally would combine cancer-cell-specific cytotoxic efficacy with a high molecular weight and a low local/systemic side effect profile - the rationale for the use of such an agent being the treatment of residual foci of disease after resection, and the

prevention of recurrence by inhibiting potential tumour cell implantation in traumatised urothelium.

The latter suggestion prompted a trial of adjuvant thiotepa chemotherapy, given intravesically following transurethral resection (Gavrell et al 1978). Patients treated with 0.5 mg/ml thiotepa twice daily for three days postoperatively had a recurrence free interval of 33 months after treatment, compared with an interval of 9.5 months before treatment. If thiotepa was continued indefinitely the recurrence free interval increased to 41 months. Thiotepa has largely fallen out of favour in the UK since the development of newer agents. In the USA, however, thiotepa is still used in some centres by means of a six to eight week course (1mg/ml) in patients with frequently recurrent tumours.

Another alkylating agent, ***Ethoglucid 1%*** (syn. Epodyl: triethylene glycodiglycerol ether - molecular weight ~250) was pursued during the 1970's in view of its theoretically lower systemic absorption and myelosuppression risk. 66% of patients responded completely or partially in one therapeutic trial (Nielson and Thybo 1979); in another 30% patients demonstrated a complete response while 32% were failures from the outset (Riddle 1982). Not surprisingly, late recurrence was found predominantly in patients whose tumours showed only a partial response during earlier treatment (Nielson and Thybo 1979). The widespread use of this agent has unfortunately been limited by its association with severe chemical cystitis, and it has now been superseded in the UK by the agents **epirubicin and mitomycin C**.

Epirubicin

Interest in the anthracycline antitumour antibiotic group grew rapidly after the discovery of **doxorubicin** *(adriamycin)* in the late 1960's (Arcamone et al 1969). A semi-synthetic development programme was introduced aimed at modifying the activity:toxicity profile of this agent and in the 1970's epirubicin was introduced into clinical practice (Cassaza et al 1978).

**Epirubicin** (molecular weight 580) is an epimer of doxorubicin (Launchbury and Habboubi 1993) and differs from the latter only in the configuration of the 4'-C atom; in epirubicin the OH attached to this group is in the equatorial configuration, whereas in doxorubicin it is in the axial configuration. This small molecular change confers some important differences in the properties of these agents.

For example this 4'-OH configuration causes hydrogen bond formation to a nearby NH2 group in doxorubicin, thereby increasing its pKa (to 8.22) and consequently lowering its lipophilicity. The converse is true in epirubicin as hydrogen bonding occurs at a different site thereby rendering the molecule more lipophilic (as a result of its lower pKa 7.7). This lipophilicity allows non-ionised epirubicin molecules to enter cells more readily than doxorubicin under the same conditions (Cantoni et al 1990).

These lipophilicity characteristics were corroborated by the findings of Groos et al. (1986) who demonstrated that in RT112 (a TCC cell line) there is a direct relationship between pH and epirubicin uptake and cytotoxicity, with cytotoxicity greater for epirubicin than doxorubicin at all pHs. Additionally this epimerisation arrangement allows epirubicin to be glucuronidated and eliminated more readily than doxorubicin, reducing the serum T1/2 from 116 hours (dox) to 50 hours (epi).

The cytotoxic effects of epirubicin appear to result from its ability to from a complex with DNA by interstrand intercalation, thus inhibiting replication and transcription. This action may be attributable, at least in part, to its interference with topoisomerase-DNA 'cleavable complex' and helicase activity. Reduction of anthracyclines to semiquinone free radicals may cause damage to DNA, cell membrane lipids and mitochondria (Plosker and Faulds 1993).

Its first use in intravesical chemotherapy was reported by Matsumura in 1986.

Mitomycin C

**Mitomycin C** is a highly water-soluble antitumour antibiotic (molecular weight 334) produced by the actinomycete *Streptomyces caespitosus,* and first described in Japan in 1960. It consists of a quinone ring linked to an indole group, with two side groups (a methoxyformamide side chain and an arizidine ring).

It acts via a metabolite produced following an NADPH-dependent intracellular bioreductive alkylation reaction. During the course of this reaction the molecule loses the methoxy group and reduction of the quinone group occurs, followed by activation of the metabolites arising therefrom (Iyer and Szybocki 1964).

The active moiety inhibits DNA synthesis, cross-links DNA in a manner proportional to the guanine/cytosine content , and exhibits highly reactive carbamate and aziridine groups. Superoxide and hydroxyl radicals are also generated with resulting cytotoxic enhancement (Lown et al 1978). Evidence also exists that single strand DNA breaks are induced by alkylation at the O-6 residue of guanine. These actions are more marked in the late G1 and early S phases of the cell cycle, but its effects are, overall, cell cycle non-specific (Lawn et al 1978).

It was first used in the intravesical chemotherapy of bladder cancer in 1980 (DeFuria et al).

Idarubicin

**Idarubicin** is a synthetic anthracycline derivative of molecular weight 497, with a pKa of 8.5 in the chloride form. It differs from daunorubicin by the absence of a methoxy group at the carbon-4 position. This arrangement results in increased lipophilicity in comparison to its parent compound, allowing greater rate of intracellular uptake and greater cytotoxicity (Ganzina et al 1984). *In vivo*, for example, idarubicin is between four and ten times as potent as daunorubicin against murine solid tumour models or experimental T cell lymphomas (Arcamone et al 1978). It is currently the only anthracycline administrable by the oral route for systemic chemotherapy (DiMarco et al 1977).

Its mechanism of action remains poorly understood. In a manner analogous to the other anthracyclines, idarubicin intercalates in a parallel fashion between DNA base pairs. It also inhibits topoisomerase II, resulting in both double- and single-strand DNA breakage. Intercalation with DNA also leads to the inhibition of DNA polymerase and of DNA-dependent RNA polymerase, leading to the inhibition of the synthesis of DNA, RNA and proteins (Kuffel et al 1992).

Additionally idarubicin may be metabolised (e.g. to *idarubicinol*) by cytochrome P450. The resulting semi-quinone promotes electron donation and production of superoxide and peroxide free radicals, which, in turn, induce oxidative injury to DNA and cell membrane lipids (Ames and Spreafico 1992) .

Currently unpublished phase III trials (personal communication - Pharmacia & Upjohn) of idarubicin as an intravesical agent were halted early in their recruitment

because of the high incidence of chemical cystitis - it has therefore failed to find a role in the intravesical context despite its promising phase II development.

Current philosophy of the role of intravesical chemotherapy

Until recently the use of intravesical chemotherapy agents was fairly empirical, and little evidence existed in the literature to persuade clinicians that its role in the management of patients with superficial TCCB had been established. Most of the published work consisted of non-randomised cohorts with small numbers of patients, and with limited follow up data.

However, it became increasingly apparent that some groups of patients with superficial TCC bladder are at particularly increased risk of developing recurrent and/or invasive tumours during the course of their lifetime (Hall et al 1994). A concerted attempt was therefore made to rationalise the use of intravesical chemotherapy agents in an effort to address this.

In 1993 Oosterlinck et al, representing the European Organization for Research and Treatment of Cancer (EORTC), reported a trial of single dose adjuvant epirubicin intravesical chemotherapy, at a dose of 80 mg/40 ml normal saline, given within 24 hours of transurethral resection in all patients (n=431) presenting with a single resectable Ta/T1 tumour (all grades). After a mean two year follow up this protocol reduced the recurrence rate from 0.32 to 0.17 (i.e. by almost 50%), regardless of stage and grade in comparison to the control group (who received sterile water).

Subsequently the UK Medical Research Council (MRC) reported seven year (median) follow up (Tolley et al 1996) in 502 patients with Ta/T1 tumours (of all grades) who had undergone transurethral resection and then been randomised into one

of three treatment arms: 1) no further treatment, 2) one instillation of mitomycin C (40mg/40ml water), and 3) one instillation at resection and at 3 month intervals for one year. In the treated groups the risk of subsequent recurrence was reduced by 34% and 50% respectively. However the additional benefit seen in the group receiving extra instillations failed to reach statistical significance ($p=0.10$). The associated recurrence rates in the control and two treatment groups were 0.82, 0.42 and 0.31 respectively ($p<0.001$) although again no statistically significant advantage was seen in the additionally treated group. However, no conclusive evidence that progression-free interval and overall survival were affected was apparent.

In response to an interim report of this work (Tolley et al 1988) and the EORTC paper Hall et al (1994) stratified patients into low, medium and high risk groups (in terms of risk of developing recurrent cancer) according to the following clinical criteria, which are currently accepted by many urologists.

*Group 1 (LOW recurrence risk (20% at one year))* - solitary tumour at presentation and no tumours at cystoscopy three months later.

*Group 2 (MEDIUM recurrence risk (40% at one year))* - solitary tumour at presentation and tumour recurrence at three months, or multiple tumours at presentation and no tumour evident at three month cystoscopy.

*Group 3 (HIGH recurrence risk (90% at one year))* - multiple tumours at presentation and recurrence at three months.

He concluded that although a progression/survival advantage might not be conferred, the tumour recurrence frequency, and therefore necessity for expensive and uncomfortable cystoscopic procedures, could be reduced in <u>all</u> groups of patients with superficial TCCB by the standardised use of a single adjuvant dose of intravesical

chemotherapy, but added that the high risk group should receive either additional subsequent doses or intravesical immunotherapy (BCG).

The efficacy of intravesical chemotherapy is, however, obviously dependent on the chemosensitivity of tumour cells to the agent employed, and the above evidence suggests that cumulative tumour recurrence rates still remain between 40 and 60% (representing all groups) even following intravesical chemotherapy with newer agents - unfortunately there is currently no available technique which will reliably predict an individual's likely response to such agents.

Tumour recurrence in such circumstances therefore represents the preexistence, or the acquisition subsequent to drug exposure, of resistant cells within a polyclonal tumour. Alternatively drug delivery to all parts of the tumour may be inadequate. However with regard to resistance to anthracyclines, and possibly to mitomycin C (see later), multidrug resistance (MDR) is the most common phenomenon mediating tumour chemoresponsiveness (Kim et al 1996).

## 1.3 Multidrug Resistance (MDR)

In 1970 Biedler and Riehm reported the effects of culturing Chinese hamster ovarian cells in gradually increasing concentrations of actinomycin D. This single agent technique of cell-selection caused additional resistance in the surviving cells to a wide range of other clinically important cytotoxic drugs, including the anthracyclines (doxorubicin and daunomycin) etoposide, colchicine and the vinca alkaloids (vincristine, vinblastine and vindesine). Then in 1979 Riordan and Ling demonstrated that cells exhibiting this phenomenon (termed *multidrug resistance* (MDR)) also exhibited a significant drug accumulation deficit, and contained much lower intracellular cytotoxic drug concentrations. They established that this drug accumulation deficit was mediated by a 170 kDa transmembrane glycoprotein which they called permeation-glycoprotein or *P-glycoprotein* (P-gp). Subsequent studies of drug transport in different MDR cell lines suggested that this accumulation deficit resulted, at least in part, from an increased rate of drug efflux occurring by an energy-dependent mechanism, since drug accumulation is enhanced by metabolic inhibitors (Inaba et al 1979). The concept then developed that P-gp might be acting as an ATP-coupled drug efflux pump (an idea originally proposed by Dano in 1973), thereby removing lipophilic compounds from multidrug-resistant cells in a cytoprotective fashion (Gros et al 1986) - see later.

There has been much speculation about the nature and complexity of MDR in clinical tumours as opposed to cancer cell lines in culture. It is accepted that MDR can both be exhibited intrinsically by tumour cells, and can also be induced/selected for in previously sensitive cells by cytotoxic drug exposure. It remains unclear, however,

how MDR mechanisms are regulated in the intratumoural environment and much of our understanding derives from cell line cancer model systems.

P-glycoproteins

P-gps are members of the family of ATP- binding cassette (ABC) transporters, whose members include the cystic fibrosis transmembrane regulator, the chloroquine transporter of *Plasmodium falciparum* and a yeast transporter (Gottesman 1993). They are also highly homologous with certain bacterial transport proteins, and studies have demonstrated their expression in normal human gastrointestinal mucosal cells, biliary canalicular cells, adrenal cells, in capillary endothelial cells in brain and testes, and in renal tubular cells - the assumption being that in physiological terms P-gp acts as a component of some tissue-specific mammalian detoxification/cytoprotective mechanism acting at vulnerable tissue interfaces (Thiebaut et al 1987) forming a channel allowing active pumping of toxins out of the cell. Rodent (hamster and mouse) P-gps have also been identified and are homologous to the human proteins (Buschman and Gros 1991).

P-gps are unusual among membrane transport molecules in that they are able to perform active transport of a variety of compounds that share little structural similarity. Most P-gp substrates are plant-derived or chemically-synthesised cytotoxic drugs/antibiotics. One of the few exceptions is progesterone, which interacts with P-gp in the pregnant mouse uterus (Yang et al 1989).

P-gp molecules are approximately 1280 amino acids long, and consist of two halves of very similar sequences. Each half of the protein includes a short highly hydrophilic N-terminal segment, a long hydrophobic region with six transmembrane segments, and another hydrophilic region which contains a nucleotide-binding site.

This site is responsible for ATP-binding and hydrolysis, and therefore for the active properties of the molecule.

There is evidence that P-gps have a role in membrane physiology, and may act as phospholipid 'flippases' facilitating phospholipid traffic within plasma membranes. For example phosphatidylcholine has been shown to be translocated from the inner to the outer leaflet of the plasma membrane in transgenic mice in the presence of human MDR3 P-gp (Smith et al 1994). The functional consequence of this behaviour remains poorly understood, but it would certainly suggest ultrastructural/physicochemical differences between the membranes of MDR and non-MDR cancer cells. Not surprisingly, therefore, a role for P-gps has also been postulated in the control of ionic charge distribution within the plasma membrane (Gollapudi et al 1992), in particular with regard to chloride channels (Hardy et al 1995) although this has been refuted by Tominaga et al (1995). In addition evidence exists for the expression of P-gp in the Golgi apparatus of MDR cells, an intracellular site where adriamycin (doxorubicin) accumulates (Molinari et al 1994). Intriguingly the horizontal transmission of MDR is hinted at by observations of P-gp expression in (normally P-gp negative) stromal cells adjacent to P-gp expressing tumour cells (Schlaifer et al 1990).

The 'classical' MDR genotype

Two genes coding for P-gps have been identified in humans, *MDR-1* (Chen et al 1986) and *MDR-3* (van der Bliek et al 1988) - three are known in mice (mdr-1 to -3) and hamsters (pgp-1 to -3). In man MDR-1 codes for P-gp 1, and MDR-3 codes for P-gp 2, as a result of a quirk of nomenclature!

Their bipartite molecular structure, and the distribution of intron position within the genes coding for P-gps, suggests that P-gps arose by the fusion of genes for two

related but independently evolved proteins (Chen et al 1990) before the evolutionary separation of animals and plants.

MDR-1

The cDNA sequence of the human MDR-1 gene is 4669 base pairs in length (Chen et al 1986) and its locus is located, with the homologous MDR-3 gene, on chromosome 7q21.1 (Callen et al 1987).

Only MDR-1 expression, and therefore P-gp 1 function, has been associated with resistance to lipophilic drugs. Several workers demonstrated, primarily using Northern blotting techniques, that intrinsic and transfected expression of MDR-1 conferred the MDR phenotype to mammalian cells (Gros et al 1986; Ueda et al 1987) - even very low levels of MDR-1 expression conferred a severalfold increase in the level of drug resistance (Chin et al 1989).

PCR technology increased the sensitivity and specificity of gene detection techniques and in 1990 Noonan et al used a quantitative PCR assay to examine MDR1 mRNA expression in a large number of normal tissues and tumours, establishing a tissue distribution pattern that had previously not been as detailed.

The regulation mechanisms of the MDR-1/P-gp 1 axis, however, are still poorly understood. Gene amplification is not invariable. For example, in clinical samples resistance levels due to P-gp 1 appear to be lower than those induced in vitro and significant amplification of MDR-1 has rarely been observed in these circumstances (Efferth and Osieka 1993). In addition, in some circumstances cells selected in vitro for lower levels of resistance do not show MDR-1 amplification. In the multidrug resistant MCF-7 (breast cancer) cell line high P-gp expressing cells express a 10-fold greater level of P-gp 1, but only a 3-fold greater level of MDR-1, than low P-gp

expressing cells, suggesting regulation at the post-translational level (Davies et al 1996).

MDR-3

Much less is known about the MDR-3/P-gp 2 axis. The MDR-3 gene is also located on chromosome 7q21.1 and it has high sequence homology with MDR-1, although it is slightly shorter at circa 3900 bp (van der Bliek et al 1988). Its tissue distribution seems more restricted than that of MDR-1, and it is found primarily in the canalicular membranes of hepatocytes (suggesting a role in the transport of phospholipid into bile) with low levels detectable in adrenal, heart, striated muscle, spleen and tonsil (Smit et al 1994).

Attempts to demonstrate that MDR-3 can confer multidrug resistance have been negative thus far (Schinkel et al 1991) although a possible contribution to anthracycline resistance in B lymphocytes has been demonstrated (Nooter et al 1990).

Multidrug resistance (associated/related) protein - MRP

Interest in other drug resistance mechanisms and markers evolved as it became apparent that MDR was not solely P-gp modulated. For example, despite the widespread occurrence of MDR in human lung tumours overexpression of P-gp was infrequent (Lai et al 1989).

In 1992 Cole et al described a new phosphoglycoprotein consisting of 1531 amino acids, which they isolated from a multidrug resistant (but P-gp negative) small cell lung cancer cell line. This 190kDa molecule, which they called MRP, also belongs to the ABC transporter molecule family, and is coded for by the MRP gene (c.5000bp on chromosome 16 p13.1) although the mechanisms which regulate this gene are

poorly understood. MRP seems to share an association with reduced intracellular cytotoxic drug accumulation (Eijdems et al 1995) and many structural features with P-gps, but the two proteins only share 15% amino acid identity (Cole et al 1994). Monoclonal antibody studies have demonstrated localisation of the MRP protein in the plasma membrane (Flens et al 1994).

Since its identification the overexpression of MRP mRNA has been shown to correlate well with the MDR phenotype in non-P-gp expressing cell lines, including T24 (TCC bladder - Hasegawa et al 1995). In addition, transfection of the MRP gene confers the MDR phenotype (Cole et al 1994) and its energy dependent nature has been confirmed. More recently it has been demonstrated that MRP transports both unaltered natural product drugs and glutathione S conjugates (Center 1995).

Zaman et al (1993) used an RNAse protection assay to demonstrate that MRP mRNA is readily detectable in a wide range of normal tissues, particularly in lung, spleen thyroid, testis, bladder, adrenal and gall bladder. It has been identified in leukaemic cells (Schneider et al 1995) and in bladder tumours with no prior exposure to cytotoxic drugs (Clifford et al 1996).

Lung Resistance-related protein (LRP)

In 1993 Scheper et al described another novel protein in a non-P-gp expressing multidrug-resistant non-small cell lung cancer cell line, which they termed LRP (measuring 110kDa) and were able to derive an LRP diagnostic monoclonal antibody (LRP-56) from it. This enabled them to demonstrate its overexpression in a number of other P-gp-negative MDR cell lines of different histogenetic origin (Scheper et al 1993)

with a characteristic punctuate cytoplasmic staining pattern. Interestingly they also established that LRP upregulation seemed to occur early during drug selection, suggesting a role for LRP in low/moderate (and therefore clinically more relevant) levels of MDR.

Most LRP expressing MDR cell lines also express MRP (Flens et al 1994) - in contradistinction most, but not all, P-gp-positive MDR cell lines are LRP negative (Scheper et al 1993). In a panel of 61 human cancer cell lines LRP and MRP were expressed in 78% and 87% respectively, as opposed to 24% expressing P-gp, suggesting that LRP might have a greater value as an MDR marker. In terms of normal tissues LRP is also widely distributed, but is found particularly in bronchus, digestive tract and keratinocytes, adrenal gland and macrophages. Among a panel of 174 clinical tumour specimens (27 tumour types) LRP was expressed in 63% (Izquierdo et al 1996).

The LRP gene lies on chromosome 16p 13-1-16p 11.2, near the MRP gene locus and its sequence is 2840 bp in length, although its regulatory mechanisms are not established.

Vaults

LRP protein sequence analysis has revealed that it is the *human major vault protein (MVP),* which is the most abundant component of *vaults*. These structures were first identified by transmission electron microscopy by Kedersha and Rome in 1986, have been isolated from various species from the lower eukaryotes to mammals, and are phylogenetically highly conserved (Kedersha et al 1990).

They are abundant in epithelial cells and comprise ribonucleoprotein particles which are composed of a MVP, three minor proteins and a small RNA molecule.

These components are arranged in a barrel-like structure of 60 x 35 nm (Kedersha et al 1991). The vault exhibits two-fold symmetry, each half of which opens into a 'flower-like' structure comprising eight 'petals' around a central ring. Most cells contain thousands of vaults, a small proportion of which are closely associated with nuclear pore complexes (NPC) on the nuclear membrane. Indeed it has been suggested that vaults constitute the central plugs of the NPC, and might mediate the transport of a variety of molecules between nucleus and cytoplasm (Chugani et al 1993).

It is well established that MDR cells exhibit an altered intracellular drug distribution as compared to parental (sensitive) cells (Gervasoni et al 1991). MDR cells redistribute epirubicin away from the nucleus into a punctuate cytoplasmic pattern, in contradistinction to the typical diffuse nuclear distribution seen in sensitive cells (Duffy et al 1996). The structures to which epirubicin is distributed in MDR cells remains unclear, but the proposal that vaults might be acting in some such regulatory capacity, mediating cytoplasmic/nucleocytoplasmic drug transport is attractive, and certainly correlates with the punctuate cytoplasmic staining pattern seen with LRP-56.

Other non-P-gp MDR-mediating mechanisms

Topoisomerase II (Topo II)

In the 1980's a pattern of multidrug resistance (termed 'atypical MDR') emerged in a leukaemia cell line that involved intracellular alterations in the DNA-repair enzyme known as *topoisomerase II* (Danks et al 1987), in the absence of P-gp/MDR-1 overexpression. Two subtypes of this enzyme have been identified, $\alpha$ and $\beta$.

Since then several MDR cell lines have been demonstrated to exhibit topo II-mediated MDR, and all share a difference in drug-induced DNA cleavable complex

formation, usually associated with both reduced levels of topo II levels and its activity (de Jong et al 1990). However this has been demonstrated to result solely from a topoII gene mutation (Deffie et al 1989) in some circumstances - in other atypical MDR cell lines, no change in topo II levels has been detectable, but a significant difference in enzyme activity was detectable (Danks et al 1987).

Glutathione-S-transferases (GST)

In 1986 Batist et al demonstrated a 45-fold increase in *glutathione-S-transferases* in an MDR subline of MCF-7 (breast cancer line) - these cytosolic enzymes are able to detoxify a wide range of xenobiotics by conjugation with glutathione, and have intrinsic peroxidase activity, thereby affording a free-radical-elimination pathway (Tew 1994). Since then it has been demonstrated that the GST $\pi$ subtype is most often overexpressed in MDR cells, and that, in these cells, the half lives of both GST $\pi$ and its mRNA are doubled in comparison to wild-type cells (Ranganathan et al 1994). In addition evidence exists for a 38kDa membrane protein that can efflux both glutathione conjugates and agents such as daunomycin and daunorubicin (Awasthi et al 1994) providing a potential link with MDR. Perhaps more importantly Muller et al (1994) have reported that glutathione is required specifically by MRP for the export of drugs from cells.

Recently interest has developed in the roles of the tumour suppressor gene *p53* (Chin et al 1992), apoptosis control genes such as *bcl-2, bax* and *bclX1* in cellular resistance to cytotoxic agents (Reed 1995), and in another ABC-transporter known as *TAP* (the transporter associated with antigen-presentation/transporter of antigenic peptides) which is known to translocate peptides from the cytosol to the lumen of the

endoplasmic reticulum (Scheper et al 1996). Additionally the induction of resistance to doxorubicin has been demonstrated to be associated with a several-fold increase in intracellular levels of heat shock proteins, in the absence of MDR proteins (Ciocca et al 1992).

## 1.4 Modulation of MDR

Since the 1980s concerted efforts have been made by many investigators to design strategies for the modulation of MDR, in order to improve chemosensitivity and therefore positively influence survival in patients with cancer. This resulted from the proposal that molecules which were able to modulate MDR might interact with the P-gp molecule, and from Tsuruo's discovery (1981 and 1982) that sensitivity to vinca alkaloids and doxorubicin could indeed be restored in MDR cells by the calcium channel antagonist *verapamil.*

In 1988 Zamora et al proposed a common P-gp 'pharmacophore' (i.e. a set of molecular structural and functional features required for 'modulator binding to P-gp') and suggested that this might consist of two planar aromatic domains and a basic nitrogen atom - such molecules included verapamil, *quinine, quinidine and reserpine*. The same group then used a series of reserpine and *yohimbine* analogues (Pearce et al 1989) to confirm this spatial arrangement, and hence these agents, as factors that could modulate MDR. Horton et al (1989) confirmed that the verapamil was also able to increase vincristine concentrations in tissues which normally express P-gp, such as liver, small intestine and kidney, and concluded that this represented functional reversal of intrinsic MDR representing binding of the modulator to P-gp.

Ramu et al (1991) then confirmed that modulation of MDR could also be achieved by *dipyridamole and tamoxifen*, and that this effect was associated with a change in cellular phospholipids, specifically an increase in cellular phosphatidylcholine content, that was not seen in sensitive cells. They suggested that *'the organisation of... plasma membrane lipids in these* (MDR) *cells must be different'*

and that this might explain differences in drug permeability. Callaghan and Higgins (1995) have since used the affinity analogue tamoxifen aziridine/azidopine to demonstrate that [3H]tamoxifen binds directly with P-gp, thereby inhibiting cytotoxic drug transport.

Woodcock and colleagues (1992) subsequently used flow cytometric evaluation of intracellular levels of daunorubicin in a leukaemia cell line to demonstrate that several pharmacologically inactive solubilising agents *(surfactants)* including cremophor, Tween 80 and Solutol were able to reverse MDR. In addition they established that different patterns of change in membrane fluidity, induced by surfactants, were seen when wild-type cells were compared with MDR cells. Further evidence of the importance of drug-lipid interactions in MDR modulation was offered by Wadkins and Houghton (1993) who used a membrane viscosity model to examine the interactions of nine MDR-reversing agents with a variety of lipids.

A large series of additional agents (largely lipophilic cationic molecules) which have MDR modulating properties have since been identified, including *progestogens* (Zibera et al 1995), *cyclosporin and its derivative PSC-833* (Boesch et al 1991).

More recently the modulation of the MDR phenotype by *anti MDR-1 ribozymes* (catalytic antisense RNAs which function as RNA cleaving enzymes) has been examined (Daly et al 1996) and resulted in reduction in MDR-1 mMRNA expression and increase in drug sensitivity in two MDR lung cancer cell lines.

Coley et al (1993) and Gervasoni et al (1991) examined the intracellular localisation patterns of doxorubicin and daunorubicin in wild-type and MDR leukaemia and breast cancer cell lines, using confocal microscopy. Taking advantage of the intrinsic fluorescence exhibited on laser excitation by the anthracyclines they

identified a reduction in anthracycline fluorescence in MDR cells, and a very characteristic difference in intracellular drug distribution. Sensitive cells showed predominantly *nuclear* uptake, with some diffuse cytoplasmic drug, whereas MDR cells contained very little drug in the nucleus, but rather a *vesicular pattern of drug distribution within the cytoplasm.* They postulated that this represented the altered drug traffic resulting from P-gp activity in the MDR cells. In addition Coley et al described a dramatic increase in nuclear drug fluorescence in MDR cells if co-incubated with verapamil in addition to the anthracyclines.

This phenomenon was also reported by Duffy et al (1996) who used the MGHU-1 (wild type) and MGHU-1R (MDR) urothelial cancer cell lines to describe punctuate or vesicular cytoplasmic epirubicin fluorescence in MDR cells, particularly in the distribution of the Golgi apparatus. Again, co-incubation of epirubicin and verapamil reversed this appearance to that of the sensitive phenotype, and this appearance correlated with increased sensitivity to epirubicin (personal communication) which approached that of wild-type cells.

## 1.5 Bladder cancer and MDR

The majority of our understanding of the mechanisms mediating MDR stem from work using cancer cell lines rather than clinical tumour material. This derives primarily from the fact that clinical material can be difficult and time-consuming to obtain, is of variable quality, poses technical difficulties in terms of processing (many cell types etc.) and has only a finite viability. In contrast cancer cell lines are readily available, of single cell type, more easily cultured, and are, by definition, immortalised. The study of MDR in relation to bladder cancer is no exception, although some information regarding putative MDR mechanisms in clinical tumours has been forthcoming.

### Evidence from cancer cell line model systems

Hagen et al described a reliable clonogenic assay to measure the in vitro sensitivities of the bladder cancer cell lines MGHU-1, MGHU-2 and RT4 to cytotoxic drugs in 1979. Subsequently, in 1984, Hepburn et al reported on the response of RT-112, a urothelial cancer cell line, to twelve chemotherapeutic drugs and they established that wild-type RT-112 was particularly sensitive to methotrexate, mitoxantrone, adriamycin (doxorubicin), mitomycin C and cisplatin in culture. They suggested that such model systems might be useful in the assessment of drug combinations for chemotherapy in bladder cancer.

Also working in 1984, Simpson et al, using the wild-type T24 bladder cancer cell line, demonstrated that the cytotoxic action of doxorubicin and thiotepa could be enhanced by both synchronous combination with and subsequent exposure to verapamil, but that verapamil exhibited no independent cytotoxic properties. This is probably the first documented use of verapamil as a combination agent in a bladder

cancer context, but although they postulated that verapamil was modulating intracellular cytotoxic drug concentrations via the 'intracellular calcium environment' the terms MDR and P-glycoprotein are not mentioned.

McGovern et al established a doxorubicin resistant human bladder cancer cell line which they called MGHU-1R, by growing MGHU-1 (a subculture of T24) wild-type cells in increasing concentrations of the agent in 1988, and demonstrated that the doxorubicin resistance could be reversed by the addition of verapamil to the growth media (Long et al 1990). In the 1990 paper the same team also established that H3-verapamil, administered by intravesical instillation in rabbits, was found in high concentrations in mucosa, less in adventitia and was absent in venous blood, suggesting its potential safety as an intravesical, rather than parenteral, combination agent. The multidrug-resistant nature of the MGHU-1R line was confirmed in 1990 by Floyd et al, who demonstrated that, in addition to its 40-fold doxorubicin insensitivity, it was 188 times more resistant to vinblastine and 13 times more resistant to etoposide than wild-type MGHU-1, whilst retaining its sensitivity to bleomycin. They also reported personally communicated evidence that MGHU-1R overexpressed the MDR-1 gene, and described the persistence of the MDR phenotype in MGHU-1R in culture even in the absence of maintenance concentrations of doxorubicin from the culture media. This contrasted with the relatively prompt reversion to a lower level of resistance found by workers using other cell lines (Roninson et al 1986). Additional evidence for the resistance of MGHU-1R to epirubicin was demonstrated by Popert et al (1994).

Another doxorubicin resistant bladder cell line (KK47/ADM) was established by similar techniques (Kimiya et al 1992), demonstrating cross-resistance to epirubicin, vinca alkaloids and etoposide, but not to cisplatin, carboplatin or, most interestingly,

mitomycin C. They confirmed the use of verapamil as an MDR-modulator in culture, and demonstrated that around 70% of the KK47/ADM cells bound MRK16, a monoclonal antibody against P-gp. Sensitivity to mitomycin C was also maintained in the MDR clone of RT-112 (doxorubicin induced) according to Seeman et al in 1995.

A more mechanistic approach was taken by Hasegawa et al in 1995, who examined the expression of MDR-1, MRP and topoisomerase II in three doxorubicin resistant, and one vincristine resistant bladder cancer cell lines which they had established *de novo*. They saw a synchronous induction of increased levels of MRP mRNA, decreased levels of topoisomerase II mRNA and decreased drug accumulation with variable MDR-1 expression during the development of MDR in these cell lines, adding to the evidence for the multifactorial nature of MDR.

Evidence in clinical tumour material

Several investigators have sought evidence of MDR in clinically obtained specimens of normal bladder mucosa and bladder tumour.

In 1993 Thomas et al published an immunohistochemical description of the expression of glutathione-S-transferase π in normal urothelium, 23 superficial bladder tumours and 26 invasive tumours (all of the latter had received platinum-based chemotherapy). They demonstrated cytoplasmic GSTπ staining in all specimens. Nuclear staining was seen in one superficial tumour and in 13/26 invasive tumours ($p=0.003$) , although there was no association between nuclear staining and (systemic) chemotherapy response in those patients with invasive disease. They therefore proposed a possible association between GSTπ expression and disease progression.

In 1994 Park et al examined the expression of P-gp in clinical bladder tumour biopsy material. They evaluated 29 cystectomy specimens (from patients with no prior

chemotherapeutic drug exposure) and bladder biopsies taken from 9 patients before undergoing intravesical chemotherapy with doxorubicin, and again at the time of recurrence, using formalin-fixed tumour sections and a polyclonal rabbit anti-human P-glycoprotein antibody. Of the cystectomy specimens 22 (75%) were P-gp positive - only three of these tumours were superficial (T1), but all three were P-gp positive. There was no correlation between P-gp expression and tumour grade, stage, morphology or the presence of carcinoma in situ.

In their small (n=9) series of superficial tumours, five had recurrent tumours after doxorubicin administration and four remained disease free during follow up (15-60 months). Of the five who failed treatment, four had tumours that were P-gp positive before the initiation of intravesical chemotherapy. All five recurrences stained P-gp positive. Of the four patients with no recurrence, three were P-gp positive before the onset of chemotherapy. Therefore, the prechemotherapy presence of P-gp failed to predict tumour sensitivity/recurrence in this series.

They concluded that a significant number of bladder cancers express P-g without prior exposure to chemotherapeutic agents, and that the incidence of intrinsic MDR (as opposed to acquired) in bladder tumours was therefore likely to be high.

The expression of the MDR-1 gene in 14 bladder cancer cell lines (10 sensitive and 4 MDR) and in tumour/normal urothelium pairs from 88 patients was assessed by Pu et al (1996) in Taiwan, by RT-PCR and Southern blotting. In addition they assessed functional P-gp activity by examining flow cytometric rhodamine-123 retention and efflux, an established P-gp model. They then correlated MDR-1 expression status with response to systemic (n=28) or intravesical (n=60) chemotherapy. None of the 60

patients in the intravesical group, and only 5 in the systemic group had received prior intravesical chemotherapy.

Only two of the wild-type cell lines were MDR-1 positive and only one was P-gp functional. Conversely all four MDR lines were MDR-1 positive and the two that were doxorubicin induced were also P-gp functional.

Of the 88 normal mucosa/tumour pairs, 62 (75%) tumours, and all 88 benign samples, were MDR-1 positive. No correlation was found between MDR-1 positivity and subsequent chemotherapy response (either systemic or intravesical).

They suggested that the striking difference in MDR-1 expression between tumour and normal mucosa might be explained by 'a form of cellular involution during carcinogenesis of TCCs in which energy exhausting cellular functions...regress.'

MRP seems similarly non-specific, but important, in the MDR phenotype in clinical bladder tumours. Clifford et al (1996) examined MRP and MDR-1 mRNA expression, using RT-PCR technology, in 24 previously untreated TCCs and five normal mucosa samples. MRP mRNA was expressed in all samples analysed but demonstrated up to 190-fold variability between tumour samples. Interestingly they established a trend towards lower MRP mRNA but higher MDR-1 levels in higher grade tumours (55%) compared with low/moderate grade (8%), but no relationship with recurrence or progression risk, and no association with chemotherapy response. Neither was there any association with MDR-1 expression, suggesting different gene regulation/expression mechanisms.

Also in 1996, Kim et al used an RT-PCR assay to examine the expression of the MDR-1, MRP, GST$\pi$ and topoII genes in 44 patients with urothelial cancers, 14 of whom had received prior chemotherapy. They obtained 52 tumours and three normal

urothelial mucosal samples from these patients and demonstrated that the mean expressions of MRP and GSTπ were higher than those for MDR-1 and topoII, and that the level of MRP expression correlated significantly with that of MDR-1 and GSTπ. They were unable to demonstrate any significant influence of chemotherapy on the induction of expression of MDR-1 or MRP, but established that the expression of GSTπ was significantly higher in those who did not receive chemotherapy. They concluded that the intrinsic and acquired resistance exhibited by urothelial cancers resulted from a multifactorial mechanism, and suggested that MRP and GSTπ expression activation occurred during tumorigenesis.

Clinical trials of MDR modulation in bladder cancer

The clinical potential of verapamil as a combination agent for intravesical chemotherapy has been addressed. Lukkarinen et al (1991) randomised seventy five patients with recurrent superficial bladder cancer after TURBT to receive either epirubicin (50mg in 50mls phosphate buffer) alone (n=40) or in combination (n=35) with verapamil (5mg added). The first instillation was performed four hours prior to surgery (TURBT), and the drug removed two hours later. Instillation was continued weekly for the first month, and then once a month for two years. The mean follow up was 21 (range 3-38) months and patients were cystoscoped at three monthly intervals.

Twenty patients (50%) in the epirubicin group and 13 (37%) in the combination group developed recurrent tumours. Before commencing instillation therapy, the recurrence rate in the first year was 16 in 30 patients treated with TURBT alone. In the epirubicin group this was reduced to 6.3, and in the combination group to 4.7 ($p<0.005$ and $p<0.001$ respectively). However there was no statistically significant difference in

recurrence rates between the two treatment arms, although clearly the numbers available are small. Interestingly the concomitant administration of verapamil did not increase serum epirubicin concentrations, but did increase epirubicin concentrations in the bladder wall and in tumour tissue.

In 1994 Tsushima et al described a trial consisting of 96 patients (48 in each of two arms) where patients with multiple (>2) Ta or T1 (G1 or 2 only) bladder tumours were randomised to receive either *A)* doxorubicin (50mg in 50ml) or *B)* doxorubicin (50mg in 40ml) with verapamil (25mg in 10 ml saline) as a two hour instillation. No information is given, however, about tumour resection. These agents were instilled daily for three days, and three such courses were given, with a four day interval between each course, for a total of nine instillations. Two weeks later the patients were recystoscoped. 40 and 39 patients were evaluable from each arm respectively.

No statistically significant differences were identifiable in residual tumours (60% in group A and 49% in group B) between the two groups as analysed for any parameter (tumour number, tumour size, grade, stage, presence of previous anthracycline exposure etc.) In addition there was no difference in disease-free period, nor in disease progression between the two groups. With the numbers available in each arm it would be surprising, however, if statistical significance had been reached unless the additional effect of verapamil had been dramatic.

The little evidence available to us from clinical trials does not, therefore, allow us to draw any real conclusions about the potential for MDR modulation in the treatment of superficial bladder cancer.

# 2. MATERIALS AND METHODS

## 2.1 General Procedures

Herein are described general procedures that are applicable throughout the work described in this thesis. Any modifications to these descriptions are discussed in greater detail in the chapters which follow.

### Drug Storage

Epirubicin (1mg/ml) and idarubicin (0.5 mg/ml) (Pharmacia Upjohn, UK) were prepared as stock solutions by dissolution in sterile water. Mitomycin C (Kyowa Hakko, UK) was prepared as a stock solution (1mg/ml) in 0.1M sodium bicarbonate. Aliquots were stored at -20°C in order to maintain drug stability, and reconstituted in tissue culture medium at the time of use.

### Cell lines.

The following cell lines were kindly donated by Prof. John Masters of University College Hospital, London, UK:

a) MGHU-1 superficial (T1 G3) transitional cell carcinoma line (Lin et al 1985) in wild-type form (non-MDR, anthracycline sensitive **MGHU-1**) and as the MDR (adriamycin induced) clone (**MGHU-1R**).

b) **MCF-7** breast cancer line (anthracycline sensitive) and its MDR counterpart (**MCF-7R**).

c) The colorectal cancer line **HT-29** and its MDR clone **HT-29R.**

Cell line culture

Cells were cultured in 25cm$^2$ and 75cm$^2$ culture flasks (Nunc, UK) in standard fashion, using Dulbecco's modification of Eagles minimal essential medium (DMEM, Sigma, UK) supplemented with 10 % fetal calf serum (FCS) (Sigma, UK) 1% antibiotic antimycotic solution (AA) (10,000 units penicillin, 10 mg streptomycin in 0.9 % NaCl stock, Sigma, UK) and glutamine (200mM stock 1% v/v). Cultures were maintained in an humidified 5% CO2-in-air atmosphere at 37°C. Subculture was achieved using 1% trypsin-EDTA solution (5g porcine trypsin and 2g EDTA in 0.9% NaCl stock, Sigma, UK).

For the purposes of flow cytometry cell suspensions were achieved using 1% trypsin-EDTA after washing with Dulbecco's phosphate buffered saline (PBS, Sigma, UK). Cells were centrifuged at 300g for three minutes and then resuspended in supplemented DMEM to neutralise trypsin. For the purposes of confocal microscopy adherent cells were subcultured as above in DMEM in 60mm diameter culture grade polystyrene Petri dishes before assay application.

Induction of new MDR variant of MGHU-1

Cells from the wild-type clone of MGHU-1 were cultured as above in the presence of sequentially increasing doses (initiation concentration 20nM to maintenance concentration 400 nM) of mitomycin C for a period of six months. Meticulous intermittent subculture technique was observed from the outset in order to ensure constant clonality within the cell line (hereinafter named **MGHU-MMC**).

Flow cytometry *(see Appendix 1)*

The flow cytometer system employed for the purposes of this work was the Becton Dickinson FACScan flow cytometer, using an argon-ion laser emitting at 488 nM. Data series collected thereby were analysed using the LYSYS II software package, allowing data display in histogram form, digital storage on optical disc, and statistical analysis, after setting an acquisition gate to include singlet cells only which was constant for all whole cell experiments. Data series were collected for samples of 5000-10000 cells/particles.

Confocal Microscopy *(see Appendix 1)*

After drug incubation (see specific methods) fluorescence was detected using the Leica TCS 4D confocal system, with the Leitz DMR BE research microscope, an air-cooled, mixed gas (argon/krypton) 3 μm fibre-optic laser emitting at 488 nm, and purpose designed software (SCANware 4.2, Microsoft, UK). The image was generated by point-scanning, detected by photomultipliers and converted to pixels for analysis and display. Multiple (serial) sections through samples were obtained at intervals of ~3μm, most often using a x50 water immersion lens.

Cell viability

Tumour colony viability was confirmed by the addition of fluoroscein diacetate (FDA) at 500 ng $ml^{-1}$ after drug assay. This molecule is actively taken up by metabolising cells and converted to fluoroscein through esterase activity - i.e. is a positive viability marker (Freshney et al 1995). Trypan blue (0.02% w/v) exclusion was used to ascertain viability of cell suspensions after flow cytometric/confocal assay

or in the presence of molecules (e.g. PKH2-GL, AnnexinV) with fluorescence profiles similar to FDA.

## 2.2 Specific Procedures

### Assessment of MDR patterns in TCCB biopsy explants

Culture of primary transitional cell culture explants

Bladder tumour specimens were obtained from patients undergoing endoscopic surgery for bladder tumours, by cold cup tumour biopsy or TURBT. Tumour specimens were transferred to sterile cold DMEM in a universal container immediately thereafter, and kept at 4°C prior to processing.

Tumours were then disaggregated by sharp dissection into <1mm explants after separation from stromal material. Explants were transferred to 60mm culture grade polystyrene Petri dishes (Nunc, UK) and held beneath droplets of DMEM supplemented with FCS and AA, to encourage cell-to-surface adhesion. Plates were maintained in this medium at 37°C in 5% CO2 (humidified) and cultures allowed to settle and adhere.

Non-adherent and non-viable cells and debris were subsequently removed by serial washing in PBS, and the adherent tumour colonies maintained in DMEM in identical conditions until assayed three to seven days thereafter (see specific methods).

Following explant senescence, or if explants failed to grow, all material was destroyed by incineration.

Epirubicin uptake assay

Colony cultures were incubated with epirubicin at $10\mu gml^{-1}$ DMEM for two hours at 37°C in 5% CO2-in-air atmosphere (humidified). Explant dishes were then maintained on ice prior to visualisation by confocal microscopy, in order to reduce active drug extrusion by resistant cells (personal communication, Duffy 1995). Where

sufficient dishes were available the assay was repeated in the presence of $100\mu gml^{-1}$ verapamil or PSC 833 (Sandoz) in an attempt to circumvent epirubicin efflux.

Colony cell labelling

PKH2-GL (Sigma) fluorescent cell linker (Horan & Slezak 1989) was incorporated into tumour explants in accordance with the manufacturers' recommendations. Following prior establishment of optimal staining concentrations, a $1x10^6$ M solution of PKH2-GL in its diluent (GL-DIL) was prepared and added to each explant dish following serial washes with PBS, and incubated at 25°C for three minutes whilst agitating. The reaction was stopped with 10% FBS and serial washes performed with DMEM. Labelled explants were maintained in ice-cold DMEM prior to subsequent confocal microscopy.

**Clonal characterisation of cell lines**

Cells from **MGHU-1, MGHU-1R, MGHU-MMC, MCF-7, MCF-7R, HT-29 and HT-29R** were taken separately into suspension, washed three times in PBS, pelleted, and taken up into 300 µl PBS in labelled 0.5 ml Eppendorf tubes.

DNA extraction

Proteinase K was added to each tube to final concentration 200µg/ml with N lauroylsarcosine (0.5%) to allow protein degradation and cell lysis. Cells were incubated at 55°C for one hour to denature DNAse. An equal volume (300 µl) of water saturated phenol was added and vortexed. Tubes were centrifuged at 13,000 rpm for three minutes. The top phase was removed and an identical phenol extraction performed thereon. The tubes were respun at 13,000 rpm for three minutes and the top phase taken. An equal volume of chloroform was added, the tube vortexed and respun at 13,000 rpm for a further three minutes. The top phase was taken and added in a fresh tube to two volumes of 100% ethanol with 3.5M sodium acetate (pH 5.3 at 10%) at -20°C to allow DNA precipitation. The tubes were inverted and placed at -80°C for 30 minutes.

Tubes were removed to room temperature and spun at 13,000 rpm for 20 minutes. The alcohol was discarded and the remaining DNA pellet gently washed with 70% ethanol at -20°C. The ethanol was removed and the pellet allowed to dry at 37°C for 30 minutes.

The DNA pellet was then resuspended in 150µl TE buffer (10mM EDTA (pH8) with 100mM TRIS HCl) and 1µl RNAse in TE (1 in 1000) added in order to lyse residual RNA.

The presence of intact DNA was confirmed by 0.6% agarose gel electrophoresis of 5µl per sample with 3µl bromophenol blue against lambdaHIND III (a marker with standardised bands demonstrating product size) run at 70mA for one hour.

Aliquots from these seven samples were used in paired fashion as described below.

Cell line characterisation by minisatellite variant repeat (MVR) analysis

Two mastermixes were made up containing the following:

20µl magnesium free buffer A
12µl 25 mM magnesium chloride (buffer B)
4µl 2% dimethyl sulphoxide (DMSO)
4µl dATP
4µl dCTP
4µl dGTP
4µl dTTP
7µl (31)A primer
1.8µl (31)tagII primer
0.4µl taq polymerase
159µl distilled water
***either*** 0.5µl (31) tag 2ac ***or*** 0.8µl (31) tag 2gt primers.

From these mastermixes, 24.5µl was taken for each 0.5µl DNA sample (total of seven DNA sample pairs). Each tube was closed, flicked up and pulse spun at 7000 rpm to mix. Samples were heated on a TECHNE Progene PCR machine according to the following protocol:

| | |
|---|---|
| One pulse of | 94°C for three minutes |
| | 52°C for 30 seconds |
| | 72°C for 15 seconds |
| then 10 pulses of | 94°C for 30 seconds |
| | 52°C for 30 seconds |
| | 72°C for 15 seconds |
| then 25 pulses of | 94°C for 30 seconds |
| | 62°C for 30 seconds |
| | 72°C for 15 seconds |
| finally one pulse of | 72°C for two minutes. |

10µl aliquots were stained with 3µl bromophenol blue and run on an 8% polyacrylamide gel (constituting 42 mls water, 6 mls TRIS-boric acid-EDTA (TBE), 12 mls bis-acrylamide, 200µl 25% APS, 50µl TEMED) at 40 mA for two hours, against 3µl ϕX Hinf I DNA marker. The gel was stained with 10 $ngml^{-1}$ ethidium bromide, and viewed under ultraviolet illumination. For result see appendix 5.

## Mitomycin C resistance (MDR Genotype and Cytotoxicity)

### 1. Expression of MDR-1 and MDR-3 by MGHU-1 and its MDR clones

mRNA extraction

Messenger RNA (mRNA) was extracted from cell pellets by means of the *RNEasy™ kit (Qiagen).* This technique involves a specialised high salt buffer system allowing up to 100μg RNA species longer than 200 bases to bind to a silca gel membrane. Lysis and homogenisation stages under highly denaturing conditions inactivate RNAses to protect intact RNA yield.

Cells from the **MGHU-1, MGHU-1R and MGHU-MMC** lines were trypsinised and taken into suspension, washed three times in PBS, and pelleted in Eppendorf tubes.

To each tube was added 350μl RLT lysis buffer (containing 1.5 mls RLT and 10μl β mercaptoethanol). Tubes were flicked up, placed on crushed ice, and spun at 13,000 rpm for three minutes. The supernatant from each tube was kept and decanted into a new tube. 350μl 70% ethanol on ice was added to each tube, which was then applied to the RNEasy column. Tubes and columns were spun at 10,000 rpm for 30 seconds, allowing RNA to remain on the column membrane. This stage was repeated until all solution was spun through the column. The column and membrane were washed with 700μl wash buffer (RWI) and respun as above. This was followed by two further wash-spin cycles with 500μl wash buffer (RPE). The column membrane was allowed to dry for two minutes at room temperature. 30μl DEPC-treated water was added and the sample spun for one minute at 10,000 rpm. Samples were stored at

-80°C.

RT-PCR to construct cDNA from mRNA templates

A mastermix was made up as follows:

28µl RT buffer (x10)
28µl DTT
14µl oligoDT primer
7µl dATP
7µl dCTP
7µl dGTP
7µl dTTP
7µl reverse transcriptase
140µl autoclaved water

32µl from this mastermix was added to 5µl of each of the mRNA samples, tubes were flicked up and a drop of mineral oil added to the surface of each to reduce evaporation. Tubes were then placed in the TECHNE Progene PCR machine and heated to 37°C for one hour, then at 94°C for three minutes.

cDNA integrity confirmation by β-actin PCR

A mastermix was made up according to the following protocol:

35µl (x10) magnesium free buffer
21µl 25 mM magnesium chloride
7µl dATP
7µl CTP
7µl dGTP
7µl dTTP
7µl upstream β-actin primer
7µl downstream β-actin primer
252µl autoclaved water
0.42µl taq polymerase

45µl of mastermix were added to 5µl of each RT-PCR cDNA sample and heated on the TECHNE Progene PCR machine for 35 cycles of the following schedule:

94°C for 30 seconds
62°C for 30 seconds
72°C for 30 seconds
72°C for 3 minutes.

10µl of each resulting product were mixed with 3µl bromophenol, and run on an 8% polyacrylamide gel (see previous constituents) at 40mA for two hours against 5µl ϕX Hae III DNA marker, and stained with ethidium bromide as above.

Gene specific primers for MDR-1 and MDR-3

Three gene specific primers were designed in order to assess the expression of MDR-1 and MDR-3 in MGHU-1, MGHU-R and MGHU-MMC. A common sequence upstream primer (MDR-U) was used in the assessment of both genes. The primer sequences are given below.

**MDR-1: --TTAgACAgCCTCATATTTTg--**

**MDR-3: --TCAgACAACCTCAAATCCTC--**

**MDR-U: --TTCTggATggTggACAggCg--**

*(A titration experiment for condition optimisation was performed at this stage - see Appendix 3)*

Expression of MDR-1 & MDR-3 in cDNA from MGHU lines

Following the identification of optimum DMSO/$MgCl_2$ conditions for the MDR primers, two further mastermixes were made up as follows:

50µl (x10) buffer
10µl dATP
10µl dCTP
10µl dGTP
10µl dTTP
5µl MDR-U
4µl MDR-1 **OR** 3µl MDR-3
40µl 25mM Magnesium chloride
0.5µl taq polymerase
301µl (for MDR-1) **OR** 302µl (for MDR3) distilled water.

44µl from the MDR-1 mastermix were aliquoted into reaction tubes 1-9, and 44µl from the MDR-3 mix into tubes 10-18. Tubes were then placed on the TECHNE Progene PCR machine and heated as follows:

| | |
|---|---|
| One cycle of | 94°C for three minutes |
| Followed by 35 cycles of | 94°C for 30 seconds |
| | 55°C for 30 seconds |
| | 72°C for 30 seconds |
| Finally one cycle of | 72°C for two minutes. |

5µl were taken from each reaction product and added to 3µl bromophenol blue and run against 3µl ϕX Hae III DNA marker on an 8% polyacrylamide gel at 40mA for two hours, stained with ethidium bromide as above.

Following identification of optimal bands, a second 8% polyacrylamide gel was run for the purposes of improved staining and photography.

Ligation of MDR-1 and MDR-3 with pGEM-T (Promega) vector

To 2µl from each of the optimal MDR-1 and MDR-3 PCR products were added:

1µl T4 DNA ligase (x10) buffer
1µl pGEM-T vector (50ng)
1µl T4 DNA ligase (1 Weiss unit per ml)
5µl distilled water

This reaction mix was then incubated at 10-12°C (crushed ice in water) for 18 hours, and stored at -4°C therafter.

Preparation of LB agar bacterial culture plates

3.2 g Lennox L agar were dissolved in 100 ml distilled water. This solution was rendered sterile by microwave. The solution was then cooled to 50°C. 100µl of 100mgml$^{-1}$ ampicillin was added, and the LB agar poured into sterile culture grade Petri dishes (Nunc, UK) and allowed to cool and set. The plates were surface flamed and dried inverted at 37°C for 15 minutes.

Plates were then smeared with 20µl of 50mgml$^{-1}$ X-Gal. Plates were then reinverted and dried at 37°C for a further 20 minutes until use.

Bacterial transformation procedure ('Cloning')

A water bath was equilibrated to 42°C. Meanwhile one vial of SOC medium was allowed to reach room temperature. The ligation reaction tubes were pulse spun at 13,000 rpm and placed on ice.

Two 50µl vials of OneShot$^{TM}$ bacteria (one per reaction) were thawed on ice. To each of these were added 2µl of 0.5mM β-mercaptoethanol and the tubes gently tapped. 1µl of each ligation reaction were pipetted directly into the competent bacteria and mixed by gently tapping. The remaining ligation mixture was stored at -20°C.

The vials were incubated on ice for 30 minutes, and then incubated in the 42°C water bath for exactly 30 seconds. Vials were removed from the water bath and immediately placed back on ice for a further two minutes.

450µl of prewarmed SOC medium were added to each vial with careful aseptic technique. The vials were then placed in a gyratory shaker-incubator at 37°C at 225

rpm. Whilst the vials were thus incubating, L-shaped bacterial spreaders were fashioned from glass pipettes.

After an hour, vials containing transformed bacteria were placed on ice. 50µl and 200µl aliquots from each transformation vial were spread on separate labelled LB agar plates, the plates inverted and placed in a 37°C incubator overnight.

The following day, transformed (white) colonies (as opposed to blue untransformed colonies) were identified and taken up onto pipette tips. These tips were placed in LB broth in sterile universal containers containing 20g Lennox L broth base per 1000mls distilled water. These were then incubated overnight at 37°C in the gyratory shaker-incubator.

Plasmid DNA extraction (Minipreps)

Transformed bacterial cultures were removed from the gyratory incubator. 1400µl were taken from each culture into Eppendorf tubes, and spun at 13,000 rpm for three minutes. 100µl TRIS-HCL EDTA (TE) (pH 8) were added to resuspend the pellet. To this were added 150µl lysis buffer (1% lauroylsarcosine) and the solution inverted until clear. 250µl 7.5M ammonium acetate were added and the tubes inverted, followed by a 10 minute spin at 13,000 rpm. The solutions were removed to new tubes. 1ml of 3.5 M sodium acetate in ethanol was added to each, the tubes inverted and incubated at -80°C for 30 minutes to precipitate DNA.

A further 20 minute spin (13,000 rpm) was performed to pellet the DNA. The pellet was washed with 50µl 70% ethanol, and air-dried at 37°C. DNA was resuspended in 21µl TE, to which were added 3µl TE RNase (1µl RNAse in 999µl TE).

A 3μl aliquot from each tube was run with 3μl bromophenol blue on a 0.6% agarose gel (0.6g in 100 ml TRIS-acetic acid-EDTA(TAE)) at 70mA for two hours, and stained with ethidium bromide as above.

Restriction digests

A 3μl aliquot was taken from each DNA miniprep tube and added in labelled Eppendorf tubes to:

0.25μl Sac-1 enzyme
0.5μl Nco-1 enzyme
2μl buffer 1
0.2μl (x100) bovine serum albumin
14μl distilled water

Reagents were mixed by flicking, and tubes pulse spun at 13,000 rpm. Tubes were incubated at 37°C for three hours, and resulting products (volume 25μl) run with 3μl bromophenol blue against 5μl ϕX Hae III DNA marker on a 8% polyacrylamide gel at 40mA for two hours, and stained with ethidium bromide as above.

QIAGEN™ preparations for high purity DNA extraction

High purity DNA extraction was required for the purposes of DNA strand sequencing, and therefore confirmation of primer/product identity. Bacterial clones in which MDR-1 and MDR-3 bands were most prominent on miniprep-restriction digest were selected.

3mls of bacterial clone were spun in Eppendorf tubes at 13,000 rpm for three minutes to pellet. The pellet was resuspended in 0.3ml buffer P1, and 0.3ml buffer P2 added. Tubes were mixed by inversion and incubated at 25°C for five minutes.

To this mixture was added 0.3ml buffer P3, tubes flicked and incubated on crushed ice for ten minutes. Tubes were mixed by inversion every three minutes whilst on ice. Tubes were then spun at 13,000 rpm for 15 minutes.

Meanwhile QIAGEN™ tips (one per sample) were preloaded with 1ml buffer QBT and allowed to drain completely. The supernatants from the sample Eppendorfs were applied individually to each tip and washed four times with 1ml buffer QC. Sample DNA was then eluted with 0.8ml buffer QF and eluted DNA solution collected into newly labelled Eppendorf tubes. DNA was precipitated by the addition of 0.56 ml isopropanol, and tubes spun at 13,000 rpm for 30 minutes. Supernatants were discarded, and DNA pellets washed with 1ml 70% ethanol, airdried at 37°C for ten minutes, and resuspended with 22µl TE with 3µl RNAse. 5µl from each sample were run (with 3µl bromophenol blue) on a 0.6% agarose in TAE gel against lambdaHIND III DNA marker at 70mA for two hours.

Restriction digests of QIAGEN™ preps

Restriction digests of pure DNA obtained by QIAGEN™ extraction were performed and analysed as already described.

MDR-1 and MDR-3 gene sequencing by the ThermoSequenase™ (Amersham) protocol

Sequencing plates, dividers and comb were washed with detergent and alcohol several times. Filter paper was cut to size and placed in the casting tray. A gel plug was cast containing

80ml distilled water
20ml bis-acrylamide
200µl 20% APS
50µl TEMED

This was poured, with electrode protection beneath water, and allowed to set.

Meanwhile, a sequencing gel was made up from

120g (8M) urea
175ml distilled water
25ml (x10) glycerol tolerance buffer
50ml bis-acrylamide (8%)

50 mls of this were taken and 50µl TEMED and 200µl 20% APS added.

Once the plug had set, this solution was poured using a 50ml bladder syringe and allowed to set. The electrode-protecting water and plug stopper were then removed and the gel plates placed in a gel tank containing glycerol tolerance buffer.

The plates were then prewarmed at 50W to 50°C 90 minutes prior to running the gel.

ThermoSequenase™ cycle sequencing reaction

All reaction stages were performed on crushed ice unless specified to the contrary.

2µl of dGTP termination mastermix (7.5µm) were taken and added in labelled Eppendorf tubes to 0.5µl of one of each of the following:

$\alpha$-$^{33}$ ddATP, $\alpha$-$^{33}$ ddCTP, $\alpha$-$^{33}$ ddGTP, $\alpha$-$^{33}$ ddTTP

Meanwhile the reaction mastermix was prepared from the following:

2μl reaction buffer (260 mM TRIS-HCl, pH9.5, 65 mM $MgCl_2$)
5μl Qiagen-prepared target DNA
2.5μl T7 primer
8.5μl distilled water
2μl DNA polymerase ($4U\mu l^{-1}$)

4.5μl of reaction mastermix was then pipetted into each labelled Eppendorf, pulse spun at 6000 rpm, and heated on the Techne Progene PCR machine to 94°C for 3 minutes, followed by 35 cycles of:

94°C for 30 seconds
55°C for 30 seconds
72°C for 45 seconds

The cycle was then completed by heating to 72°C for two minutes.

Reactions were then stopped with 4μl stop solution, and tubes pulse spun a 6000rpm. Tubes were finally heated to 95°C for one minute and then loaded into the sequencing gel, which was run at 50W for two hours.

The gel was then fixed in 10% methanol and 1% acetic acid in water for 20 minutes, adsorbed onto filterpaper, clingfilm wrapped and dried at 80°C for one hour in a vacuum-condenser gel drier. Clingfilm was then removed and the gel exposed to Kodak X-Omat radiography film for 16 hours, and developed in standard fashion thereafter.

## 2. Cytotoxicity of epirubicin and mitomycin C in MGHU cell lines

Cytotoxicity experiments were performed using the ***MTT*** (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay. Cells from lines MGHU-1, MGHU-1R and MGHU-MMC were taken into suspension as described previously, and seeded into 96-well culture-grade microtitre plates (Nunc, UK) and allowed to settle overnight (initial plating concentration $10^3$ $well^{-1}$) in standard conditions using 100µl supplemented DMEM per well.

Dilutions of epirubicin and mitomycin C were made up (fom stock solutions, diluted in supplemented HEPES buffered DMEM) to obtain final assay concentrations of 0.6, 1.25, 2.5, 5.0, 10, 20, 40 and 80 µg $ml^{-1}$.

Cells were then exposed to either agent and incubated at 37°C for one hour. Cytotoxic drug-containing medium was then removed, and the cells cultured for a further 72 hours in drug free DMEM. Subsequently the plates were incubated with MTT (0.2 mg MTT $ml^{-1}$ HEPES buffered DMEM, 250 µl $well^{-1}$) for four hours, and theresultant formazan crystals solubilised with 100% dimethyl sulphoxide (DMSO).

The optical density (representing remaining viable cell biomass) was determined on a Bio-Tek EL312e microplate reader and calculated as a percentage against control wells containing cells which had not been exposed to either cytotoxic agent.

**Membrane Characteristics of MDR Cell Line**

MGHU-1 and MGHU-1R cells were cultured to 90% confluence in standard fashion on glass coverslips:

Coverslips were prewashed in PBS and fixed by immersion in cold 100% acetone (4°C for five minutes). The following antibody assays were performed against controls substituting 1 or 2% FBS in PBS for primary antibody. Coverslips on glass slides (Citrifluor mountant) were then viewed and antibody expression assessed by confocal microscopy (FITC filter set).

Expression of P-glycoproteins (P-170s/P-gps) using JSB-1 monoclonal antibody

60µl JSB-1 monoclonal antibody (Harlan Sera Lab, Crawley Down, UK) were diluted 1:20 in 1% FBS in PBS. 100µl was pipetted onto each coverslip and incubated for 90 minutes at 25°C in humidified atmosphere. Three PBS washes were followed by incubation of each coverslip for 1 hour (25°C) with 100µl (diluted 1:100 in PBS) of goat-anti-mouse IgG-FITC (Sigma, UK).

Expression of multidrug-resistance related protein (MRP) using MRPm6 monoclonal antibody (Avidin/Biotin complex method)

MRPm6 monoclonal antibody (TCS, Buckingam UK) was diluted to 1:20 in 2% FBS in PBS and incubated on coverslips for 90 minutes at 25°C in humidified atmosphere. Secondary antibody binding was achieved by incubation with 100µl of biotinylated goat-anti-mouse IgG (1:100 in 2% FBS in PBS). Tertiary streptavidin

(conjugated with Cy3) was then diluted 1:100 in PBS and incubated on the coverslips for one hour at 25°C.

Expression of lung resistance protein (LRP) using LRP-56 monoclonal antibody

Incubation concentrations and conditions were employed as for P-gp, with the primary monoclonal antibody LRP-56 (TCS, Buckingham, UK) rather than JSB-1.

Transmission electron microscopy (TEM) of MGHU-1R nuclear envelope

MGHU-1R cells were trypsinised into suspension, washed three times with PBS and pelleted by centrifugation. Cells were fixed by overnight incubation in 3% glutaraldehyde in 0.1M cacodylate buffer, and then 'stained' with 2% osmium tetroxide in 0.1M cacodylate for two hours.

Following a distilled water rinse cells were exposed to aqueous uranyl acetate for 30 minutes, rewashed and dehydrated in an ethanol series (70-100%). Cells were then exposed to a 50:50 mixture of Histosol$^{TM}$ (Life Sciences International Ltd.) and Spurr's epoxyresin, and then incubated in neat epoxyresin for 24 hours. The resin was polymerised by incubation at 60°C for 16 hours in Eppendorf tubes. 60-90 nm sections were then cut on a Reichert OMU III microtome (diamond knife) and taken up onto 100μm copper grids before further 'staining' with Reynold's lead citrate. Grids were then viewed on a Philips 201 transmission electron microscope.

Membrane labelling by the phospholipid analogue PKH2-GL in cell suspensions/adherent cells

The fluorescent cell linker kit PKH2-GL (Sigma, UK) allows the incorporation into the lipid bilayer of the plasma membrane by selective partitioning of a negatively-charged aliphatic reporter molecule containing a fluorochrome group, thereby serving as a cell labelling technique.

For the purposes of flow cytometry, PKH2-GL was incorporated into both MGHU-1 and MGHU-1R in suspension according to the manufacturers' instructions. An initial solution of $2x10^{-6}$ molar PKH2 in its diluent (GL-DIL) was prepared. Doubling dilutions were then performed to obtain 1:2 ($1x10^{-6}$ M) and 1:4 ($5x10^{-7}$ M) solutions of PKH2 in its diluent.

Cells suspended in diluent were then added to each PKH2 dilution and incubated at 25°C for five minutes. The reaction was stopped with 10% fetal calf serum and serial washes performed with DMEM. Cells were resuspended in ice-cold DMEM and examined by flow cytometry.

Adherent cells for confocal microscopy were labelled *in situ.* The 1:2 dilution was incubated with each monolayer and incubated as above whilst agitating. The reaction was again stopped with fetal calf serum and serial washes performed with DMEM. Labelled cells were then maintained in ice-cold DMEM prior to examination by confocal microscopy.

*P-gp pump suppression.* The possible role of P-gp mediated probe efflux was assessed by examining PKH2-GL (1:2) binding in suspensions of both sublines held at 0°C (on ice) prior to ice-cold incubation as described above.

*MDR (functional P-gp) reversal.* PKH2-GL (1:2) was incorporated into the MDR subline as above and after prior incubation of the cell suspension with verapamil (MDR reversing agent) at 100μg/ml in HEPES buffered MEM at 37°C for one hour.

Membrane labelling by Annexin V in cell suspensions/adherent cells

Fluorescein isothiocyanate conjugated Annexin V was obtained from Biowhitaker, UK. Serial dilutions were performed in McCoy's 5a modified medium to obtain optimal binding concentrations for the MGHU-1 and MGHU-1R cell lines. Following harvest by trypsinisation, cells from each line were then resuspended in Annexin V (80 nM) in serum free McCoy's 5A medium with molar HEPES for ten minutes at 25°C. Cell suspensions were then kept on ice and examined by flow cytometry.

Cells for confocal microscopy were again viewed *in situ* adherent to plastic Petri dishes, after incubation with 80 nM Annexin V for ten minutes at 25°C and subsequent maintenance on ice.

Membrane labelling of MDR cells by anti-Tissue Factor (TF) monoclonal antibody

Fluorescein conjugated murine monoclonal IgG1 antibody (MAb) against human tissue factor (American Diagnostica Inc.) [100μg/ml in PBS] was incubated with adherent MGHU-1 and MGHU-1R cells in plastic Petri dishes for 30 minutes at 4°C.

Dishes were held on ice, and viewed immediately therafter by confocal microscopy.

**Anthracycline uptake by cell fractions**

1. Nuclear fractionation from cells of the MGHU line

Cells from sublines MGHU-1 and MGHU-1R were subcultured into 60mm Petri dishes and allowed to attain 80% confluence. Cells were then washed three times in PBS containing protease- and phosphatase-inhibitors (PBSI), comprising:

9.2mM $Na_2HPO_4$
10mM $NaH_2PO_4$
0.15M NaCl
1mM $Na_3VO_4$
1mM PMSF (phenylmethylsulphonyl fluoride)
5$\mu gml^{-1}$ Leupeptin
1$\mu gml^{-1}$ Aprotinin

Immediate release of nuclei from cells was then initiated in 2 mls lysis buffer (10mM HEPES, 1.5mM $MgCl_2$ pH5.5) by the addition of 10 drops of Zaponin (Coulter Electronics, UK). The supernatant was pipetted off immediately and layered over a 20% solution of sucrose in PBSI in polythene centrifuge test tubes. These tubes were spun at 800G for five minutes, and the top phase discarded. A further single PBSI wash was performed, and the suspension respun through 20% sucrose/PBSI at 800G for five minutes, and the top phase again discarded. The presence of fractionated nuclei was confirmed by phase contrast light microscopy (method from Holt et al 1994).

2. Confirmation of fractionated nuclear integrity by scanning electron microscopy (SEM)

Nuclei obtained as described above were fixed in suspension in 1ml 4% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). They were then washed in 0.1M cacodylate buffer with 0.23M sucrose. Thereafter nuclei were exposed for two hours to

2% osmium tetroxide in 0.1M cacodylate, and dehydrated through graded ethanol. Critical point drying was performed in a Balzers CPD030 from ethanol using liquid $CO_2$ as the exchange medium, and the samples stub-mounted. Nuclei were coated with gold-paladium using a Polaron E5100 sputter coater, and examined using a Hitachi 5800 field emission scanning electron microscope.

3. Chromosome extraction from cells of the MGHU lines

Cells from each line were cultured in the usual fashion to 50% confluence in 60 mm Petri dishes. Cells were incubated overnight (~12 hours) with 0.05μg/ml Colcemid (Sigma) in DMEM to achieve metaphase arrest. Cells were then taken into suspension by trypsinisation, neutralised in supplemented DMEM and spun at 100G for ten minutes. This wash step was repeated. The tube was tissue-dried and the cells taken up in 5ml hypotonic solution (75mM KCl) and incubated at 25°C for 30 minutes. Cells were again pelleted by spinning at 100G for ten minutes.

Meanwhile x10 chromosome isolation buffer (CIB - 200mM NaCl, 800mM KCl, 150mM Tris-HCl, 5mM EGTA, 20mM EDTA, 1.5% mercaptoethanol, 2mM spermine, 5mM spermidine) was added to 9 mls water and 12mg digitonin, magnetic stirred and pH corrected to 7.2 by the addition of aliquots of 4M NaOH. This solution was filtered through a 0.22μm filter and placed on ice.

The pellets were then incubated in ten times pellet volume (~3mls) CIB and stored at 4°C.

4. Assessment of anthracycline uptake by isolated nuclei and chromsosomes

Isolated cell nuclei and chromosomes were obtained as described above and pelleted by centrifugation. Separate solutions of epirubicin and idarubicin (10μg/ml) were made up at pH 6, 7.4 and 8 (achieved in PBS by the addition of aliquots of 27M HCl or 4M NaOH).

0.5mls of each solution were incubated separately with pelleted nuclei/chromosomes at room temperature for one hour, placed on ice and examined by confocal microscopy. Nuclear suspensions were also submitted to flow cytometry to allow corroborative fluorescence quantitation.

5. Assessment of anthracycline uptake by avian (chicken) erythrocytes

Avian erythrocytes were obtained by chicken wing vein whole blood venesection, collected into standard EDTA haematology tubes. 1ml was spun down at 300G for five minutes and the supernatant discarded. The pellet was resuspended in 9 mls PBS and respun to remove EDTA by again discarding supernatants.

Erythrocyte pellets were then resuspended in 1ml epirubicin or idarubicin at 10μg/ml in PBS, with pH adjusted as before to 6, 7.4 or 8, and incubated at 37°C in a 5% CO2 in air atmosphere for two hours. Tubes were then placed on ice.

Anthracycline fluorescence was then assessed by confocal microscopy and flow cytometry.

6. Comet assay - single cell DNA gel electrophoresis

Apoptotic thymocytes were induced in infantile 50g rats by the intraperitoneal injection of 5mg/kg prednisolone hemisuccinate (Sigma) and live incubation for four hours. Animals were sacrificed, thymuses dissected and cell suspensions ($10^4$ $ml^{-1}$) obtained in 2mM EDTA and washed in cold PBS. 0.5ml cell suspension were placed in a 3ml tube and 1.5ml 1% low gelling temperature agarose (Sigma type VII in distilled water at 40°C) added. The contents were rapidly pipetted onto a glass microscope slide and allowed to gel for one minute. Viable lymphocytes (controls) were obtained from peripheral human blood and prepared identically.

Comet assays were performed according to the method described by Olive et al in 1992. For neutral cell lysis (for the detection of *double stranded DNA breaks*) slides were submersed in lysing solution containing 30mM EDTA, 0.5% sodium dodecyl sulphate, pH 8.0 and the temperature was raised to 50°C for four hours Slides were washed free of detergent in a large volume of TBE buffer ( 90 mM Tris, 2mM EDTA, 90mM boric acid, pH 8.5) for 16 hours prior to electrophoresis in TBE buffer at 0.6 $Vcm^{-1}$ for 25 minutes.

For the detection of *single stranded DNA breaks* slides were immersed in an alkaline lysis solution containing 1M NaCl, 0.03M NaOH and 0.1% sarkosyl for one hour at 25°C. Slides were rinsed by submersion for one hour in 0.03M NaOH, 1mM EDTA. Alkaline electrophoresis was conducted in a fresh solution of 0.03M NaOH, 2mM EDTA at 0.6 $Vcm^{-1}$ for 25 minutes and slides subsequently neutralised with a large volume of water.

Slides were stained for 30 minutes either in 2.5$\mu gml^{-1}$ propidium iodide, 100$\mu gml^{-1}$ epirubicin or 100$\mu gml^{-1}$ idarubicin and resultant 'comets' viewed by confocal microscopy and compared with controls.

## 3. Assessment of MDR Patterns in Bladder Tumour Explants

Hypothesis

Patterns of epirubicin uptake seen in MDR cells are also seen in human bladder cancer explants.

Aims & Objectives

1. To establish a methodology for the culture of human bladder tumour explants obtained ex vivo.
2. To examine patterns of epirubicin uptake in these colonies by confocal microscopy.
3. To compare cellular epirubicin handling within explants with that seen in MDR and epirubicin-sensitive cell lines.
4. To examine any correlation between epirubicin handling and relevant clinical parameters such as previous cytotoxic drug exposure, tumour grade and stage etc.
5. To examine the potential for MDR modulation in tumours exhibiting resistant features.

Demographics

Biopsy material was obtained from 57 patients with clinical evidence of bladder tumours - 44 men and 13 women (ratio 3.4 : 1). The mean age at time of biopsy was 74 (range 46 to 93) years. 36 (63%) patients admitted to a current or past history of cigarette smoking. Two (male) patients had identifiable occupational risk factors (one having been an industrial chemist, the other a marine engineer) but both were also smokers.

In patients with a previous bladder tumour history, the mean time for which the patients had had TCC bladder was 3.5 (range 0 - 20) completed years. *See Table 1.*

Tumour histopathology

Specimens were obtained from 21 newly presenting patients. Previous bladder tumour histology reports were available for 29 patients, and in the remaining seven patients with previous tumours this information was unavailable. Current tumour histopathology was available from 43 (75%) patients but was not obtainable for the remaining 14.

In two patients from whom a specimen was obtained there was no evidence of malignancy (inflammatory change only), despite a clinical suspicion thereof. All remaining patients, with histologically confirmed tumours, had transitional cell carcinomata except three (two adenocarcinomata, one squamous carcinoma).

The stage/grade analysis of the current tumours with available histology is given in table 1. Thirteen tumours were identified as Ta, sixteen were T1 and nine were T2. Twelve were graded G1, thirteen were G2 and thirteen were G3. Six tumours were described as T1G3. Only one patient had evidence of carcinoma in situ (CIS), and this was in the presence of a T1G3 tumour.

Table 1 summarises current and also previous (that for which the last histopathological report was available) tumour pathology. In seven patients there was evidence of progression (i.e. increase) in either stage or grade.

| Patient | Age | Smoking | Sex | Culture | Assay | Tumour | Prev tum | Tum yrs | DXT | Chemo |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 78 | ex | m | n | n | NO | n/a | 15 | no | no |
| 2 | 60 | yes | m | n | n | Squamous | no | 0 | no | no |
| 3 | 73 | no | m | n | n | T2G3 | T2G3 | 0 | no | no |
| 4 | 74 | yes | m | y | y | n/a | T1G3 | 17 | no | Epi/BCG |
| 5 | 84 | yes | m | y | n | T1G2 | T1G2 | 5 | no | Epi |
| 6 | 91 | no | f | n | n | n/a | T1G1 | 9 | no | no |
| 7 | 73 | yes | f | y | y | n/a | TaG1 | 15 | no | no |
| 8 | 85 | yes | m | y | n | n/a | T2G3 | 1 | no | no |
| 9 | 72 | ex | m | y | y | n/a | T1G2 | 11 | no | Epodyl |
| 10 | 74 | ex | m | y | y | n/a | T1G2 | 1 | no | Epi |
| 11 | 76 | no | f | y | y | T1G1 | TaG1 | 6 | no | Epi/BCG |
| 12 | 93 | yes | f | y | y | Adeno | no | 0 | no | no |
| 13 | 83 | ex | m | n | n | n/a | TaG1 | 3 | no | Epi |
| 14 | 91 | no | m | y | y | n/a | no | 0 | no | no |
| 15 | 70 | ex | m | y | y | T1G2/3 | no | 0 | no | no |
| 16 | 61 | yes | m | n | n | T2G3 | T2G3 | 0 | no | CMV |
| 17 | 83 | no | f | n | n | Adeno | no | 0 | no | no |
| 18 | 72 | yes | m | y | n | T1G2 | n/a | 0 | no | Epi |
| 19 | 73 | yes | f | y | y | T1G2 | T1G2 | 9 | no | Epi/BCG |
| 20 | 74 | ex | m | n | n | T1G3+cis | T1G3+cis | 6 | no | BCG |
| 21 | 82 | ex | m | y | y | T1G3 | T1G2 | 5 | yes | Epi |
| 22 | 84 | no | m | y | y | T2G2 | no | 0 | no | no |
| 23 | 75 | yes | m | n | n | n/a | n/a | n/a | no | n/a |
| 24 | 75 | ex | m | y | y | T2G2 | no | 0 | no | no |
| 25 | 80 | no | m | y | y | T1G3 | T1G2 | 1 | yes | no |
| 26 | 60 | ex | m | n | n | T2G3 | no | 0 | no | no |
| 27 | 74 | yes | m | y | y | n/a | T1G1 | 12 | yes | Epi |
| 28 | 70 | yes | m | n | n | n/a | n/a | 5 | no | no |
| 29 | 82 | yes | f | y | y | T2G2 | n/a | n/a | no | n/a |
| 30 | 82 | no | m | n | n | T1G2 | no | 0 | no | no |
| 31 | 67 | ex | m | y | n | T1G2/3 | no | 0 | no | no |
| 32 | 85 | ex | m | y | y | TaG1 | no | 0 | no | no |
| 33 | 67 | yes | m | y | y | TaG1 | TaG2 | 0 | no | no |
| 34 | 72 | ex | f | y | y | TaG1 | no | 12 | no | no |
| 35 | 67 | no | m | y | y | TaG1 | no | 0 | no | no |
| 36 | 62 | ex | m | y | y | n/a | TaG2 | 2 | no | Epi |
| 37 | 75 | no | f | y | y | TaG1 | no | 0 | no | no |
| 38 | 59 | no | m | y | y | T1G1 | no | 0 | no | no |
| 39 | 79 | ex | m | y | y | TaG2 | TaG2 | 13 | no | Epo+Epi |
| 40 | 75 | ex | m | y | n | TaG2 | T1G2 | 1 | no | Epi |
| 41 | 83 | no | f | n | n | T1G2 | no | 0 | no | no |
| 42 | 75 | no | m | y | y | T1G1 | no | 0 | no | no |
| 43 | 46 | ex | f | y | n | T1G1 | TaG1 | 4 | no | Epi |
| 44 | 67 | no | m | n | n | n/a | TaG2 | 1 | no | Epi |
| 45 | 73 | no | m | n | n | T2G3 | no | 0 | no | no |
| 46 | 52 | no | m | y | y | TaG2 | TaG1 | 1 | no | no |
| 47 | 52 | no | m | y | y | T2G3 | T2G3 | 0 | no | no |
| 48 | 78 | no | m | y | y | TaG1 | TaG1 | 1 | no | no |
| 49 | 81 | ex | m | n | n | NO | no | 0 | no | no |
| 50 | 76 | ex | m | y | y | T1G3 | T1G2 | 0 | no | no |
| 51 | 85 | no | m | n | n | T1G2 | n/a | 20 | no | no |
| 52 | 66 | yes | m | y | y | TaG1 | n/a | 11 | no | Epo+Epi |
| 53 | 76 | ex | m | y | y | T2G3 | no | 0 | no | no |
| 54 | 72 | no | f | y | y | TaG1 | T1G1 | 0 | no | Epi |
| 55 | 74 | yes | m | y | y | n/a | no | 0 | no | no |
| 56 | 83 | no | f | y | y | T2G3 | T1G3 | 0 | no | Epi |
| 57 | 76 | ex | m | y | y | TaG2 | T1G2 | 4 | no | Epi |

**Table 1: Patient and tumour demographics**

Previous adjuvant therapy

Nineteen patients had received previous intravesical chemotherapy with either epirubicin (16), epodyl (1) or both agents metachronously (2). One patient had previously received CMV (cisplatin, methotrexate and vinblastine) systemic chemotherapy for invasive disease. Four patients had received previous intravesical immunotherapy with BCG (Bacillus Calmette-Guerin), one as a single agent, and three in metachronous combination with epirubicin. Three patients had previously undergone external beam radiotherapy to the pelvis during the course of their disease (see Table 1).

Tumour explant culture

Explants were initially successfully cultured from 40/57 patients, equivalent to 70% total culture success.

Primary explant growth failed in the tumours from the remaining 17 patients. This figure includes specimens which were predominantly stromal and disaggregated poorly. In those where explant growth failed 11 failures occurred during the first half of the experimental period, partly, therefore, reflecting changing expertise in culture techniques. Four of these failed tumours represented those obtained from four patients (all male) undergoing cystectomy (two TCC's, one squamous carcinoma and one with no available histology report) rather than transurethral surgery. Two further failed explants had been obtained from the patients with no evidence of malignancy.

Successful cultures were grown from 13/13 (100%) Ta tumours, from 12/16 (75%) T1 tumours and from 5/9 (55.5%) T2 tumours respectively (see Table 1). A

similar relationship existed between tumour grade and culture success - 12/12 (100%) G1 tumours, 10/13 (77%) G2 tumours and 8/13 (61.5%) G3 tumours yielded explants in culture. All six tumours exhibiting stage/grade progression were cultured successfully, with no failures. In contrast 31/45 (69%) non-progressing tumours yielded successful cultures, with failures in the remaining 14.

Culture success was obtained in the explants from 75% (27/36) current or ex-smokers, and from 62% (13/21) non-smokers. Previous exposure to cytotoxic agents had no detrimental effect on colony growth, with 16/18 (89%) cultures successful in those previously exposed to epirubicin, 3/3 with prior epodyl exposure and 3/4 with prior BCG exposure. No explants were grown from the tumour with previous exposure to systemic CMV. All three tumours from patients who had received external beam radiotherapy yielded successful cultures.

Explant morphology

Successful explants developed epithelial 'skirts' over one to three days. Colony development to this stage was largely by cell migration with some cultures then going into a period of expansion by division. All became senescent by one month, and were then destroyed.

Four examples are shown in figure 1 as phase contrast photomicrographs. A common feature is the raised or rolled periphery, of which figure 1A is a dramatic example. Figure 1B shows a large explant with a small skirt; figure 1 C a more diffuse pattern of small interlacing colonies more closely resembling culture-adapted cells. Figure 1D illustrates a large proliferating colony with small remnants of original explant.

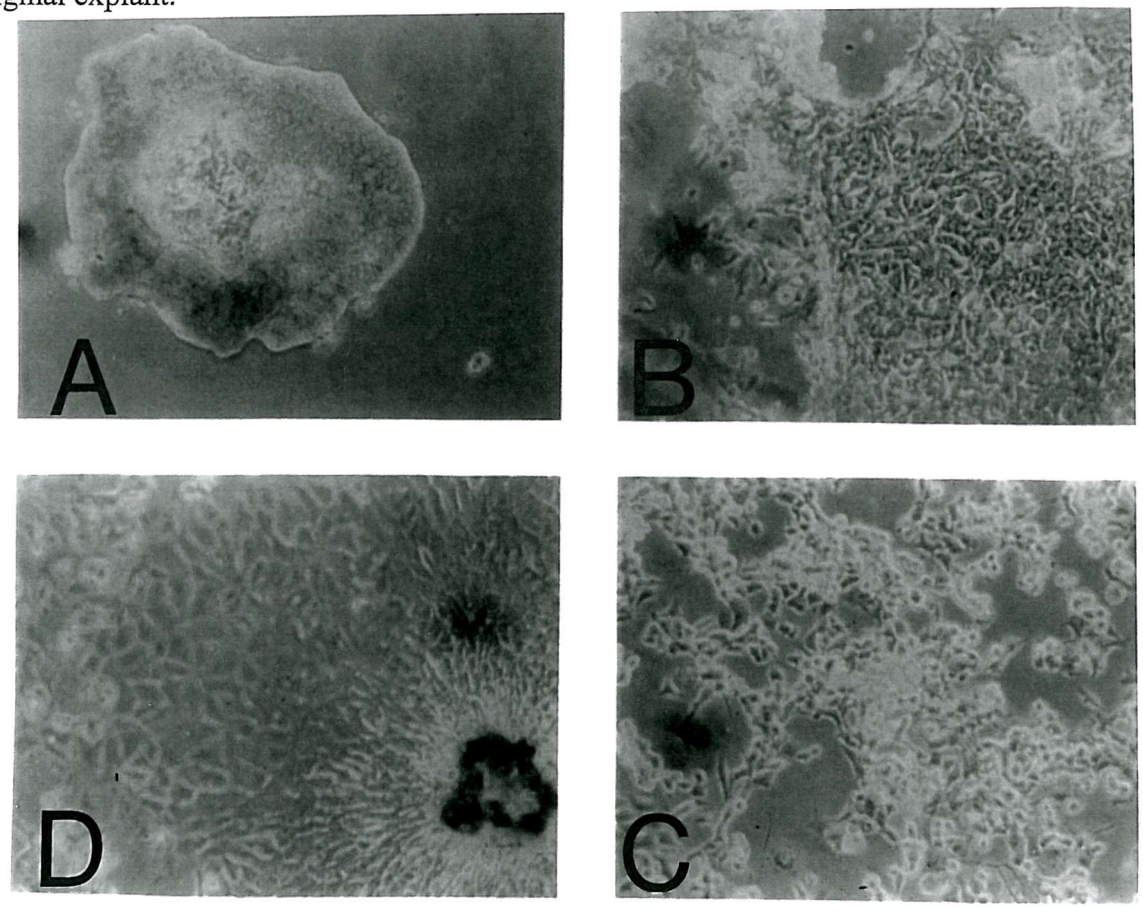



Figure 1 - Phase contrast photomicrograph of explant colonies - see text

Figure 2

Colony topography - a 3D reconstruction in rotation (four images A to D). Epirubicin fluorescence.

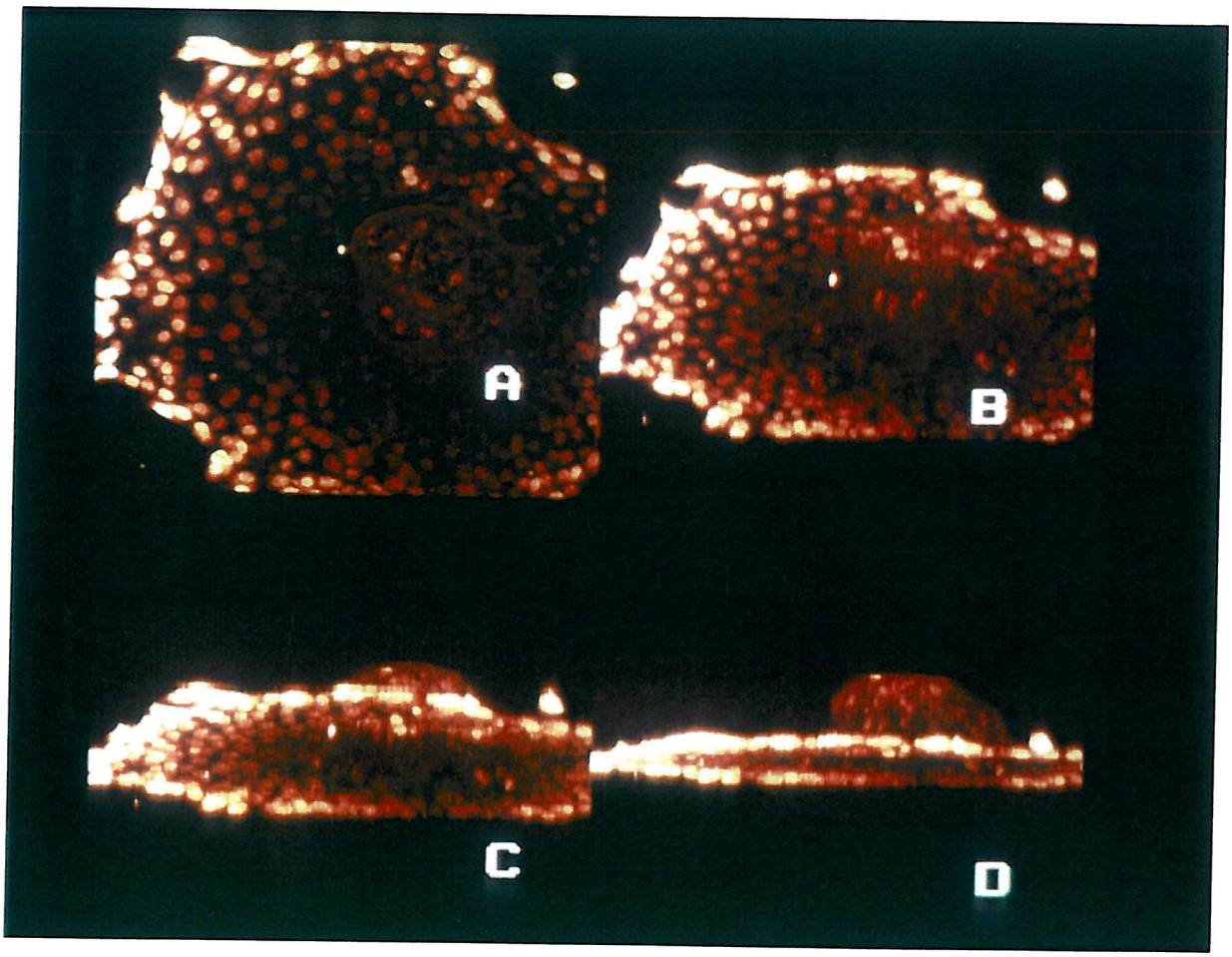
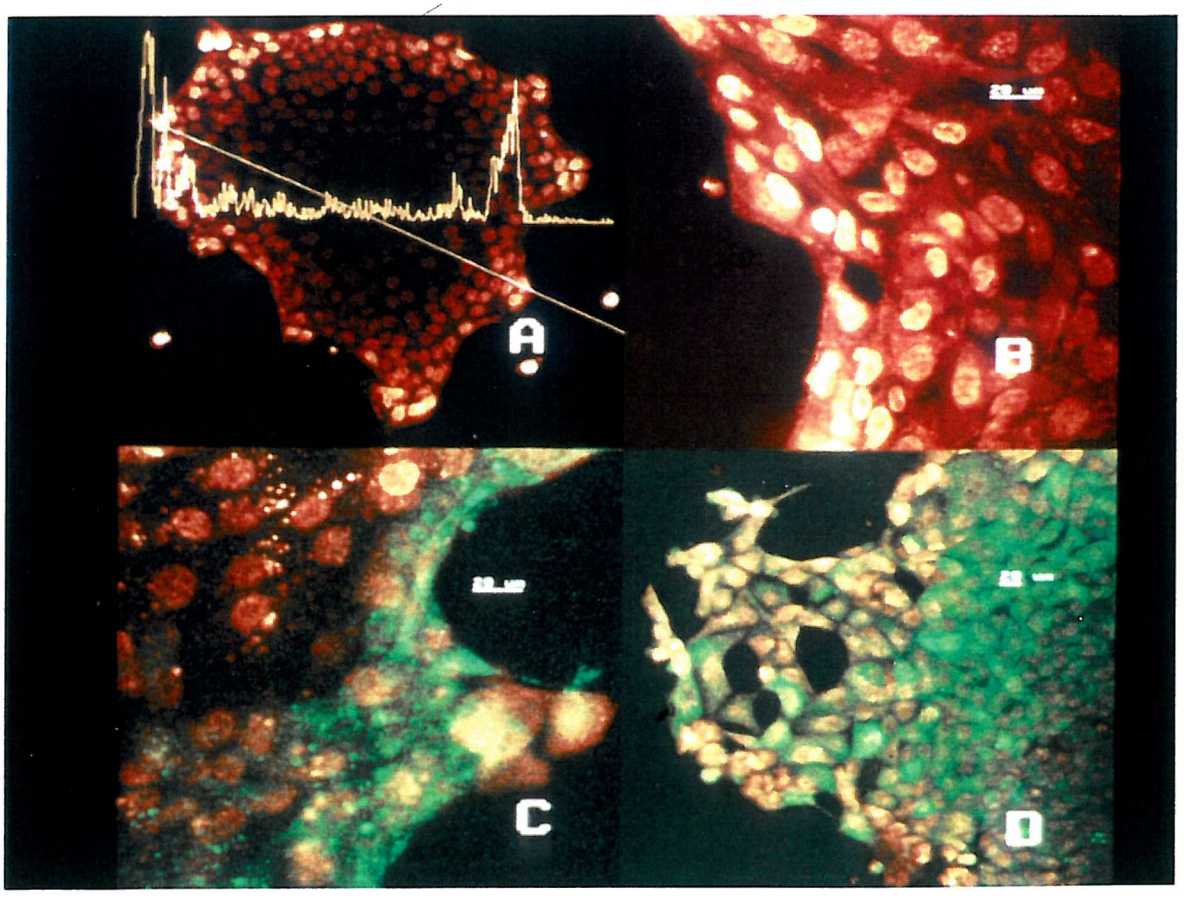


Figure 3

Tumour colony features - *A* epirubicin fluorescence with fluorescence density profile overlaid; *B* same feature magnified; *C* PKH2-GL fluorescence at colony edge; *D* FDA uptake confirming viability.

Scale bars indicate 20 microns.



Confocal colony topography and associated phenomena

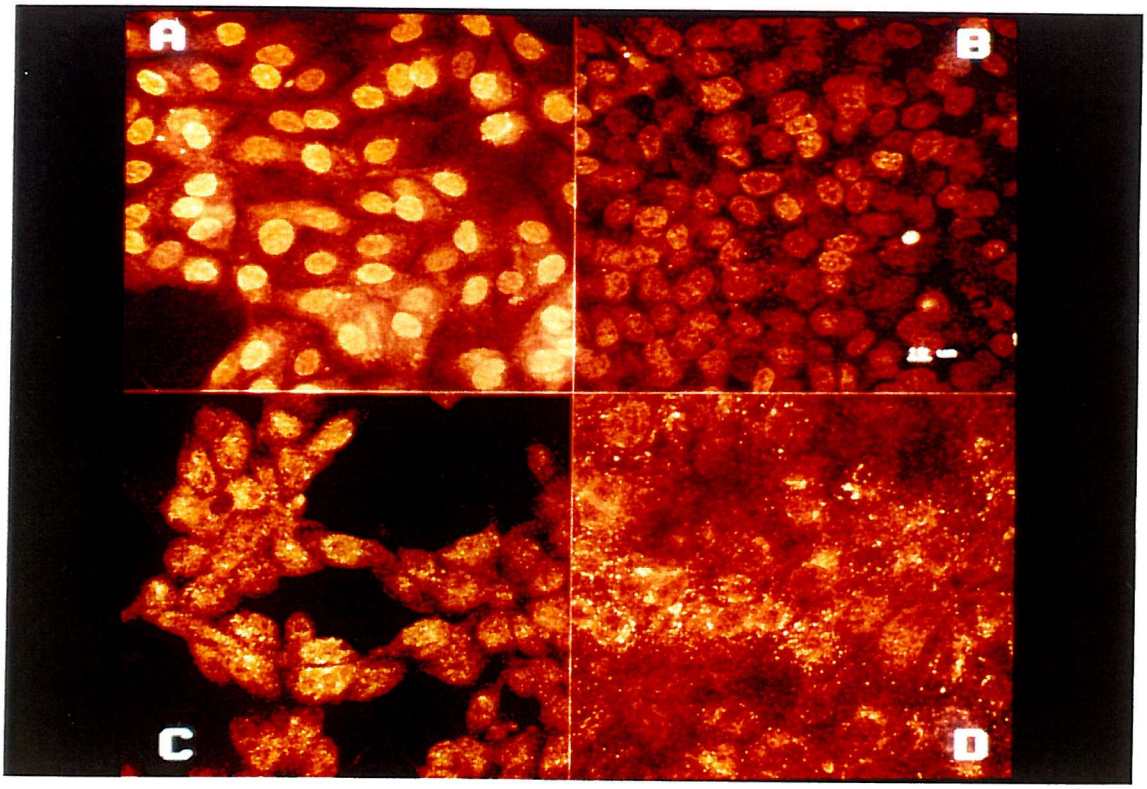
The topography of a typical colony with central explant is demonstrated by figure 2, a confocal 3-D reconstruction viewed laterally, visualised by its uptake of (fluorescent) epirubicin, showing a bright rolled edge to the organised monolayer epithelial skirt around the raised central explant, which is largely denuded of cells.

Figure 3A is a single confocal section across a typical colony viewed from above, with a fluorescence density profile overlaid. It illustrates a common feature whereby cells at the expanding edge take up more drug (usually in the sensitive pattern) then cells in the interior of the epithelial colony, regardless of residual stroma. A single confocal slice through another edge is shown at higher magnification in figure 3B. All integrated and adherent cells in a colony are viable. Dead or dying cells detach, appearing only in the uppermost confocal slices and accumulating nuclear drug rapidly even at ambient temperature, in the fashion of a propidium iodide viability assay.

A similar 'edge effect' was obtained after prior incubation with the fluorescent membrane phospholipid analogue PKH2-GL (figure 3C). In contrast, the viability marker FDA entered all cells within the colony (figure 3D), although in this illustration some FDA fluorescence is masked by the dominant epirubicin fluorescence at the colony edge. MGHU-1 and MGHU-1R cells, even grown to confluence, never exhibit this edge effect for either epirubicin or PKH2-GL. Interestingly, scoring across a colony with a soft instrument 0 - 24 hours before drug exposure induces a new edge effect in cells which were previously centrally situated. Additionally, small clumps of non-adherent tumour cells immobilised in agarose also appear uniformly bright.

Figure 4

Tumour colony MDR scoring - examples of colonies scoring S1 (top left) S2 (top right) M3 (bottom left) and R4 (bottom right). Epirubicin fluorescence.



Primary confocal MDR assay

34/40 (85%) of cultures yielded at least one primary assay (39 assays in total), representing 34/57 (60%) patients (see Table 2). Subsequent technical difficulties (culture infection, incubator malfunction, confocal microscope malfunction etc.) precluded completion of the assay in the remaining six cases.

Explants were scored according to the following schema (see figure 4):

**S1** *sensitive, with all cells demonstrating nuclear epirubicin uptake.*

**S2** *predominantly sensitive, with >50% uptake into nuclei.*

**M3** *mixed pattern, with some features of both phenotypes.*

**R4** *resistant, with minimal nuclear epirubicin uptake and granular cytoplasmic uptake predominant.*

No tumour colonies were entirely 'resistant' throughout, some were entirely 'sensitive'.

Of the 39 completed assays 9 (23%) were scored S1, 16 (41%) S2, 9 (23%) M3 and 5 (13%) R4 colonies. In five cases sufficient cultures were available to perform two primary assays. In no case did the two assays correspond exactly (see table 2) but the pairs were all either intermediate and sensitive, or intermediate and resistant.

| Patient | Tumour | Prog | Chemo | Result 1 | Colonies | Result 2 | Colonies | Wks | Pavement |
|---|---|---|---|---|---|---|---|---|---|
| 1 | NO | no | no | nr | | | | | |
| 2 | Squamous | no | no | nr | | | | | |
| 3 | T2G3 | no | no | nr | | | | | |
| 4 | n/a | no | Epi/BCG | S1 | 1 | no | | | |
| 5 | T1G2 | no | Epi | nr | | | | | |
| 6 | n/a | no | no | nr | | | | | |
| 7 | n/a | no | no | S2 | 1 | no | | | |
| 8 | n/a | no | no | nr | | | | | |
| 9 | n/a | no | Epodyl | S2 | 1 | S2 | 1 | 1 | |
| 10 | n/a | no | Epi | S1 +M3 | 2 | no | | | |
| 11 | T1G1 | yes | Epi/BCG | S1 | 1 | S2 | 2 | 1 | |
| 12 | Adeno | no | no | S2 | 3 | M3 | 1 | 3 | |
| 13 | n/a | no | Epi | nr | | | | | |
| 14 | n/a | no | no | R4 | 1 | no | | | |
| 15 | T1G2/3 | no | no | S2 | 1 | no | | | |
| 16 | T2G3 | no | CMV | nr | | | | | |
| 17 | Adeno | no | no | nr | | | | | |
| 18 | T1G2 | ? | Epi | nr | | | | | |
| 19 | T1G2 | no | Epi/BCG | S1 | 1 | S1 | 4 | 1 | yes |
| 20 | T1G3+cis | no | BCG | nr | | | | | |
| 21 | T1G3 | yes | Epi | S1+2 | 1 | no | | | |
| 22 | T2G2 | no | no | S2+M3 | 1 | R4 | 1 | 2 | |
| 23 | n/a | ? | n/a | nr | | | | | |
| 24 | T2G2 | no | no | M3 | 1 | S1 | 2 | 2 | |
| 25 | T1G3 | yes | no | S1 | 1 | S1 | 2 | 3 | |
| 26 | T2G3 | no | no | nr | | | | | |
| 27 | n/a | no | Epi | S1 | 1 | no | | | |
| 28 | n/a | ? | no | nr | | | | | |
| 29 | T2G2 | ? | n/a | S1 | 1 | no | | | |
| 30 | T1G2 | no | no | nr | | | | | |
| 31 | T1G2/3 | no | no | nr | | | | | |
| 32 | TaG1 | no | no | M3 | 1 | no | | | |
| 33 | TaG1 | no | no | R4 | 1 | S2 | 3 | 2 | |
| 34 | TaG1 | no | no | S2 | 1 | R4+S1 | 2 | 1 | |
| 35 | TaG1 | no | no | S2 | 1 | no | | | |
| 36 | n/a | no | Epi | M3 | 2 | S2+S1 | 2 | 1 | |
| 37 | TaG1 | no | no | S2 | 1 | S2 | 1 | 1 | yes |
| 38 | T1G1 | no | no | S1 | 1 | no | | | |
| 39 | TaG2 | no | Epo+Epi | R4 | 3 | no | | | |
| 40 | TaG2 | no | Epi | nr | | | | | |
| 41 | T1G2 | no | no | nr | | | | | |
| 42 | T1G1 | no | no | R4+M3 | 2 | no | | | yes |
| 43 | T1G1 | yes | Epi | nr | | | | | |
| 44 | n/a | no | Epi | nr | | | | | |
| 45 | T2G3 | no | no | nr | | | | | |
| 46 | TaG2 | no | no | R4 | 4 | no | | | yes |
| 47 | T2G3 | no | no | M3+S2 | 2 | no | | | yes |
| 48 | TaG1 | no | no | S2 | 1 | no | | | |
| 49 | NO | no | no | nr | | | | | |
| 50 | T1G3 | yes | no | M3 | 1 | no | | | |
| 51 | T1G2 | ? | no | nr | | | | | |
| 52 | TaG1 | ? | Epo+Epi | S2 | 1 | no | | | yes |
| 53 | T2G3 | no | no | S2 | 1 | S2 | 1 | 1 | |
| 54 | TaG1 | no | Epi | S2 | 1 | no | | | |
| 55 | n/a | no | no | M3 | 2 | S2+M3 | 2 | 3 | |
| 56 | T2G3 | yes | Epi | S2 | 1 | S2 | 1 | 1 | |
| 57 | TaG2 | no | Epi | S2 | 1 | no | | | |

**Table 2: Pathology, colony growth, MDR scores and associated features**

MDR score was examined for any correlation with *tumour stage*, as shown below:

| | S1 | S2 | M3 | R4 |
|---|---|---|---|---|
| **Ta** | 0 | 7/11 (64%) | 1/11 (9%) | 3/11 (27%) |
| **T1** | 5/10 (50%) | 2/10 (20%) | 2/10 (20%) | 1/10 (10%) |
| **T2** | 1/8 (12.5%) | 4/8 (50%) | 3/8 (37.5%) | 0 |

**Table 3**

Although emphasising the tendency towards phenotypic sensitivity no significant correlation of score and stage was seen.

Similarly no apparent correlation existed between MDR phenotype score and *tumour grade*, as shown below:

| | S1 | S2 | M3 | R4 |
|---|---|---|---|---|
| **G1** | 2/12 (17%) | 6/12 (50%) | 2/12 (17%) | 2/12 (17%) |
| **G2** | 2/8 (25%) | 2/8 (25%) | 2/8 (25%) | 2/8 (25%) |
| **G3** | 2/9 (22%) | 5/9 (56%) | 2/9 (22%) | 0 |

**Table 4**

No apparent relationship existed between tumour score and *progression*, in the 37 explants where these data were available. In the tumours which progressed 3/6 were S1, 2/6 S2 and 1/6 M3 with no resistant tumours. In the non-progressing tumours 5/31 were scored S1, 13/31 were S2, 8/31 were M3 and there were 5/31 R4 tumours.

Primary MDR score was examined in relation to *previous chemo/immuno/radiotherapy*, as shown below:

**Table 5**

| | S1 | S2 | M3 | R4 |
|---|---|---|---|---|
| **Epirubicin** | 6/14 | 5/14 | 2/14 | 1/14 |
| **Epodyl** | 0/3 | 2/3 | 0/3 | 1/3 |
| **BCG** | 3/3 | 0/3 | 0/3 | 0/3 |
| **CMV** | 0 | 0 | 0 | 0 |
| **DXT** | 3/1 | 1/4 | 0 | 0 |
| **No therapy** | 2/23 | 10/23 | 7/23 | 4/23 |

No discernible difference in MDR score was detectable between these subgroups although 4/6 (67%) R4 and 7/9 (78%) M3 tumours respectively had received no prior exposure to cytotoxic agents, implying intrinsic MDR phenotypic tendencies.

The *male/female and smoking/non-smoking* MDR score distributions were comparable, although no M3 or R4 tumours were grown from women, as shown:

**Table 6**

| | S1 | S2 | M3 | R4 |
|---|---|---|---|---|
| **Male** | 6/29 (21%) | 9/29 (31%) | 9/29 (31%) | 5/29 (17%) |
| **Female** | 3/10 (33%) | 7/10 (70%) | 0 | 0 |
| **Smoker** | 6/23 (26%) | 9/23 (39%) | 6/23 (26%) | 2/23 (9%) |
| **Non-smoker** | 3/16 (19%) | 7/16 (44%) | 3/16 (19%) | 3/16 (19%) |

For those explants from which *sequential MDR assays* were done, the tumour pathology, primary and secondary MDR scores and time lapsed between first and second assay are summarised below:

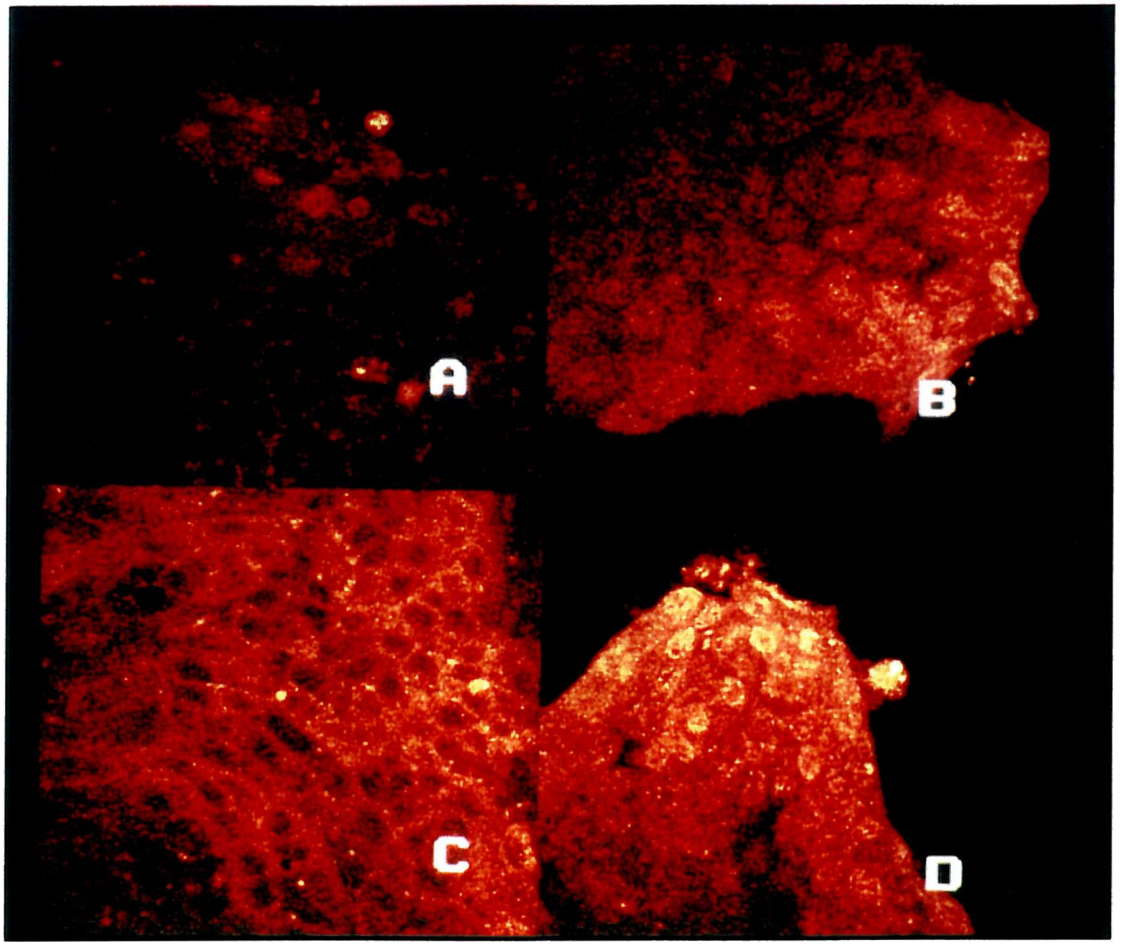
| Patient | Pathology | Score 1 | Score2 | Time lapse (weeks) |
|---|---|---|---|---|
| 9 | n/a | S2 | S2 | 1 |
| 11 | T1G1 | S1 | S2 | 1 |
| 12 | Adeno | S2 | M3 | 3 |
| 19 | T1G2 | S1 | S1 | 1 |
| 22 | T2G2 | S2+M3 | R4 | 2 |
| 24 | T2G2 | M3 | S1 | 2 |
| 25 | T1G3 | S1 | S1 | 3 |
| 33 | TaG1 | R4 | S2 | 2 |
| 34 | TaG1 | S2 | R4+S1 | 1 |
| 36 | n/a | M3 | S2+S1 | 1 |
| 37 | TaG1 | S2 | S2 | 1 |
| 53 | T2G3 | S2 | S2 | 1 |
| 55 | n/a | M3 | S2+M3 | 3 |
| 56 | T2G3 | S2 | S2 | 1 |

**Table 7**

7/17 colonies scored the same, 4/17 scored more sensitive, and 6/17 more resistant on second assay. The time lapsed between assays had no effect on outcome.

Figure 5

Confocal photomicrographs of MDR reversal phenomena - *A* resistant colony with epirubicin, *B* resistant colony with epirubicin and verapamil, *D* resistant colony with PSC-833 and C example of pavement phenomenon.



Assay of MDR modulation by verapamil

Ten explants (11 colonies) underwent confocal epirubicin localisation assay in the presence of verapamil 100μg/ml.

| Patient | Tumour | Prog | Chemo | R: no ver | R: +ver | Trend |
|---|---|---|---|---|---|---|
| 11 | T1G1 | yes | Epi/BCG | S1 | S1 | no |
| 22 | T2G2 | no | no | S2+M3 | S1 | to sens |
| 32 | TaG1 | no | no | S2 | S2 | no |
| 39 | TaG2 | no | Epo+Epi | R4 | S1 | to sens |
| 46 | TaG2 | no | no | R4 | M3 | to sens |
| 47 | T2G3 | no | no | M3+S2 | S2 | no |
| 48 | TaG1 | no | no | S2 | S1 | to sens |
| 50 | T1G3 | yes | no | M3 | S1+S2 | to sens |
| 52 | TaG1 | ? | Epo+Epi | S2 | S2 | no |
| 56 | T2G3 | yes | Epi | S2 | S2 | no |

**Table 8**

Five (50%) tumours were graded more sensitive by the assay in the presence of verapamil, when compared to the epirubicin-alone tumours. Epirubicin uptake in the remaining 50% remained unaffected. No tumour was graded more resistant after co-incubation with verapamil. An example of colony MDR modulation by verapamil is shown in figure 5.

Of the seven colonies with initially more sensitive uptake characteristics (graded S1 and S2) two were graded more sensitive with verapamil.

Of the five colonies with initially intermediate/resistant characteristics (M3, R4) all were graded more sensitive with verapamil. The most dramatic change was from R4 to S1 in one tumour.

No significant correlation was apparent between either tumour grade/progression or previous cytotoxic exposure and MDR modulation effect. Interestingly, MDR modulation was only demonstrable in one of the four tumours with previous cytotoxic drug exposure.

Assay of MDR modulation by PSC 833

Eight explants underwent confocal epirubicin localisation assay (see table 9) in the presence of PSC 833 (Sandoz).

| Patient | Tumour | Prog | Chemo | R:no PSC | R:+PSC | Trend |
|---|---|---|---|---|---|---|
| 22 | T2G2 | no | no | S2+M3 | S1 | to sens |
| 42 | T1G1 | no | no | R4+M3 | M3 | no |
| 46 | TaG2 | no | no | R4 | S2 | to sens |
| 48 | TaG1 | no | no | S2 | S1 | to sens |
| 50 | T1G3 | yes | no | M3 | S1 | to sens |
| 52 | TaG1 | ? | Epo+Epi | S2 | S2 | no |
| 55 | n/a | no | no | M3 | M3 | no |
| 56 | T2G3 | yes | Epi | S2 | S2 | no |

**Table 9**

Four (50%) tumours were graded more sensitive by the assay in the presence of PSC 833, when compared to the epirubicin-alone tumours. Epirubicin uptake in the remaining 50% remained unaffected. Again no tumour was graded more resistant after co-incubation with PSC 833. An example of colony MDR modulation by PSC 833 is given in figure 5.

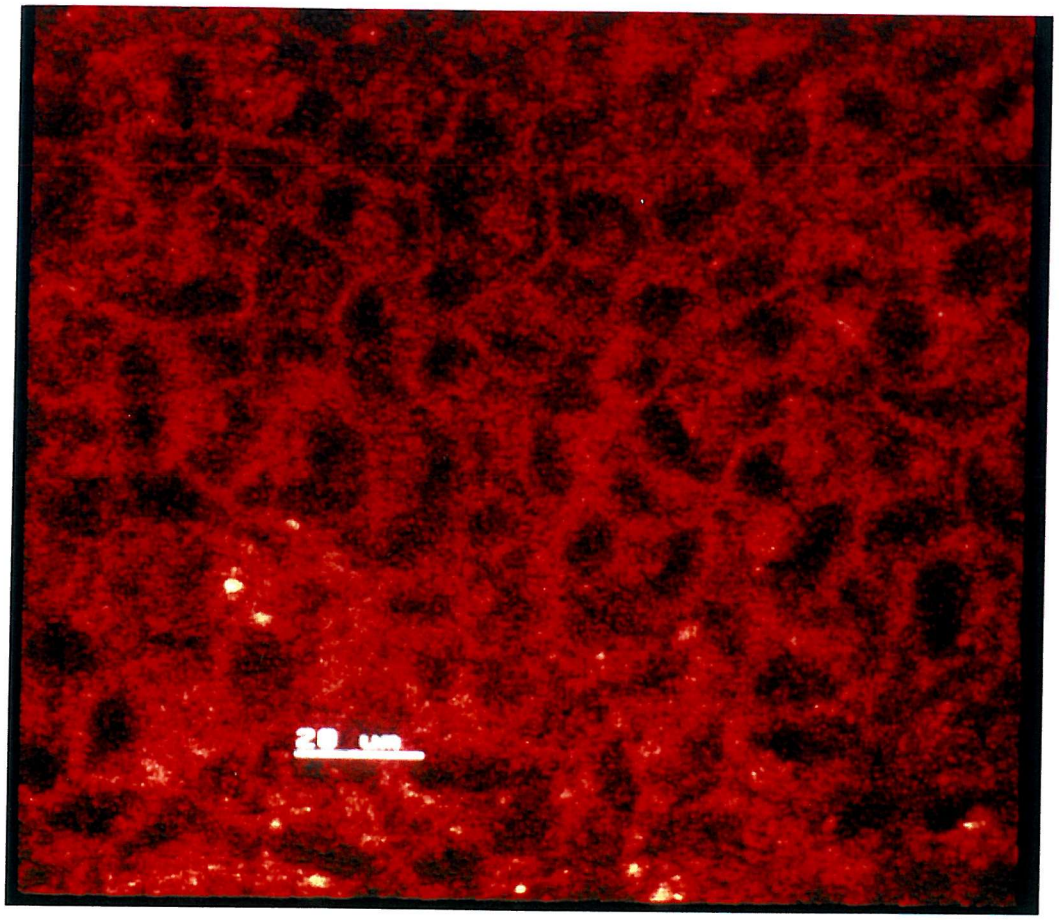
Of the four colonies with initially more sensitive uptake characteristics (graded S1 and S2) two were graded more sensitive with PSC 833.

Of the six colonies with initially intermediate/resistant characteristics (M3, R4) five (83%) were graded more sensitive with PSC 833.

No significant correlation was apparent between either tumour grade/progression or previous cytotoxic exposure and MDR modulation effect, although MDR modulation was not demonstrated in either tumour from the patients who had previously received intravesical chemotherapy.

Figure 6

Pavement phenomenon - fluorescence of membrane bound or intercellular epirubicin seen centrally in some colonies. Scale bar 20 microns.



Pavement phenomenon

The phenomenon of intercellular (perhaps membrane fixed), rather than intracellular epirubicin fluorescence was seen towards the centre of six larger and more proliferative explant colonies (four primary and two secondary assays) as summarised in table 10, and illustrated in figure 6.

In these cells total fluorescence is low, but faint nuclear fluorescence is occasionally seen. This fluorescence pattern has not been described in association with cell line cultures.

**Table 10**

| Patient | Tumour | Prog | Chemo | Sex | Smoking | Result 1 | Result 2 | Pavement |
|---|---|---|---|---|---|---|---|---|
| 19 | T1G2 | no | Epi/BCG | f | yes | S1 | S1 | yes |
| 37 | TaG1 | no | no | f | no | S2 | S2 | yes |
| 42 | T1G1 | no | no | f | ex | R4+M3 | no | yes |
| 46 | TaG2 | no | no | m | no | R4 | no | yes |
| 47 | T2G3 | no | no | m | no | M3+S2 | no | yes |
| 52 | TaG1 | ? | Epo+Epi | m | yes | S2 | no | yes |

No association was seen between this phenomenon and any patient/tumour characteristic, other than the fact that no tumour exhibiting pavement fluorescence had progressed in terms of stage or grade since its last histopathological assessment.

Assessment of MDR Patterns in Bladder Tumour Explants - Discussion

The multidrug resistant (MDR) phenotype is known to be associated with a marked reduction in intracellular drug concentrations (Coley et al 1993). This has been demonstrated for several drugs, including epirubicin and other anthracyclines, in many cancer models including the MGHU-1 cell line (Duffy et al 1996) where the MDR phenotye is characterised by the absence of drug from the nucleus, and a predominantly cytoplasmic (perhaps Golgi apparatus) drug distribution. This is in marked contrast to the distribution of drug in non-MDR cells where little cytoplasmic drug is seen and nuclear drug accumulation is profound.

Multidrug resistance is complex and controversy abounds about the relative roles of P-gp, multidrug resistance associated protein (MRP) and other cellular systems (eg. cytoplasmic vaults, associated with lung resistance protein - LRP).
One important functional hypothesis for MDR was made by Izquierdo et al in 1996. They suggested that, as MDR cells distribute anthracyclines primarily into the perinuclear region in a punctate cytoplasmic fashion, it was possible that a perinuclear organelle might have a role in this phenomenon. Schuurhuis had demonstrated in 1991 that reduced nuclear accumulation of daunorubicin occurred in the cell line 2R120, which was subsequently confirmed to overexpress LRP. Izquierdo therefore postulated that, although the perinuclear and cytoplasmic structures to which anthracyclines were distributed in such cells are not known, vaults appeared to be a possible candidate. These are large organelles and are found in the cytoplasm in significant numbers. In addition there is evidence that they may act as plugs of nuclear pore complexes. Izquierdo therefore suggested that vaults might act to regulate the cytoplasmic redistribution and nucleocytoplasmic transport of drugs. Until now, however, little study has been made of anthracycline distribution patterns in human tumours, as almost all such work has involved the use of cancer cell lines.

Superficial bladder cancers offer an ideal tumour type for such work - they are readily accessible, are rarely fatal in the medium term, are frequently recurrent in

nature, and seem to be readily culturable *in vitro* using standard tissue culture techniques and conditions.

The explant technique described above resulted in the successful culture of 70% of explants derived from tumour biopsy specimens. One feature significantly negatively associated with culture success was the apparent extent of diathermy artefact to the biopsy specimen - of 38 specimens documented as being obtained by cold cup biopsy (ie. not resected or diathermised) 33 grew successfully. Interestingly, the occasional necessity for overnight refrigerated (4°C) specimen storage, rather than immediate processing, was not detectably detrimental to the primary culture outcome (indeed the initial cultures from these specimens seemed less contaminated with blood elements and effete squames) although this phenomenon was not explored further.

The inverse relationship between increasing tumour stage and culture success might be explained by the likely presence of more stromal and connective tissue in the higher stage tumours. Similarly, the apparently inverse relationship between increasing tumour grade and culture success may imply that cell-surface and cell-cell adhesion mechanisms may be altered/inhibited in less well differentiated tumours.

Indeed the degree of heterogeneity of epirubicin uptake within an epithelial tumour explant sheet (edge effect) may also reflect the degree of maturity of mutual cellular adhesion and anchorage in more centrally placed cells. These may be more polarised and have assembled a "luminal" surface more able to resist drug or PKH2-GL penetration, yet be permeable to the small (soluble) FDA molecules. If such heterogeneity extrapolates legitimately to tumour *in vivo* it is possible to envisage implications for the design of modified chemotherapy regimes.

It is interesting but not surprising that tumour grade, stage, progression, and prior chemotherapeutic drug exposure did not correlate to MDR phenotype score. Pu et al (1996) demonstrated that although 70% of clinical tumours expressed varying degrees of the MDR-1 gene no correlation existed between its expression and chemotherapy response, although they made no comment about tumour stage and

grade. Similarly Park et al (1994) evaluated P-glycoprotein expression in 29 cystectomy specimens with no prior exposure to chemotherapaeutic agents. They demonstrated that P-gp was detectable in 75% of these specimens, suggesting a high incidence of intrinsic *(de novo)* rather than induced MDR. In addition they established no correlation between P-gp expression and grade stage or tumour morphology, nor any predictive value of the presence of P-gp for likely chemoresponsiveness. Kim et al (1996) studied fifty two urothelial tumour samples using RT-PCR techniques, and demonstrated detectable levels of MDR-1, MRP, GST-π and topoisomerase II, but were again unable to identify any correlation between gene expression and either tumor grade/stage or chemotherapy status, although the expression of GST-π in patients not receiving chemotherapy was higher than in those who did. In addition, evidence exists that the surface cells in a normal established transitional cell epithelium may express P-glycoprotein on the plasma membrane, suggesting a possible role in urothelial protection.

The absence of resistant-phenotype explants in tumours obtained from women is likely simply to reflect chance and the small numbers of such tumours in this series. In addition the variability of the MDR scores where sequential assays were performed seems likely to represent the heterogeneous nature of the explants obtained from tumours rather than any culture-specific tissue adaptation. There is little pattern to such variability, which is also apparent in five of the primary MDR explant assays.

The coincubation of tumour explants with verapamil or PSC 833 in addition to epirubicin resulted in either no change (50%) or a trend towards increased sensitivity, with no tumour being graded more resistant. Although this trend is encouraging for proponents of MDR reversal strategies it should be interpreted with some caution. It was not technically feasible for the same explants to be used for both epirubicin-only and coincubation assays, so this may also represent a heterogeneity phenomenon alone or in concert with MDR reversal.

An unusual pattern of drug uptake occurred immediately surrounding the more central regions of larger and more proliferative explant colonies. This appearance is of apparently intercellular or membrane fixed epirubicin fluorescence, and is termed the pavement phenomenon. In these cells total intracellular drug fluorescence was low, but faint nuclear fluorescence was occasionally seen. This phenomenon was commonly seen through the microscope eyepiece using mercury vapour illumination, but was difficult to capture in the confocal images illuminated from above with laser light, even with extended focus. This phenomenon is most probably related to the polarized nature of laser light but is not seen in cell line cultures. It may perhaps reflect an additional feature of drug distribution in cells with a degree of luminal differentiation, where production of urothelial surface uroplakins (Su et al 1997) may exhibit a protective effect.

In summary, these data confirm that it is possible to culture, with a reasonable degree of reliability, human bladder tumour explants obtained ex vivo. Epirubicin uptake in these colonies is observable when examined by confocal microscopy. Patterns of epirubicin handling within some explants correlate with those seen in MDR and epirubicin-sensitive cell lines, but clearly the phenomenon is more heterogeneous in the former. Importantly, no comment can be made about quantifiable drug sensitivity/resistance in tumours demonstrating 'resistant' features, as no currently available reliable technique exists which would allow for this in such small volumes of tumour material.

No correlation was demonstrable between epirubicin handling and relevant clinical parameters such as previous cytotoxic drug exposure, tumour grade and stage etc. However, numbers in this study are comparatively small and it is possible that such correlations might be observable in a larger series. In addition more information might

be expected to be obtained from patients recruited to this sort of study in a prospective fashion. MDR characterisitics seen in tumours prior to chemotherapeutic drug exposure could then be compared with those from any residual tumour found after resection and intravesical chemotherapy, and thereby more closely correlated with recurrence/progression. This next phase of the study is pending in our department as part of a trial of pH buffered epirubicin intravesical chemotherapy, currently recruiting patients across Wessex (see later).

The potential for MDR modulation in tumours exhibiting resistant features is only hinted at from this study and no more comment can be made with regard to this.

Explant cultures viewed by fluorescence microscopy - perhaps not always necessarily confocal - may therefore be useful in studying, determining and monitoring clinical drug resistance, particularly in tumours with behavioural characteristics such as superficial bladder cancer. It seems unlikely, however, that any one tumour will appear uniformly either sensitive or resistant - indeed it seems likely that tumours may contain some intrinsically resistant cells which will have a survival advantage over drug-sensitive cells (clonal expansion theory), and that other cells within the tumour may develop (acquire) resistance consequent to drug exposure. The effect of cellular organisation on drug handling is of potential interest both to the basic cellular oncologist and to the clinician, who might consider developing strategies to abrogate the reduced uptake illustrated by central cells of *in vitro* colonies, and by cells of the MDR phenotype.

## 4. Mitomycin C Resistance

Hypothesis

Induction of resistance to mitomycin C in a urothelial cancer cell line confers the MDR genotype and phenotype.

Aims & Objectives

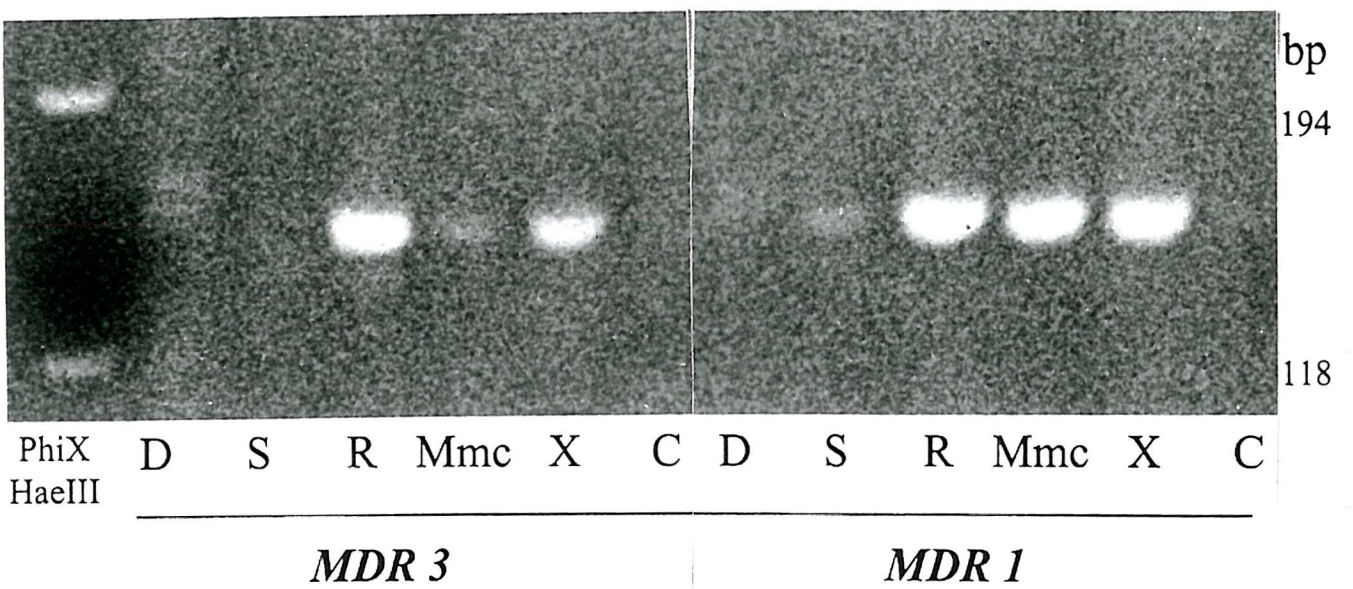
1. To induce resistance to mitomycin C in wild type MGHU-1 cells.
2. To examine the expression of MDR-1 and MDR-3 in wild-type (MGHU-1), established MDR (MGHU-1R) and mitomycin C resistant cells (MGHU-MMC).
3. To examine the sensitivity of these cell lines to epirubicin and mitomycin C.
4. To seek, by implication, evidence that mitomycin C is an MDR class drug.

Expression of MDR-1 and MDR-3 by MGHU-1 related cell lines

The expressions of MDR-1 and MDR-3 mRNA (coding for P-gp 1 and P-gp 2 respectively) by MGHU-1, MGHU-1R and MGHU-MMC are shown in Figure 8 overleaf. The 8% non-denaturing polyacrylamide gel had been run at 40mA for two hours, stained with ethidium bromide and viewed under ultraviolet illumination. Lane 1 $\phi$X 174RF Hae III DNA marker. Visible band sizes are, as indicated, 194 and 118 bp. Lanes 2-7 represent MDR-3 expression. Lanes 8-13 represent MDR-1 expression. Lanes D contain commercial DNA controls, lanes C contain water (negative controls). Lanes marked S, R and Mmc contained DNA from MGHU-1, MGHU-1R and MGHU-MMC respectively. Lanes X represent mixed S, R and Mmc DNA (positive controls).

Figure 8

MDR-1 and MDR-3 expression in MGHU-1 and its MDR clones (see preceding text).



The expected product size for both MDR-1 and MDR-3, using the primers designed for this project, was *145 base pairs*. It can be seen from figure 8 that the product length falls almost exactly between 118 and 194 bp according to the standard Hae III DNA marker.

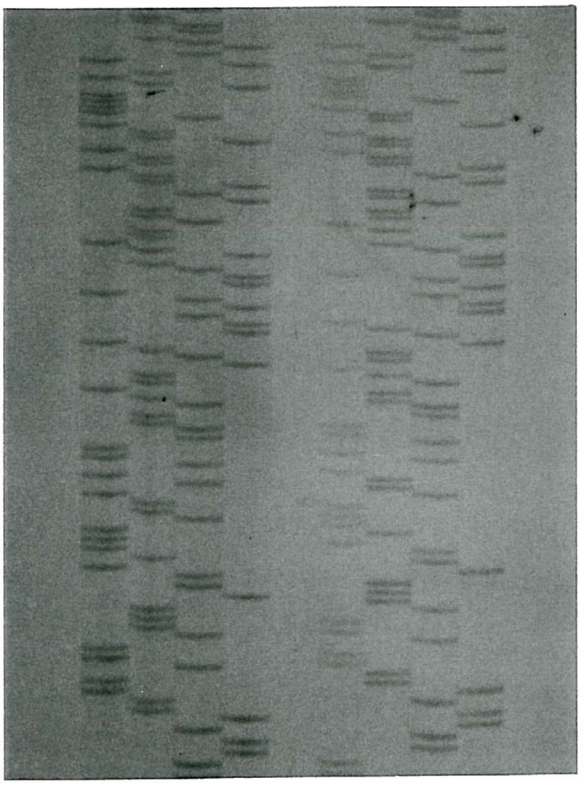
**MDR-1** is significantly overexpressed by both MGHU-1R and MGHU-MMC. It is also present in low concentrations in wild-type MGHU-1. It is absent in the control DNA and, by definition, in the negative controls.

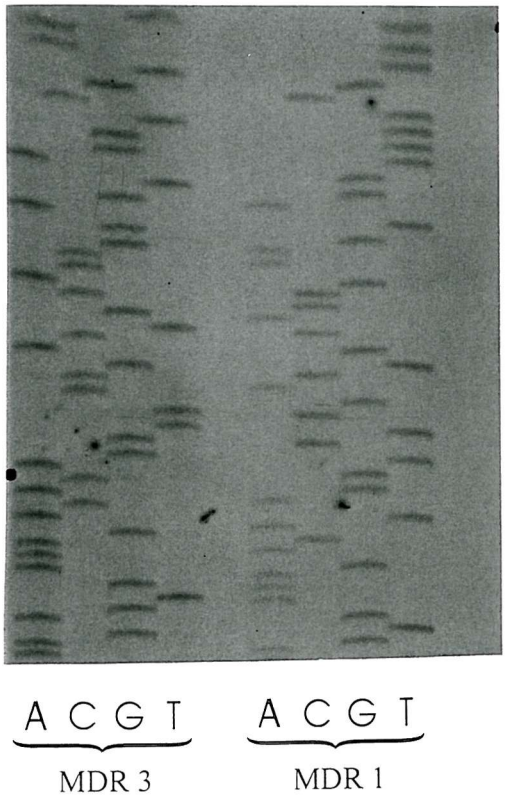
In contrast, **MDR-3** is significantly overexpressed by MGHU-1R only. It is present, but in very low concentrations, in MGHU-MMC, and seems to be entirely absent in wild-type MGHU-1. Again it is absent in negative controls and control DNA.

Confirmation that these bands represented the MDR-1 and MDR-3 genes respectively was achieved by DNA fragment cloning and sequencing, as described previously. A representative example of part of MDR-1 and MDR-3 sequencing gels is shown in figure 9.

Figure 9

Sections from sequencing gel of MDR-1 and MDR-3 - upper section demonstrating homology, and lower section heterologous base pair distribution elsewhere in sequences. Lanes A, C, G, T indicate base type.



Cytotoxic effect of epirubicin and mitomycin C in MGHU-MMC, MGHU-1 and MGHU-1R

Figures 10 to 15 illustrate in graphical form (data tables given in *appendix 4*) the effect of increasing doses of either epirubicin or mitomycin C on cultured cells from the cell lines MGHU-MMC (newly induced MMC resistance), MGHU-1 (wild-type) and MGHU-1R (adriamycin-induced MDR). Note the non-linear X axes denoting drug concentration by serial dilution.

The dotted lines on each chart indicate the IC50 - that is, the concentration of cytotoxic drug at which 50% of the cell population are killed.

It can be seen in figures 10 and 11 that, although there is a comparable dose related cytotoxic response in MGHU-MMC cells to both epirubicin and mitomycin C, it is negligible, and the IC50 is not reached for either drug at the concentrations used for these experiments. This contrasts dramatically with the effects of the same concentrations of drug in MGHU-1 cells ( figures 12 and 13) where the IC50 for mitomycin C is reached at 1μg/ml and that for epirubicin at 2μg/ml.

Interestingly, in the MGHU-1R cell line (figures 14 and 15) a similar pattern to that in MGHU-MMC is seen, whereby a dose response is still present but the IC50 for epirubicin is only approached at 80μg/ml. However MGHU-1R cells do seem slightly more sensitive to mitomycin C, where the IC50 is reached at 35μg/ml.

Figure 10

The cytotoxic effect of gradually increasing doses *mitomycin C* on cells of the MGHU-MMC line (one hour exposure). Bars are 95% confidence intervals. Dotted line is drawn at 50% residual biomass. RVB - residual viable biomass.

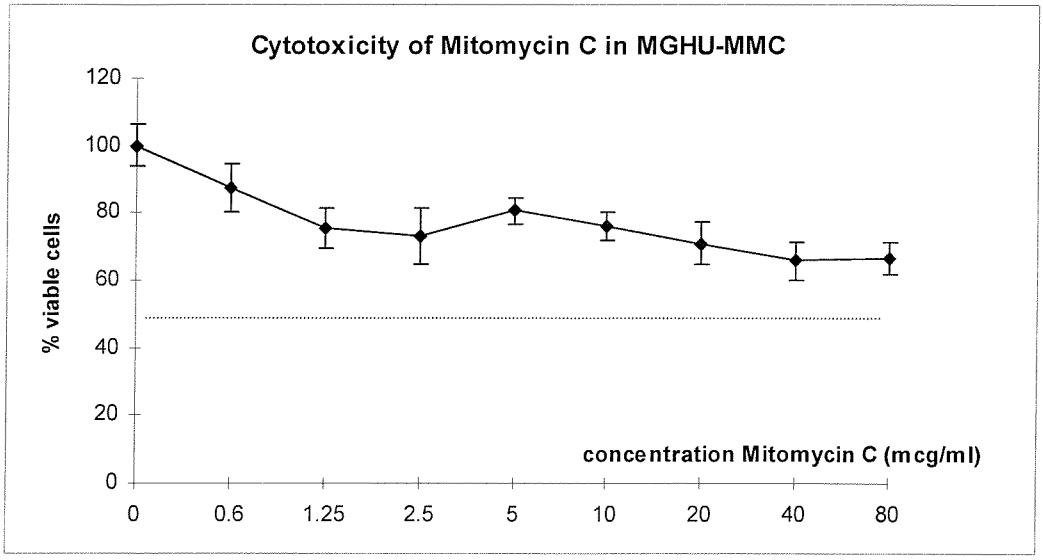



Figure 11

The cytotoxic effect of gradually increasing doses of *epirubicin* on cells of the MGHU-MMC line (one hour exposure). Bars are 95% confidence intervals. Dotted line is drawn at 50% residual biomass.

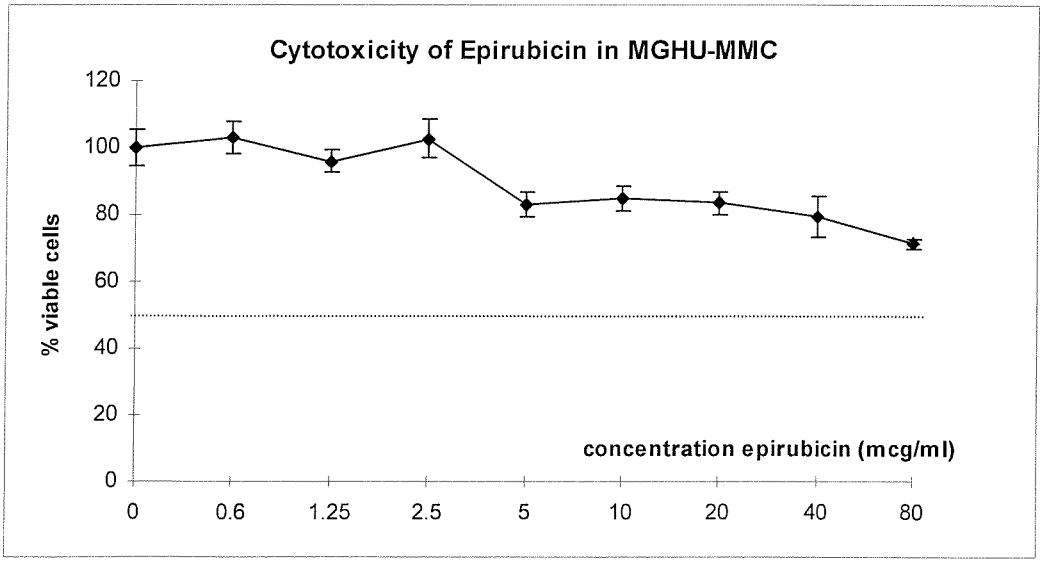


Figure 12

The cytotoxic effect of gradually increasing doses *mitomycin C* on cells of the MGHU-1 line (one hour exposure). Bars are 95% confidence intervals. Dotted line indicates IC 50. RVB - residual viable biomass.

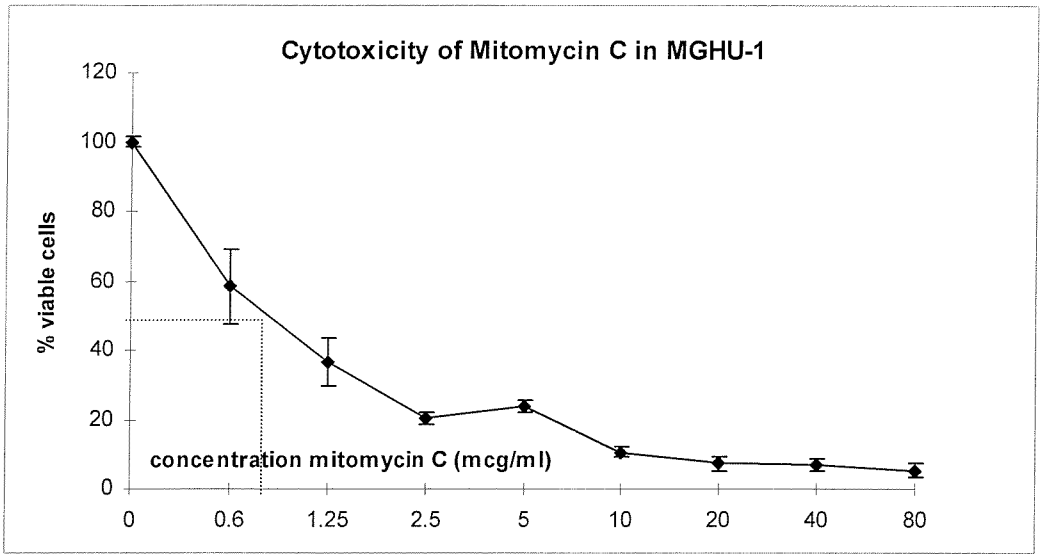



Figure 13

The cytotoxic effect of gradually increasing doses *epirubicin* on cells of the MGHU-1 line (one hour exposure). Bars are 95% confidence intervals. Dotted line indicates IC 50.

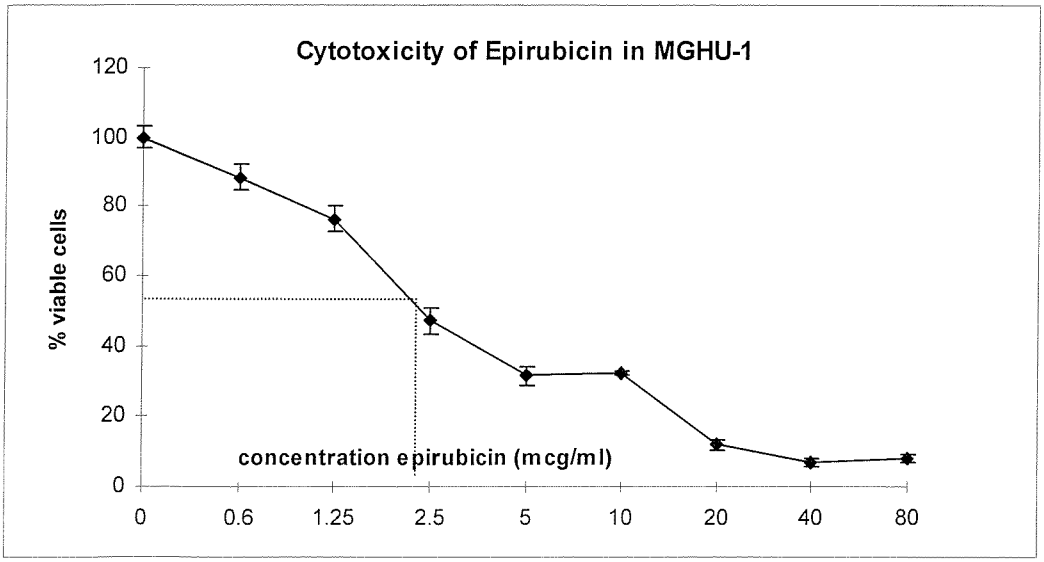


Figure 14

The cytotoxic effect of gradually increasing doses *mitomycin C* on cells of the MGHU-1R line (one hour exposure). Bars are 95% confidence intervals. Dotted line indicates IC 50. RVB - residual viable biomass.

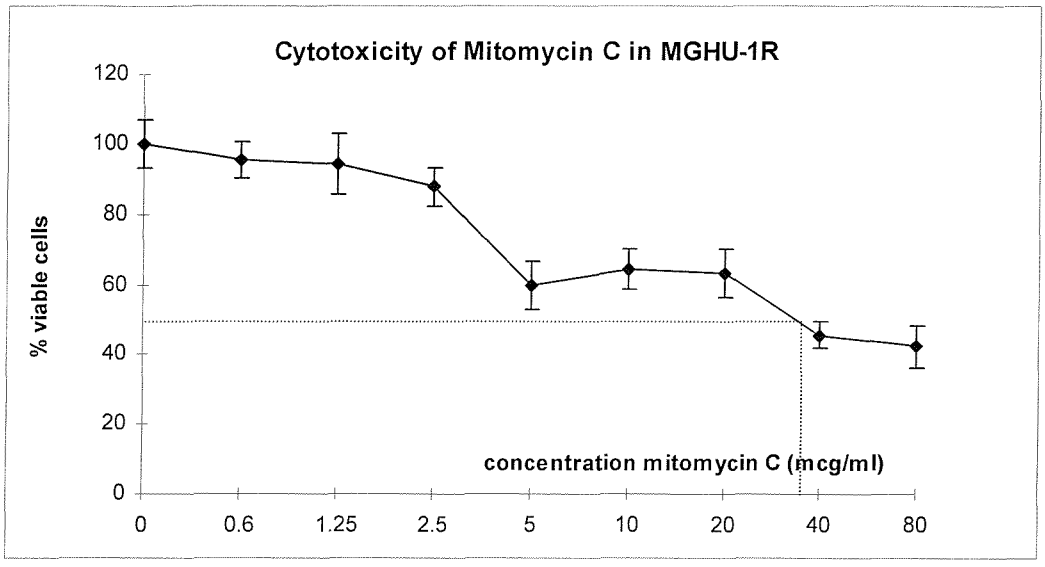
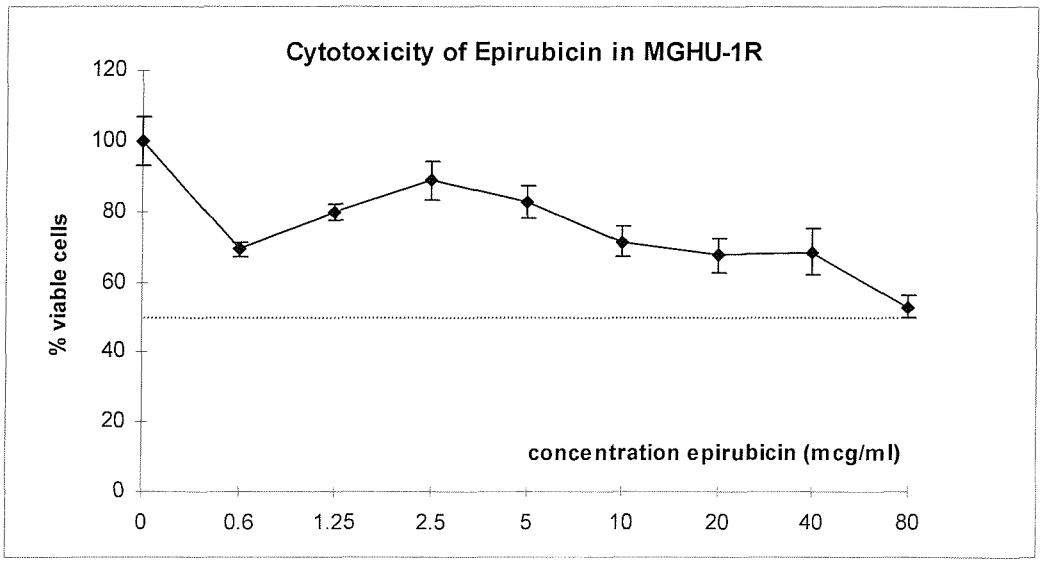



Figure 15

The cytotoxic effect of gradually increasing doses *epirubicin* on cells of the MGHU-1R line (one hour exposure). Bars are 95% confidence intervals. Dotted line indicates IC 50.



Mitomycin C Resistance - Discussion

Both anthracyclines and mitomycin C cause DNA damage indirectly via intracellular free radical production and directly by interstrand intercalation, and are thus maximally effective during S phase of the cell cycle. Epirubicin is independently cytotoxic, whereas mitomycin C requires intracellular modulation via a bioreductive alkylation reaction (catalysed via the DT-diaphorase and NADPH cytochrome P450 reductase pathways (Iyer and Szybocki 1964)) to the active hydro/semiquinone metabolite to achieve cytotoxic efficacy.

Classical P-glycoprotein mediated multidrug resistance (MDR) undoubtedly modulates resistance to the anthracyclines (Gervasoni et al 1991) and alkylation (drug activation) failure is primarily responsible for much resistance to mitomycin C (Hoban et al 1990). However, despite its comparable clinical resistance pattern the status of mitomycin C in the MDR family, and the extent to which MDR mcehanisms modulate resistance to it, remains equivocal and the literature is inconclusive.

For example, MDR characteristics and the overexpression of membrane P-glycoprotein have been demonstrated in a clone derived from the leukaemia cell line (L1210) which exhibited cross resistance with mitomycin C, anthracyclines and vinca alkaloids (Dorr et al 1987). In addition the P-glycoprotein expressing subline of K562, a doxorubicin-resistant erythroleukaemia clone, exhibits cross-resistance to epirubicin and mitomycin C (Hu and Chen 1994). Alterations in topoisomerase II expression (which facilitates DNA repair mechanisms) have also been identified in association with adriamycin-induced multidrug resistance in Chinese hamster ovary cells which were cross-resistant to topoisomerase II inhibitors (Hoban et al 1992). These cells were also more than 80-fold resistant to mitomycin C when compared to the parental cell line. A resistant subline selected from rat hepatoma AH130 cells after exposure to

adriamycin also demonstrated marked cross-resistance to mitomycin C, porfiromycin and vinblastine (Wakusawa et al 1997). The use of 1,3,5-triazacycloheptanes as MDR reversing agents were investigated with regard to P-gp mediated MDR in mouse P388 leukaemia cells (Sawanashi et al 1995) - these molecules potentiated the cytotoxic effect of vinblastine, adriamycin and mitomycin C in these cells.

On the other hand overexpression of π-type glutathione transferase, implying enhanced intracellular drug conjugation/inactivation, has been demonstrated in a bladder cancer clone (derived from J82) which exhibits cross-resistance between mitomycin C and cisplatin - the latter is not a drug to which resistance is mediated by classical (P-gp associated) MDR (Singh et al 1995). Ohga et al (1996) demonstrated that a nuclear factor (MDR-NF1) which is involved in the regulation of the MDR-1 gene is identical to the Y box-binding protein (YB-1) which binds to the control region of many genes. Transfection of a YB-1 antisense construct into human epidermoid cancer KB cells resulted in increased sensitivity to cisplatin and mitomycin C but not to the established MDR drugs vincristine, doxorubicin or etoposide. Shibata et al (1995) established two mitomycin C resistant sublines of PC-9 (non-small cell lung cancer) by continuous exposure. These were not resistant to the MDR drugs vindesine, etoposide and adriamycin amongst others, and the authors concluded that deficient drug activation (demonstrated by decreased cytosolic DT-diaphorase activity) was the resistance mechanism at work.

In these experiments resistance to mitomycin C was induced in an established transitional carcinoma cell line, and established cytotoxicity and RT-PCR assays were applied. By implication the possibility of cross-resistance to both epirubicin and mitomycin C was sought in a bladder cancer model.

Resistance to mitomycin C was invoked by persistent low-dose exposure (400 nM) of the agent to wild-type (non MDR) MGHU-1 cells in culture. This is very different to the intermittent single high-dose (1mg/ml) exposure seen in the clinical scenario. Clearly it as a matter of speculation whether the development of MDR in these circumstances represents the operation of 'instructive' or 'selective' mechanisms - that is to say direct gene upregulation in previously sensitive cells, or clonal expansion of a previously small population of already resistant cells. Nevertheless from these cytotoxicity data it seems that persistent low-dose exposure not only results in significant increase of the IC 50 of MGHU-MMC for mitomycin C but also for epirubicin.

The presence of detectable levels of MDR-1 in wild type cells is to be expected, as it can be identified by RT-PCR in up to 70% of bladder tumours and 50% samples of normal urothelium (Clifford et al 1996). The overexpression of MDR-1 and MDR-3 in MGHU-1R, but only of MDR-1 in MGHU-MMC, may represent the interaction of different regulatory elements or binding affinites for each drug by the cell line. Alternatively it is possible that overexpression of MDR-3 in addition to MDR-1 in MGHU-1R is a reflection of established functional MDR, in contrast to the newly induced MDR in MGHU-MMC.

Intracellular epirubicin concentrations (as detected by flow cytometry) and localisation patterns (confocal microscopy) are very similar both in MGHU-MMC cells and in MGHU1-R cells (which exhibit established P-glycoprotein mediated epirubicin resistance). The reversibility of these patterns in the presence of verapamil (MDR reversor) is similar both for MGHU-1R and MGHU-MMC (personal communication Dr A J Cooper).

Clearly extending these techniques to other cell lines (particularly urothelial) would give a greater understanding of the role of MDR in resistance to mitomycin C, as extrapolating from one cancer model alone can be misleading. It would in addition be instructive to establish the possible roles of MRP, LRP and the atypical resistance mechanisms in mediating resistance to mitomycin C across a selection of such cell lines. Transfection of MGHU-1 with MDR-1, rather than inducing mitomycin C resistance by drug exposure, might be considered to be a more representative model for the purist, but this technique was not available in our laboratory at the time of these experiments. Furthermore, we sought to mimic the clinical exposure of bladder cancer to intravesical agents in an in vitro model in the selection of this technique.

Anecdotally it seems that some patients with recurrent tumour after exposure to one agent will benefit from substitution single agent intravesical chemotherapy, although no clinical trials to date have sought to validate this practice. These data, however, suggest that an element of cross-resistance between these agents might be expected. Such suspicions are supported by the similar clinical response/recurrence rates seen in most clinical trials of both agents. It also, however, demonstrates that the phenomenon of MDR reversal, seen in the presence of verapamil and other agents which modulate P-glycoprotein mediated drug resistance, may be applicable both to epirubicin and mitomycin C. Extrapolation of cell line work to the clinical scenario is, however, at best optimistic and at worst frankly misleading, and further clinical trials are required to examine the role of MDR reversing agents in combination with intravesical chemotherapeutic drugs directed against superficial bladder cancer.

## 5. MDR Membrane Characteristics

Background

During the course of earlier experiments, examining the possible transfer of the MDR phenotype between neighbouring populations of cells, it became apparent that MGHU-1R cells were relatively resistant to labelling by the fluorescent cell marker PKH2-GL, in contrast to their wild-type counterparts. An explanation for this was sought by examining possible differences in plasma membrane structure in these cells.

Hypothesis

The plasma membrane of MGHU-1R cells differs from that of its parental clone.

Aims & Objectives

1. To assess the expression of membrane (P-gp & MRP) and intracellular (LRP) MDR proteins, thereby to confirm that overexpression of MDR-1 in MGHU-1R cells was associated with P-gp overexpression.
2. To quantify, by means of flow cytometry, the difference in PKH2-GL binding in parental and MDR cells, and to examine by confocal microscopy its cellular uptake patterns.
3. To examine the binding of Annexin V (a phosphatidylserine-binding protein) to MDR and wild-type cells.
4. To examine the binding of a monoclonal antibody directed against tissue factor (a membrane protein associated with MDR and tumorigenicity).

Expression of P-glycoproteins using JSB-1 monoclonal antibody

Fluorescence in wild type cells (Figure 16B) approaches the low levels seen in negative controls (Figure 16A and 17A). In contradistinction the expression of P-gp in MGHU-1R cells (Figure 17B) is much higher and is concentrated in the plasma membrane, although some cytoplasmic fluorescence is also seen. At higher magnification, individual points of fluorescence are seen along the plasma membrane of MGHU-1R cells suggesting foci of P-gp.

Expression of multidrug-resistance related protein (MRP) using MRPm6 monoclonal antibody

Fluorescence in wild type cells (Figure 16D) matched the low levels seen in negative controls (Figure 16A and 17A) suggesting non-specific secondary antibody activity. Fluorescence in MGHU-1R cells (Figure 17D) was identical implying no overexpression of MRP in MGHU-1R.

Expression of lung resistance protein (LRP) using LRP-56 monoclonal antibody

Fluorescence in wild type cells (Figure 16C) approximated to the low levels seen in controls (Figure 16A and 17A) confirming absence of LRP expression. However in MGHU-1R cells (Figure 17C) LRP was overexpressed, and its cytoplasmic distribution was notably punctuate. In addition some punctuate peri- and intranuclear staining is seen, as demonstrated in Figure 18.

Figure 16

MDR protein immunocytochemistry in MGHU-1

*A* control; *B* P-glycoprotein; *C* lung resistance protein; *D* multidrug resistance associated protein.

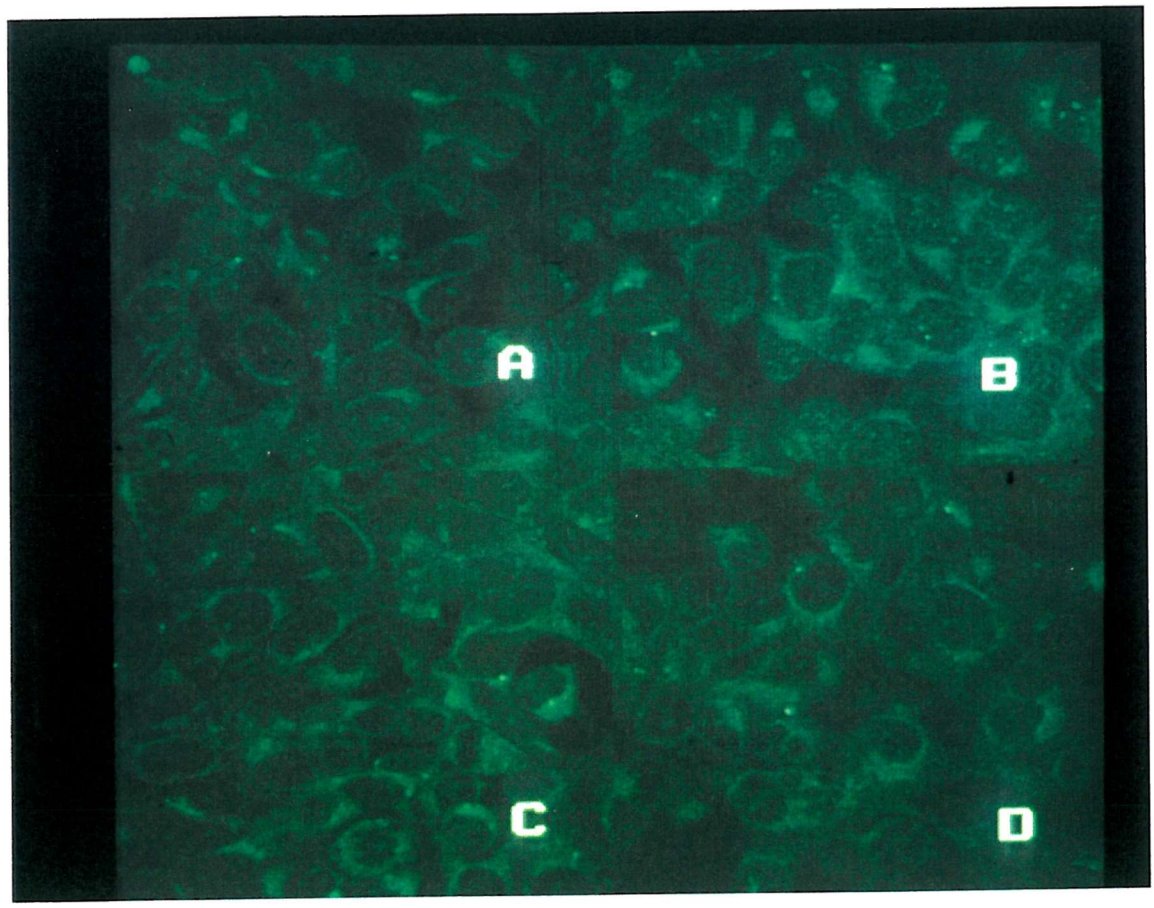


Figure 17

MDR protein immunocytochemistry in MGHU-1R

*A* control; *B* P-glycoprotein; *C* lung resistance protein; *D* multidrug resistance associated protein.

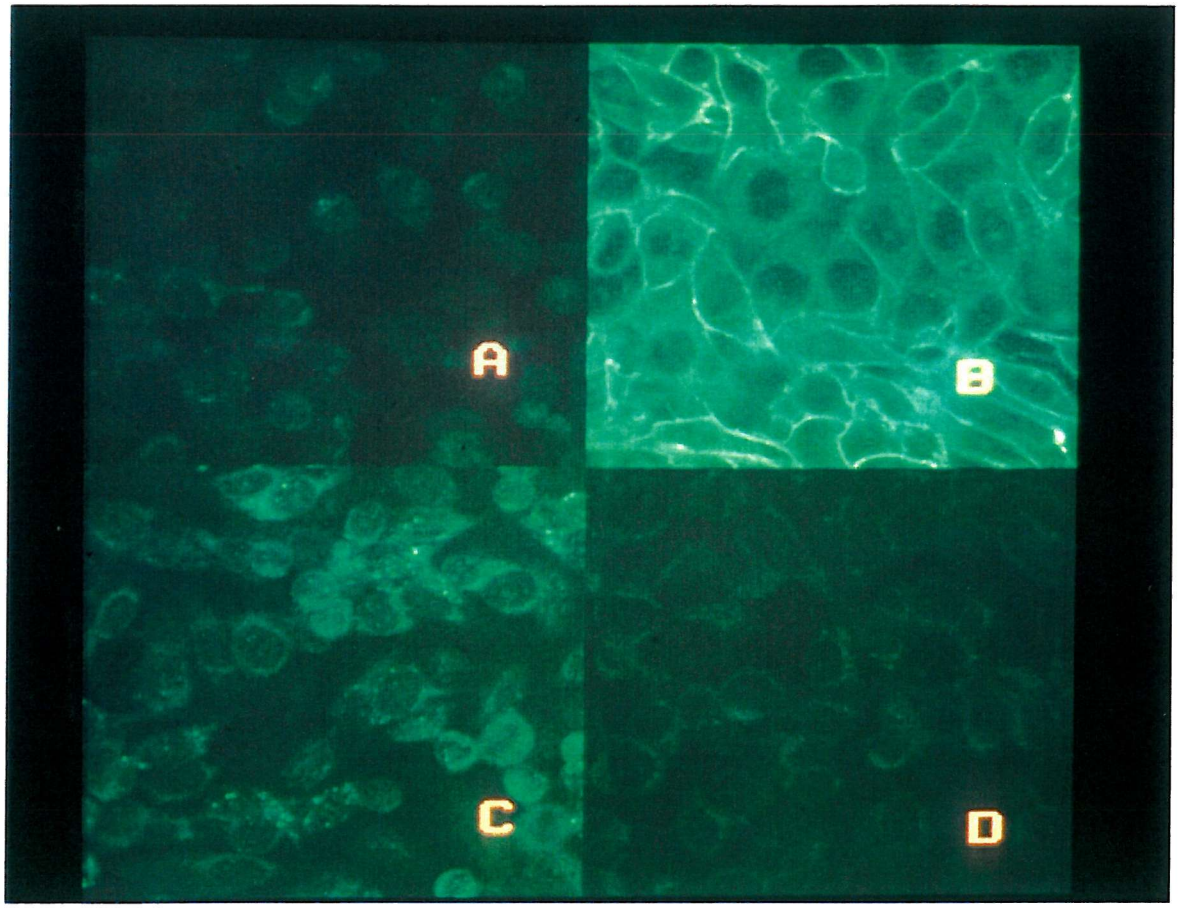
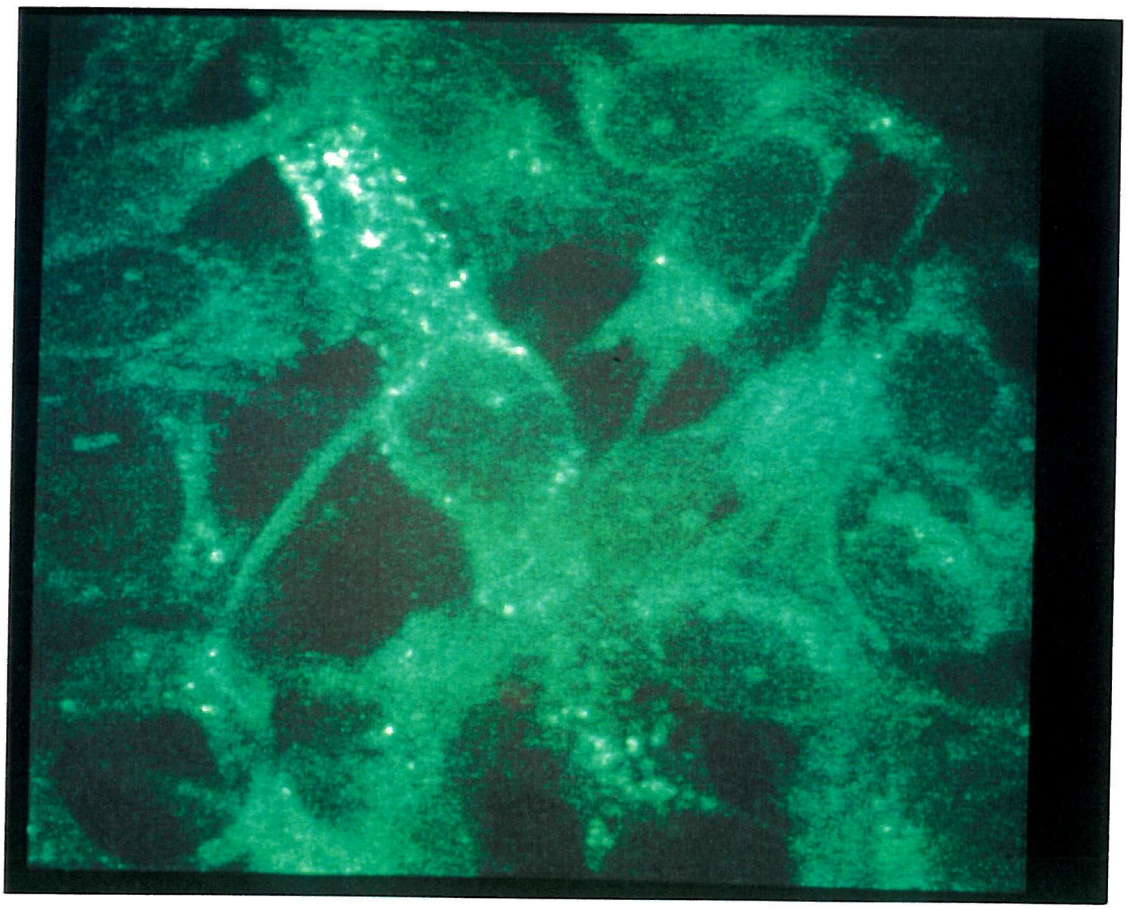


Figure 18

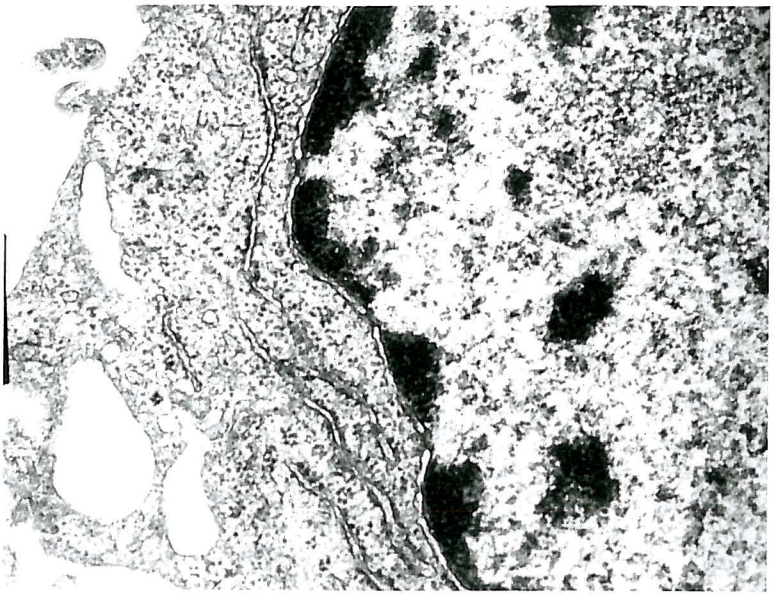
Lung resistance protein expression in MGHU-1R.



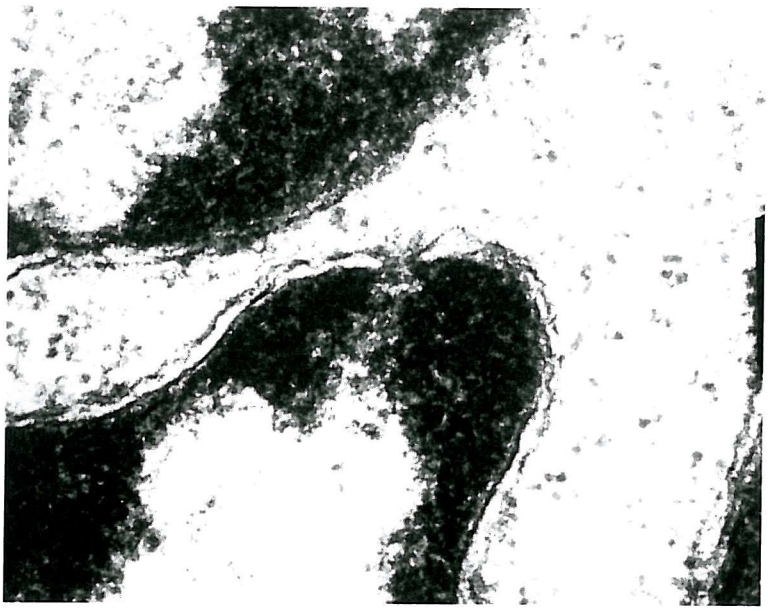
Figures 19, 20 and 21

Nuclear pore complexes - electron photomicrographs at x30000, x70000 and 150000 respectively.

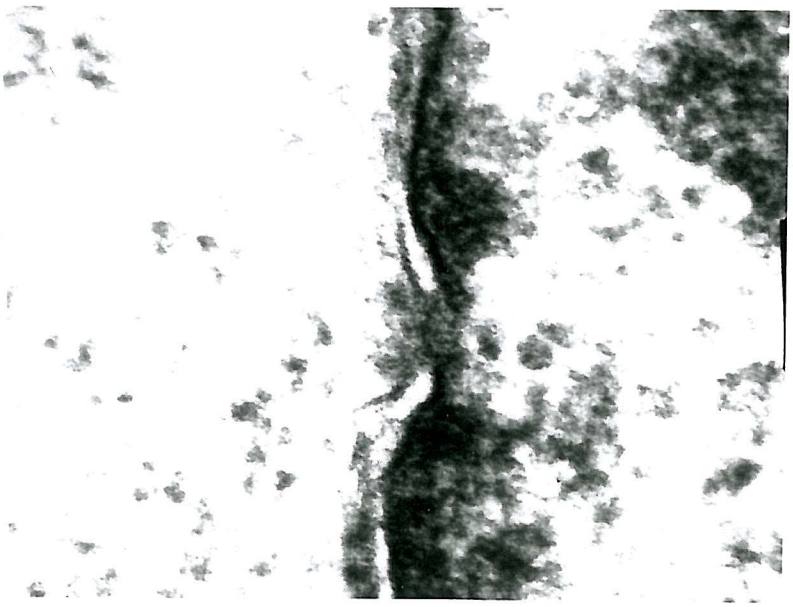
19.



20.



21.



Transmission electron microscopy (TEM) of MGHU-1R nuclear envelope

Nuclear pore complexes (arrowed) were identified in the nuclear membrane at magnification x30 000 (Figure 19), x70 000 (Figure 20) and x150 000 (Figure 21). Granular extranuclear cytoplasm, nuclear pores and dark intranuclear chromatin are well seen but vaults and vault complexes were not identified. Material bridging the nuclear pore and apparently extending from the cytosol into the nucleus is seen in Figure 20.

Membrane labelling by the phospholipid analogue PKH2-GL in cell suspensions/adherent cells

Drug sensitive cells in suspension incorporated PKH2-GL in a dose dependent fashion. Using standard flow cytometric machine parameters fluorescence in MDR cells remained close to the baseline (Tables 21& 22). On adjusting the fluorescence detectors (FL1) to bring the MDR fluorescence to a peak on the X axis the difference in incorporation could be measured consistently over one hundredfold (Figure 22). Statistical comparisons between curves in the same experiment are made using the D value in the Kolmogorov-Smirnov two sample test (K/S-D) by the LYSYS II software (Young 1977). For plots comprising 5000 points each K/S-D >0.04 implies a difference between the curves at the 99.9% confidence level. Between-run comparisons are by the Wilcoxon paired rank sum test on not less than seven data sets of both mean and peak FL1 channel number. K/S-D values were all >0.80 comparing PKH2-GL between resistant and sensitive cells, for the Wilcoxon test, $p<0.01$. Confocal microscopic imaging of PKH-2 labelled adherent cells was consistent with the flow cytometry data. Drug sensitive cells labelled in both plasma and intracellular membrane systems. Intracellular fluorescence of PKH2-GL appeared most marked in the perinuclear region, and may represent Golgi apparatus or cytoskeletal labelling. Drug resistant cells, conversely, exhibited much less PKH2-GL associated fluorescence with negligible intracellular probe traffic (Figure 23A & B).

Figure 22

Flow cytometric fluorescence histograms of PKH2-GL binding in MGHU-1 (S) and MGHU-1R (R). Y axis - number of events, X axis - log fluorescence intensity.

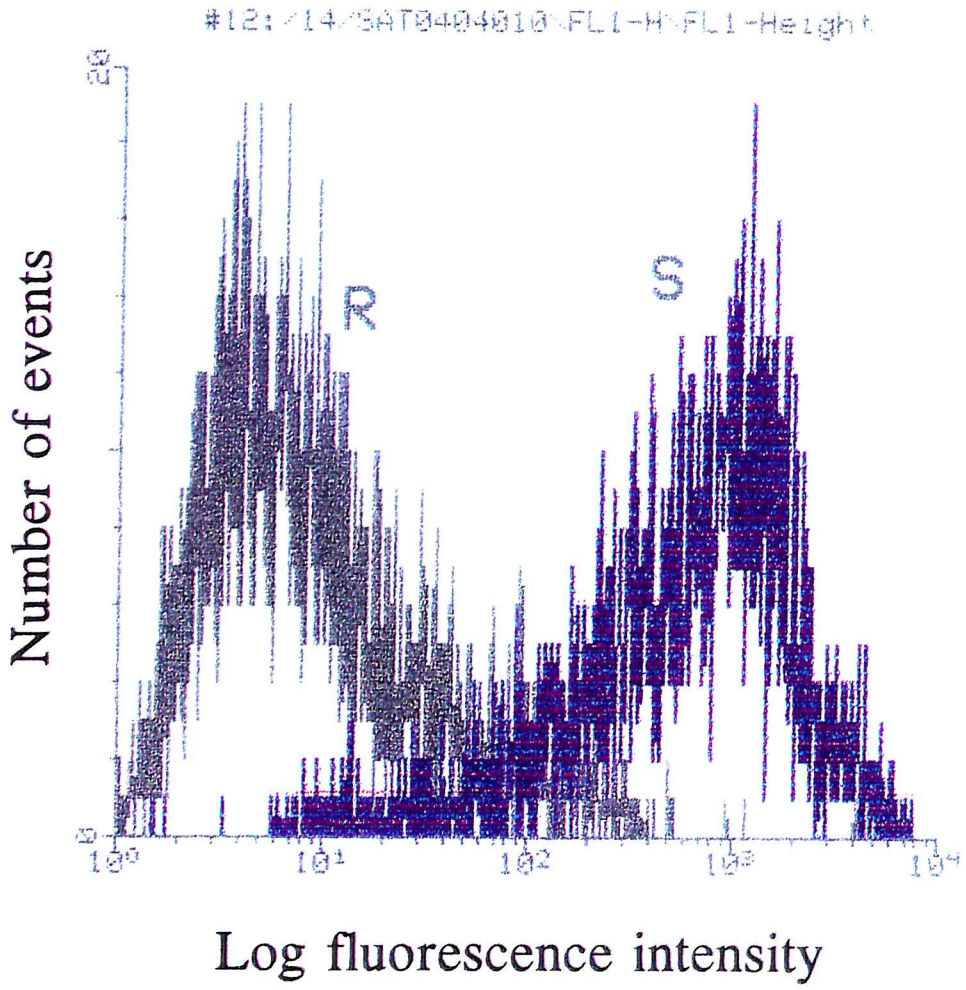
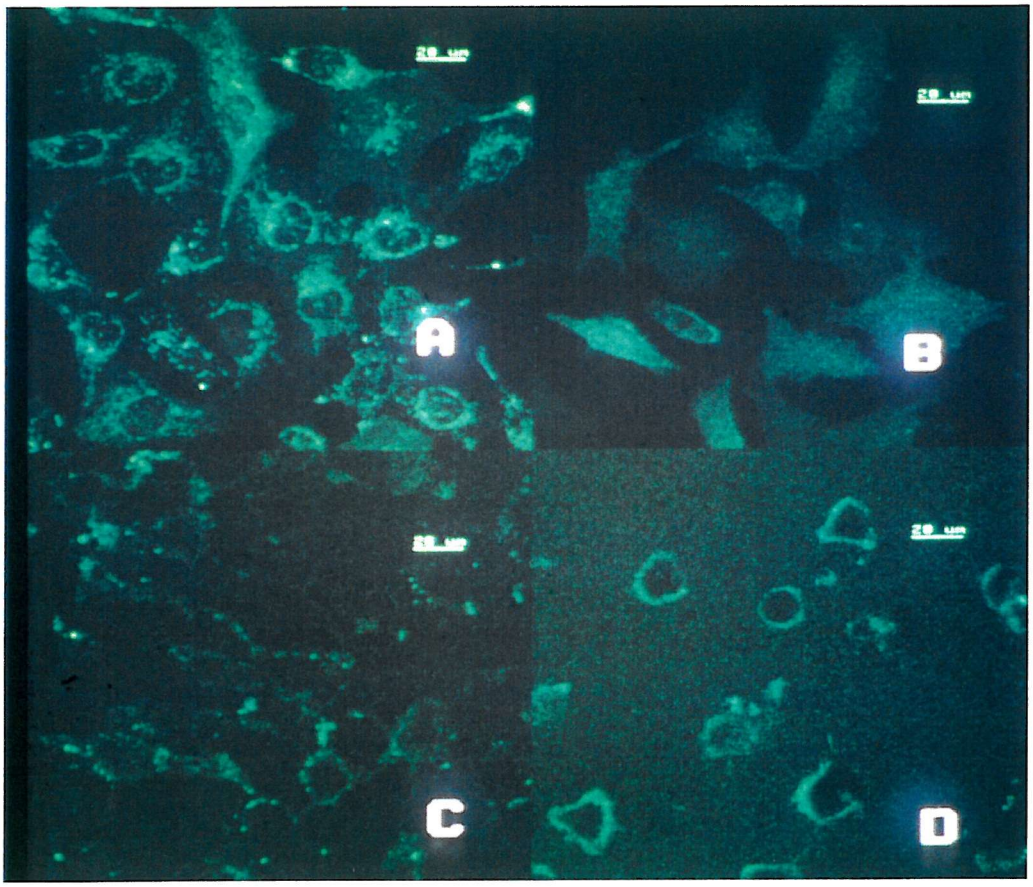


Figure 23

Confocal photomicrographs of *A* PKH2-GL fluorescence in MGHU-1; *B* PKH2-GL fluorescence in MGHU-1R; *C* Annexin V binding in MGHU-1; *D* Annexin V binding in MGHU-1R. Scale bars 20 microns.



*P-gp pump suppression.*

PKH2-GL incorporation at 0°C was reduced five-fold in cells of the sensitive clone compared to controls (Table 22). No such effect was demonstrated in cells of the MDR clone, where PKH2-GL fluorescence remained negligible.

*MDR (functional P-gp) reversal.*

Incubation of MDR cells with verapamil prior to PKH2-GL labelling evoked no increase in PKH2-GL fluorescence (Table 23) which remained near the baseline.

Membrane labelling by Annexin V in cell suspensions/adherent cells

Fluorescence associated with Annexin-V seen in cells from the MDR subline was approximately twice (mean 2.11, range 1.3 - 2.68) that seen in sensitive cells (Figure 24). This difference in fluorescence was also repeatable and significant (K/S-D >0.40; Wilcoxon $p<0.02$).

Imaging of adherent cell sublines following incubation with Annexin-V *in situ* confirmed binding in resistant cells, with diminished Annexin-V fluorescence in the sensitive subline (Figure 23C & D).

Membrane labelling of MDR cells by anti-tissue factor (TF) monoclonal antibody

Using online SCANware 4.2 (Leica) confocal image analysis software, cells of the MGHU-1R line demonstrated plasma membrane fluorescence around 1.5-fold higher than that in MGHU-1 cells after incubation with anti-tissue factor antibody, suggesting increased tissue factor expression in MGHU-1R cells (figure 25). A greater differential binding effect can be seen in MCF-7 and its MDR clone.

Figure 24

Flow cytometric fluorescence histograms of Annexin V binding in MGHU-1 (S) and MGHU-1R (R). Y axis - number of events, X axis - log fluorescence intensity.

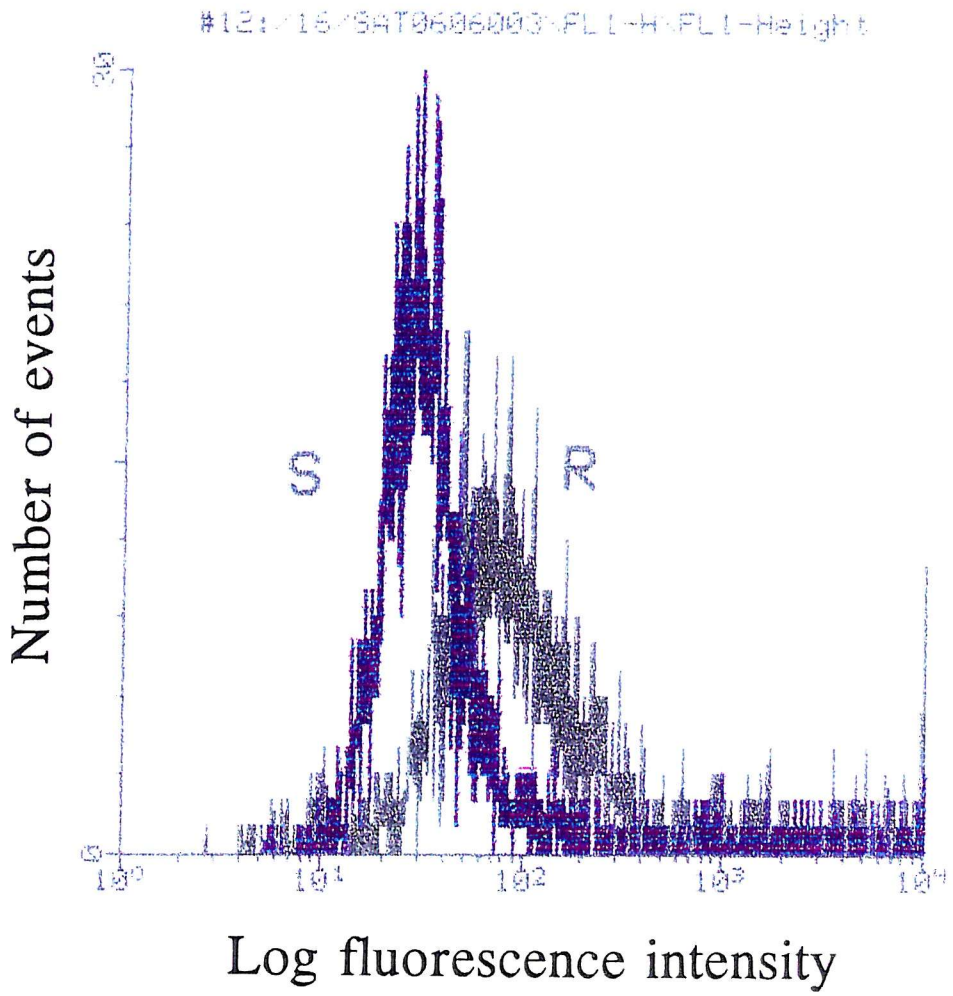
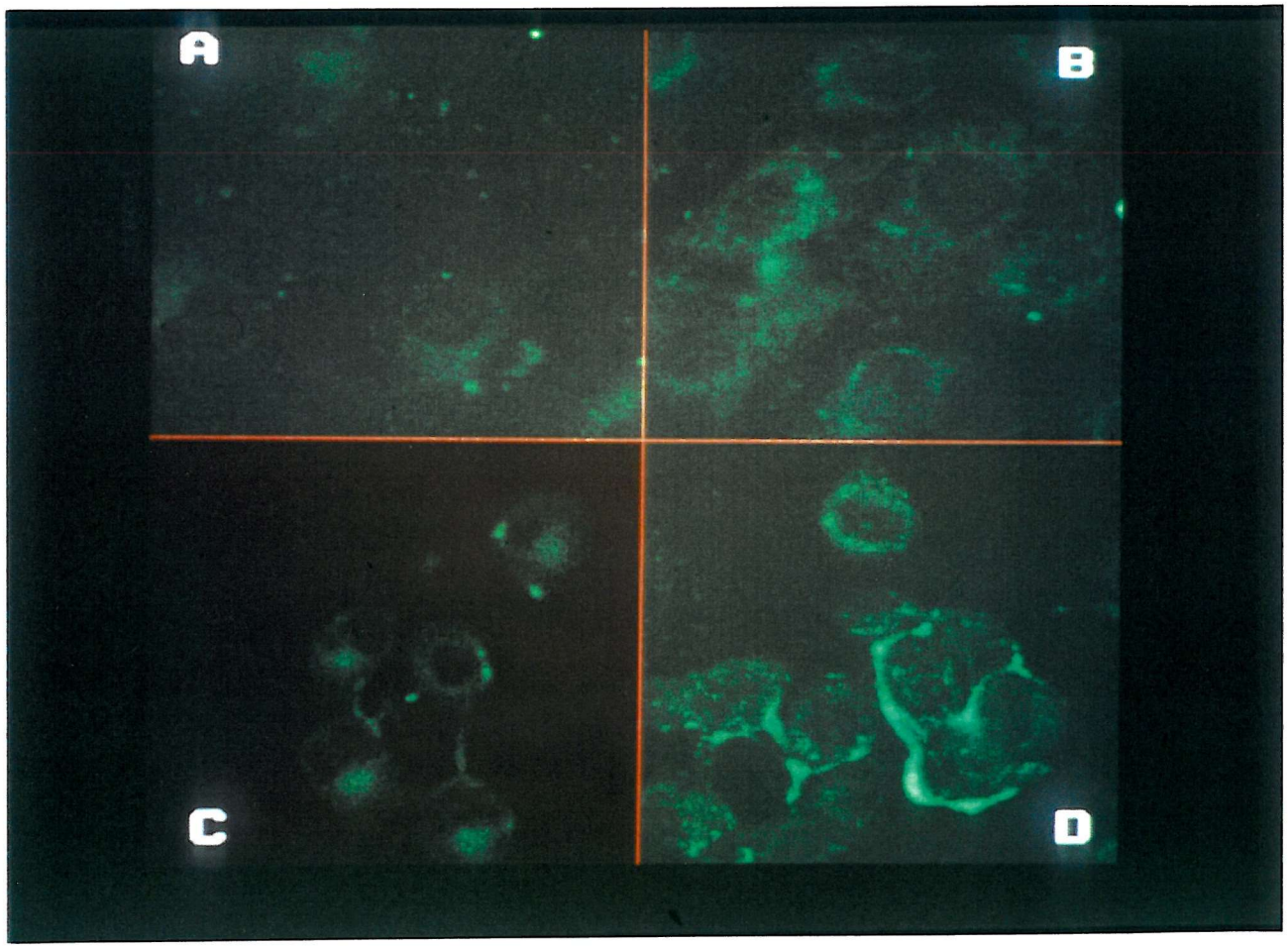


Figure 25

Tissue factor binding in *A* MGHU-1, *B* MGHU-1R, *C* MCF-7 and *D* MCF-7R.



MDR Membrane Characteristics - Discussion

The expression of MDR proteins was examined in order to elucidate the likely mechanisms mediating MDR in the MGHU-1R cell line. Both P-glycoprotein (P-gp) and lung resistance protein (LRP) were significantly overexpressed in MGHU-1R cells, but no such overexpression of multidrug resistance associated protein (MRP) was identified. It seems likely, therefore, that both P-gp and LRP at least contribute to the MDR phenotype in this cell line.

LRP expression in MGHU-1R cells was primarily punctuate and perinuclear, but also stained the cytoplasm in a granular fashion. LRP has significant structural homology to the major vault protein which is widely distributed in the cytosol, and is also structurally very similar to the transporter of the nuclear pore complex to which vaults are known to associate (Chugani et al 1993). It is conceivable, therefore, that the absence of nuclear drug staining in MGHU-1R cells might be explained by vaults/LRP either extruding nuclear drug into the cytoplasm or preventing drug incorporation into the nucleus from the outset. The absence of MRP staining in MGHU-1R suggests that it plays no part in the mediation of MDR in these cells. In the MRP containing MDR lung cancer cell line H69AR drug accumulation differences and efflux were not observed, and thus appeared not to have a role in the MDR phenotype (Cole et al 1991).

P-glycoprotein is well studied and much is known about its postulated role in the MDR phenomenon. It has been demonstrated both to reduce cytotoxic drug influx and increase efflux, perhaps by forming a transporter molecule energy dependent membrane channel (Gottesman 1993). Increased membrane trafficking to the plasma membrane from the intracellular environment occurs in MDR cell and may result in drug extrusion (Sehested et al 1987). In addition P-glycoprotein is expressed on Golgi apparatus (Willingham 1987) which also implies an intracellular membrane trafficking role. Evidence exists that P-glycoproteins can influence ion channel regulation

(Altenberg et al 1994) and there is also evidence that they may act as a phospholipid flippase (Smith et al 1994). Studies on total membrane phospholipids in MDR cells have proved inconclusive but are suggestive of differences in phospholipid distribution (Kiss et al 1994). Phosphatidylcholine has been shown to be translocated from the inner to the outer leaflet of fibroblast plasma membranes in transgenic mice in the presence of human MDR3 P-gp (Smith et al 1994) but the functional consequence of this phenomenon remains poorly understood.

The marked differential incorporation of PKH2-GL into the membranes of sensitive and MDR sublines of the MGH-U1 cell line is consistent with such a membrane change. We have, unfortunately, been unable to obtain detailed information about the physicochemical characteristics of PKH2-GL and its diluent as they are patented products. PKH2-GL is known to be a cationic aliphatic molecule with a fluoroscein-like fluorochrome reporter group (Horan and Slezak 1989). This is incorporated into the cell membrane lipid bilayer by selective partitioning, and undergoes subsequent traffic through the cytoplasm. Differential labelling of human lymphocyte subsets by PKH2-GL has previously been described (Festin 1992) and the authors similarly postulate a causative difference in lipid composition of lymphocyte cellular membranes, but this phenomenon has not previously been reported in association with multidrug resistance.

Interestingly it seems that this PKH2-GL binding differential is not mediated directly by P-gp associated molecule efflux. Preincubation of MDR cells with verapamil (MDR reversing agent) invokes no apparent increase in PKH2-GL binding to cells of the MDR clone. Similarly no increase in its binding is seen when incubated with MDR cells at 0°C, at which temperature ATP-dependent P-gp mediated efflux might be expected to be negligible. The five-fold reduction in PKH2-GL binding in sensitive cells at 0°C (compared to incubations at room temperature) probably reflects temperature-dependent membrane fluidity effects which are inapparent in the MDR cells where fluorescence is already negligible.

Changes in plasma membrane phospholipid asymmetry in MGH-U1 MDR cells are further suggested by differential Annexin-V binding. Annexin-V is a member of a family of proteins that bind to negatively charged phospholipids in a Ca++ dependent manner (Meers and Mealy 1993, Trotter et al 1995). The physiological role of the annexins is as yet undetermined but they are probably involved in membrane fusion and exocytosis (Creutz 1992). They have been shown to interact with cytoskeletal proteins, and to have anti-coagulant and mitogenic properties. Annexin-V forms a Ca++ selective ion channel in phospholipid bilayers, and binds most preferentially to phosphatidylserine at the plasma membrane surface (Koopman et al 1994) including, for example, during apoptosis (Homburg et al 1995). The higher apparent affinity of Annexin-V for the plasma membrane of cells of the MDR subline might therefore represent greater expression of phosphatidylserine on the outer leaflet of the membrane of these cells compared to their sensitive counterparts. These results also suggest that charge may play an important role in the interactions between MDR cells and the molecules that surround them, although a charge theory alone would be insufficient to explain the PKH2-GL binding differential.

The increased affinity of anti-tissue factor antibody for the plasma membrane of MGHU-1R, in comparison to its wild-type parental cell line, is additional evidence of altered membrane biochemistry in these MDR cells. Tissue factor is a 30 kD single chain membrane glycoprotein which functions as the principal initiator of coagulation by complexing with plasma factors VII and VIIa to activate factors X and IX, resulting in thrombin formation and fibrin deposition (Nemerson and Bach 1982). It has, for some time, been recognised as a potential diagnostic aid in cancer, the tests being colorimetric determinations of tissue factor on monocytes or in urine (Dashamapatra et al 1987). More recently the role of tissue factor in tumour neovascularisation and angiogenesis has been described (Contrino et al 1996) where tissue factor was found to be overexpressed both by vascular endothelial cells and cancer cells in close proximity to them. It has also been proposed as an important mediator of normal tissue remodelling, where tissue factor de-encryption is associated with apoptosis (Greeno et

al 1996). Tissue factor expression is frequently present but functionally unavailable ('encrypted') within the cell membrane (Maynard et al 1975, Greeno et al 1996) - this seems particularly to apply to cells in direct contact with blood where the constant exposure of a potent functional procoagulant would be disadvantageous. De-encryption of functional tissue factor on normal cells is often a reflection of states of activation, for example of monocytes with bacterial endotoxin. In addition cancer cells frequently express abnormally high levels of tissue factor, a finding which has resulted in the proposal that tissue factor may have a role in cancer development and metastasis (Zhang et al 1994, Folkman 1996). It has also been reported that a (adriamycin induced) P-glycoprotein expressing MDR subline of the MCF-7 breast cancer cell line expresses 10-fold more tissue factor mRNA than its wild-type parental counterpart (Hu et al 1993). The MDR subline was found to be significantly more tumorigenic than the wild-type, indicated by mouse flank inoculation. Interestingly, however, the same investigators were unable to demonstrate an increase in tissue factor expression in wild-type MCF-7 cells which had been transfected with the MDR-1 gene suggesting that, at least in adriamycin induced MDR MCF-7 cells, increased tissue factor activity is not directly associated with MDR expression alone - the phenotype is, as always, more complex than the genotype.

It is conceivable, therefore, that in a similar fashion MGHU-1R cells, which express more tissue factor antigen, may have more tumorigenic and potentially metastatic potential than their wild type parental line. There certainly seems little doubt that many of the characteristics of their plasma membranes differ considerably, offering many areas worthy of further investigation.

PKH2-GL, Annexin-V and tissue factor may therefore prove to be useful tools for studying mechanisms in MDR, particularly in relation to P-glycoprotein and its functional relationship with the plasma membrane, not only in cell lines but in human cancers.

## 6. Anthracycline uptake by cell fractions

Background

In MGHU-1 and MGHU-1R cells the uptake of idarubicin differs from that of epirubicin. In wild-type cells epirubicin uptake is intranuclear, in contrast to MDR cells where uptake is primarily cytoplasmic. However in MGHU-1 idarubicin fluorescence appears predominantly perinuclear and cytoplasmic, with an area of intense perinuclear fluorescence which may represent the Golgi apparatus. There is relatively little nuclear drug fluorescence. The distribution of idarubicin in MGHU-1R cells is comparable, at a slightly lower level of fluorescence (Duffy et al 1996).
The following experiments were performed in order to establish whether this phenomenon represents differences in the binding of these anthracyclines to nuclear and subnuclear structures, and the effect of differing physicochemical environments.

Hypothesis

Epirubicin and idarubicin interact differently with cell nuclei and subnuclear structures.

Aims & Objectives

1. To examine anthracycline binding in isolated human urothelial cell nuclei.
2. To examine anthracycline binding in isolated human chromosomes.
3. To examine anthracycline binding in avian erythrocytes (in which 'vestigial' nuclei are not actively transcribing).
4. To examine anthracycline binding in DNA comets by means of gel electrophoresis.

Isolated nuclei

Uptake of epirubicin or idarubicin into isolated nuclei of MGHU-1 (sensitive) and MGHU-1R (resistant) was assessed at pH 6, 7.4 and 8. Both epirubicin and idarubicin fluorescence was detectable within nuclei at all pHs by flow cytometry.

The median *epirubicin* fluorescence was 313.4 and 282.0 at pH 7.4 for sensitive and resistant nuclei respectively ('arbitrary units of fluorescence'). Median *idarubicin* fluorescence was 349.1 and 302.3 at pH 7.4 for sensitive and resistant nuclei respectively.

No meaningful differences in pH-related uptake of either anthracycline were demonstrable for nuclei from either cell line. Fluorescence histograms pertaining to these experiments are given in figure 26 and data values given in tables 11 and 12 (appendix 4).

Confocal microscopy of isolated nuclei incubated in an identical fashion with epirubicin or idarubicin was also performed. Again nuclear fluorescence of both anthracyclines was confirmed, with foci of increased fluorescence suggesting chromatin/nucleolar binding (see figure 27). No difference in nuclear anthracycline uptake was seen in relation to the cell phenotype of origin.

Chromosomes

Brightly fluorescent disaggregated chromosomes were detected by confocal microscopy after extraction and incubation with either epirubicin or idarubicin, confirming binding of both anthracyclines to intact human chromosomes *in vitro* (see figure 27).

Figure 26

Flow histograms for epirubicin (EPI) and idarubicin (IDA) fluorescence with isolated sensitive (S) and resistant (R) nuclei. Y axis - number of events, X axis - log fluorescence intensity.

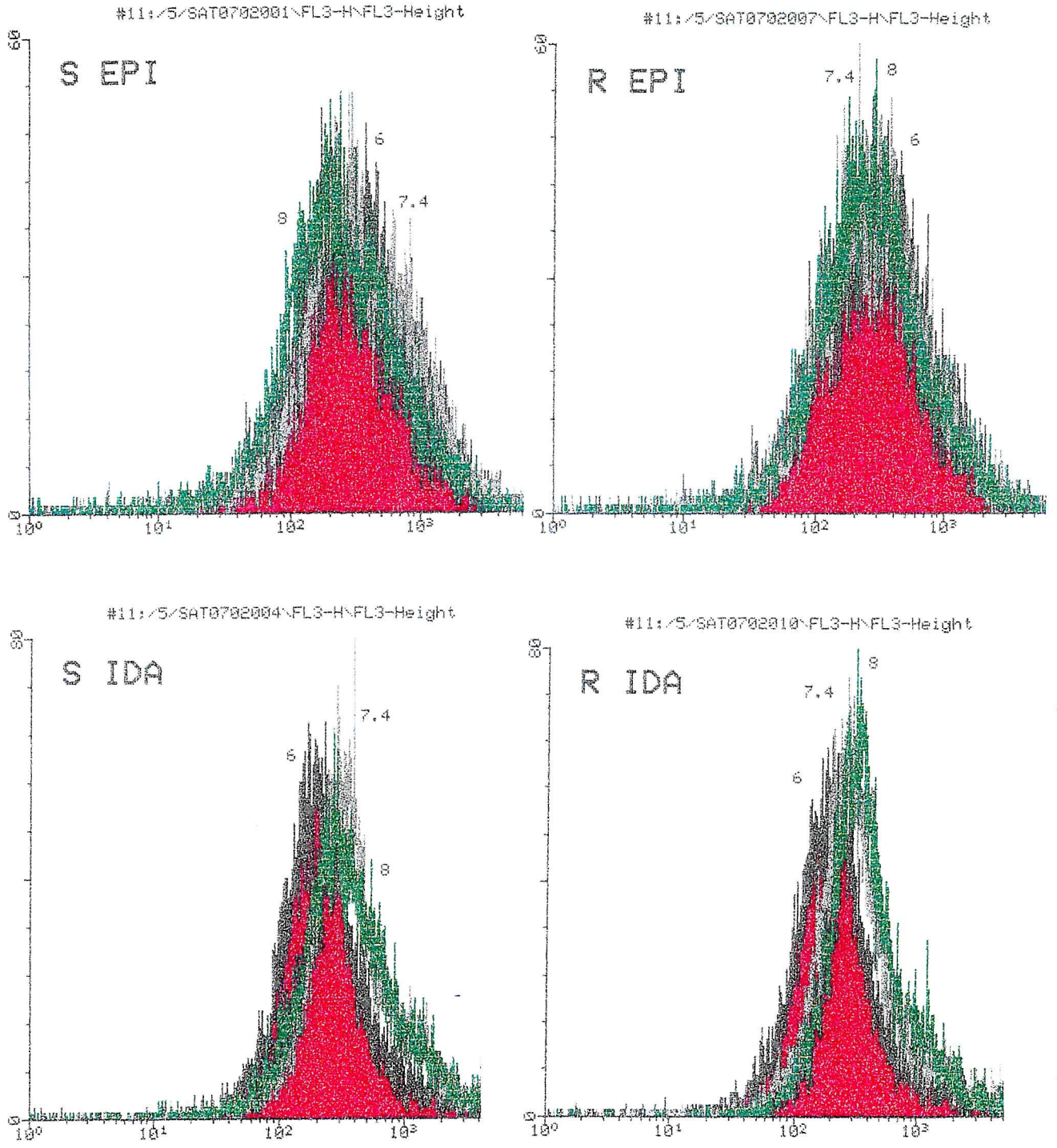


Figure 27

Confocal photomicrographs of *A* epirubicin and *B* idarubicin fluorescence in chromosomes, *C* epirubicin and *D* idarubicin fluorescence in isolated nuclei.

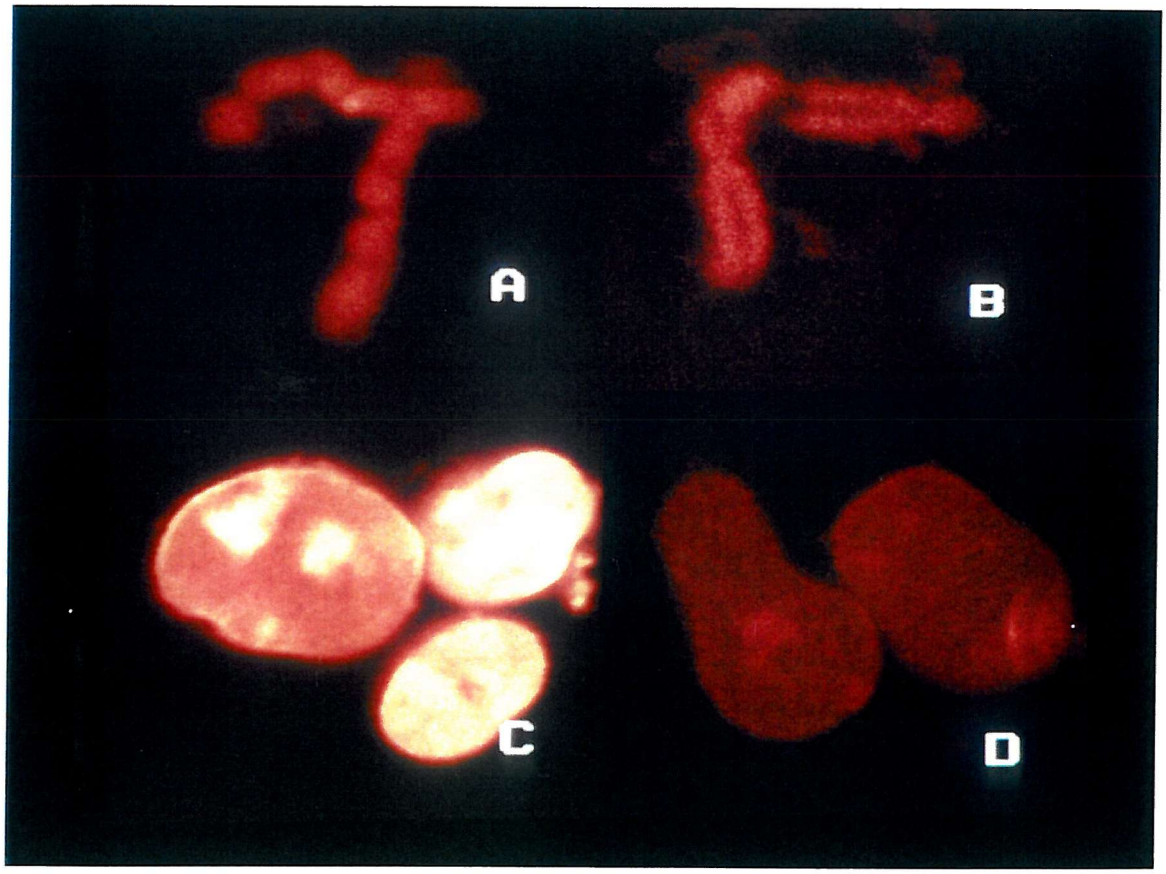


Figure 28

Flow cytometry histograms of epirubicin (EPI) and idarubicin (IDA) binding in avian erythrocytes at pH 6.0, 7.4 and 8.0. Y axis - number of events, X axis - log fluorescence intensity.

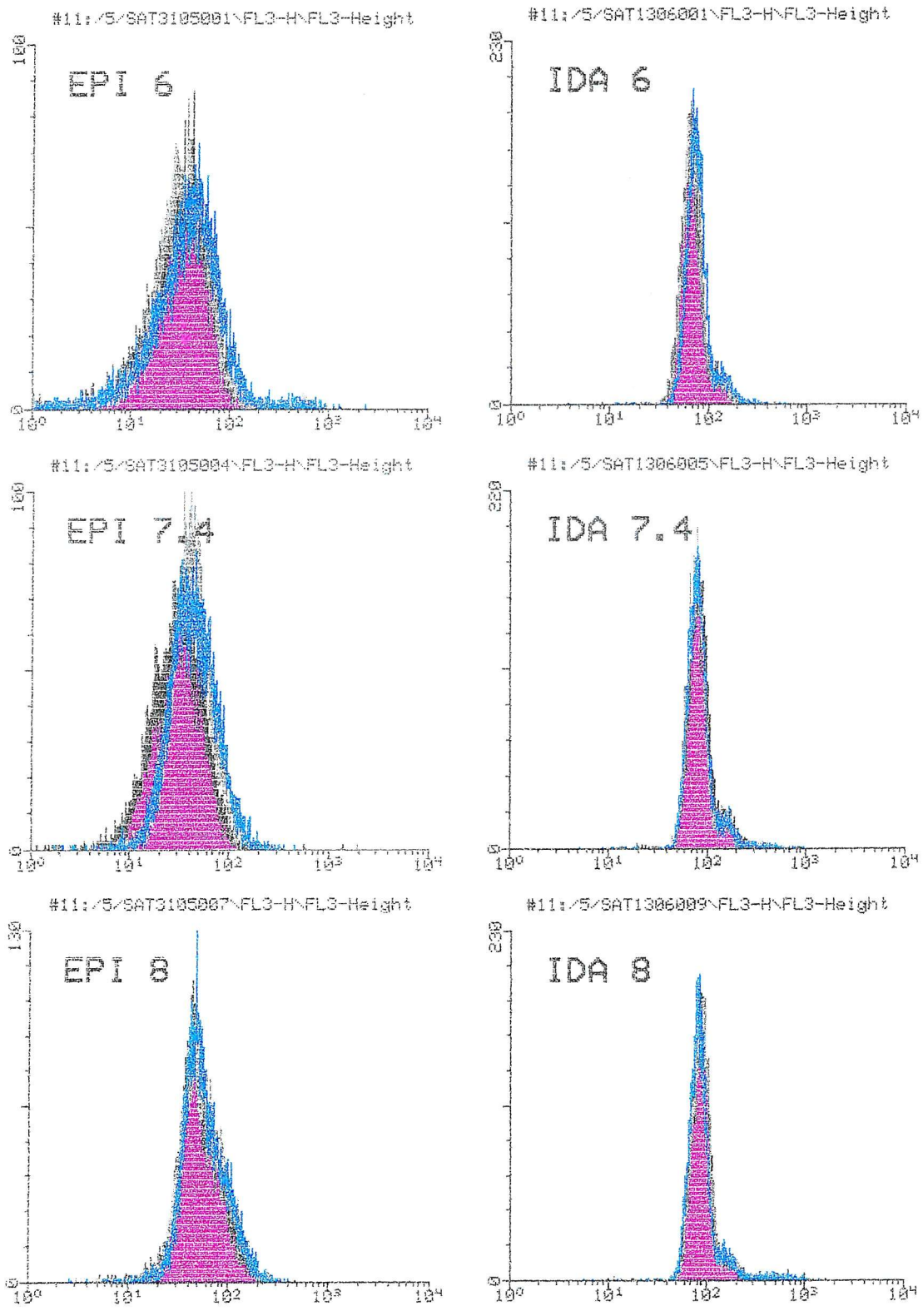
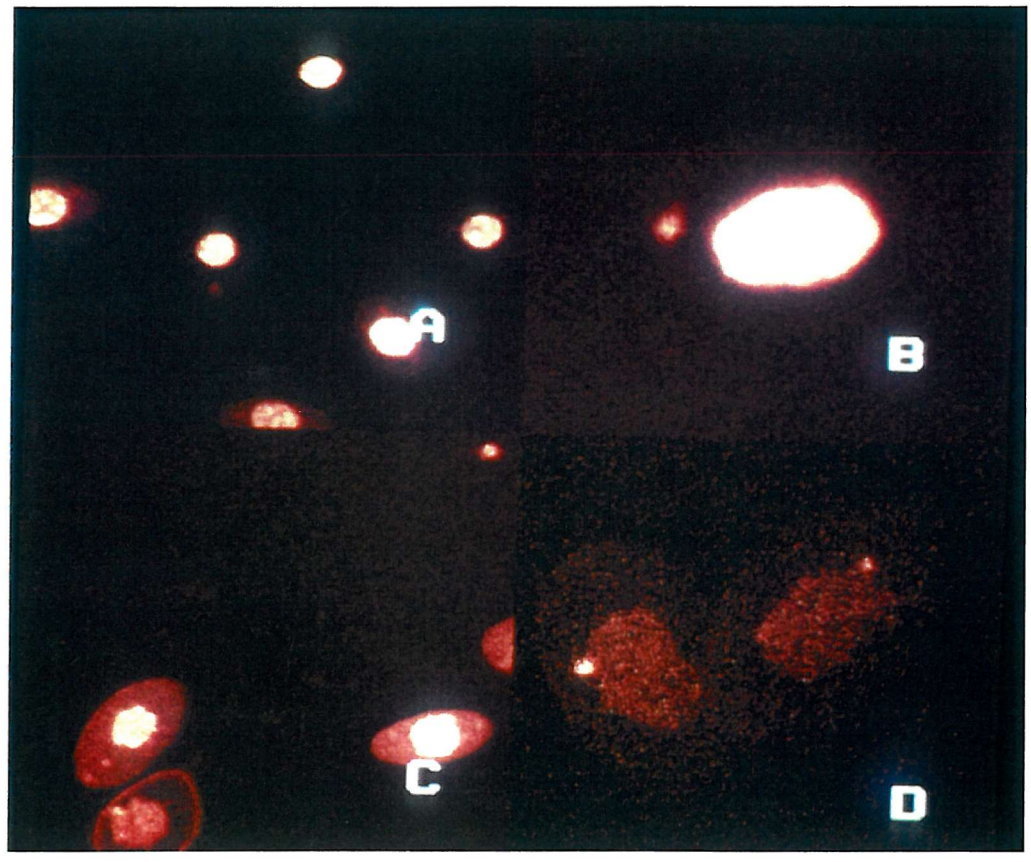


Figure 29

Confocal photomicrographs of anthracyline binding in avian erythrocytes.

*A* low power and *B* high power - epirubicin; *C* low power and *D* high power - idarubicin.



Avian erythrocytes

Avian erythrocytes are nucleated and were therefore chosen as a model for anthracycline binding in a quiescent nucleus surrounded by cytoplasm, in contrast to the isolated human epithelial nuclei described above. Experiments were again performed at pH 6.0, 7.4 and 8.0.

For epirubicin (table 13) there was a threefold increase in uptake at pH 8.0 (median fluorescence 425.5) compared with pH 6.0 (median fluorescence 127.5).

For idarubicin (table 14) there was also a trend towards increased uptake at pH 8.0 (median fluorescence 88.2) compared with pH 6.0 (median fluorescence 66.7). Flow cytometry histograms are given for these data in figure 28.

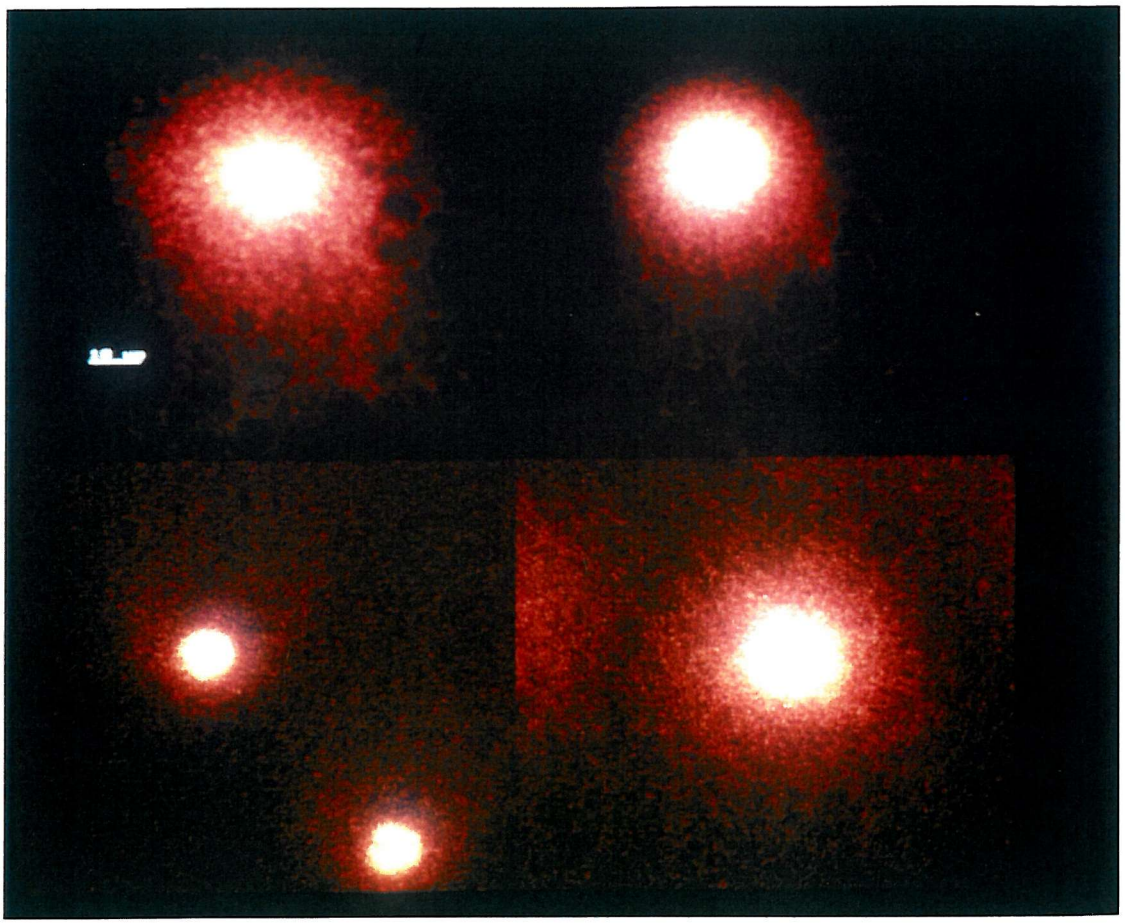
Confocal microscopy of avian erythrocytes incubated with epirubicin or idarubicin confirmed primarly nuclear drug uptake for both anthracyclines, with brighter epirubicin than idarubicin fluorescence (see figure 29).

Comet assays

Both neutral and alkaline comet assays produced no visible comets in controls (human peripheral lymphocytes) when stained with epirubicin, idarubicin or propidium iodide. However, in lysed apoptosed rat thymocytes, in *neutral* conditions, comets were seen after staining with epirubicin, idarubicin and propidium iodide (Figure 30). Conversely in *alkaline* conditions comets were seen when stained with propidium iodide, but not with epirubicin or idarubicin.

Figure 30

Confocal photomicrograph of (top) epirubicin, (bottom left) idarubicin and (bottom right) propidium iodide fluorescence in DNA comets. Scale bar 10 microns.



Anthracycline uptake by cell fractions - Discussion

The mechanism of action of anthracyclines remains poorly understood, although a number of possibilities exist. These molecules certainly bind to DNA and intercalate in a parallel fashion between adjacent base pairs (Plosker and Faulds 1993). Such binding has been shown to induce DNA stiffening, bending and elongation, and to inhibit DNA polymerase and DNA-dependent RNA polymerase (Booser 1994). Additionally DNA breakage leads to inhibition of topo II activity and binding to topo II cleavable complexes, resulting in subsequent DNA repair defects. Evidence also exists that free radical formation (probably after cytochrome P450 metabolism) induces oxidative injury to lipids in the cell membrane. The anthracyclines are intrinsically fluorescent and molecular uptake characteristics are therefore amenable to study by confocal laser scanning microscopy and flow cytometry. It is established, however, that binding of anthracyclines to DNA results in fluorescence quenching on a concentration-dependent basis, and quantitative measurements must be interpreted accordingly.

*Epirubicin* was first synthesised in 1980, as an epimer of doxorubicin (Adriamycin). Epirubicin differs from doxorubicin only in the configuration of the 4'-C atom; in epirubicin the 4'-OH attached to this group is in the equatorial, rather than axial, configuration (Launchbury and Habboubi 1993). This small change in molecular structure results in equipotent anti-tumour efficacy, but, for primarily physicochemical reasons, less toxicity in both animals and humans (Mouridsen 1990).

The 4'-OH configuration prevents interaction with a nearby amino group, resulting in greater lipophilicity. This is also associated (by means of internal hydrogen bonding

characteristics) with a lower pKa (7.7-8.08 compared with 8.22-8.34 for doxorubicin). This lower pKa (i.e. the pH at which 50% of the substance is ionised) means that less anthracycline will be electrically charged at physiological pHs and (since ionised drug is very poorly lipid soluble and is less able to enter cells across the lipid membrane) theoretically higher intracellular concentrations will be achieved. It is reassuring to note, however, in the context of intravesical chemotherapy that a study by Mross et al (1987) confirmed that the systemic uptake of epirubicin after intravesical instillation is almost undetectable and is of no clinical significance.

In 1986 Groos et al demonstrated that, for the RT112 transitional carcinoma cell line, a direct relationship existed between pH of the medium and uptake of both epirubicin and doxorubicin. In addition they confirmed that epirubicin exhibited greater cytotoxicity (reflecting its lower pKa) throughout the pH range studied (pH 5 to 10). This relationship between pH and epirubicin cytotoxicity has been confirmed in our laboratory for the MGHU-1 cell line and its MDR subline (MGHU-1R), and has been shown to correlate closely with increasing epirubicin uptake (both by flow cytometry and confocal microscopy) across a similar pH range (personal communication Mr P M Duffy & Dr A J Cooper).

*Idarubicin* is a synthetic 4-demethoxy-anthracycline analogue of daunorubicin. In a similar fashion to the epirubicin epimer, the absence of a methoxyl group at the 4' position in idarubicin results in greater lipophilicity in comparison to its parent molecule - the pKa of idarubicin is around 8.5 (personal communication Pharmacia Upjohn). Idarubicin is a more potent inhibitor of topoisomerase II than daunorubicin, and as result induces a higher number of double and single strand DNA breaks than its parent molecule (Capranico et al 1987). It is extensively metabolised to idarubicinol,

which is also a potent inhibitor of cell proliferation - this metabolite may therefore exert a significant clinical effect.

In cells of the MGHU-1 line and its MDR subline MGHU-1R the uptake of idarubicin differs significantly from epirubicin. In sensitive MGHU-1 cells epirubicin uptake is almost entirely nuclear, in contrast to MGHU-1R cells where uptake is cytoplasmic, punctuate and perinuclear. However in sensitive MGHU-1 cells idarubicin fluorescence appears predominantly perinuclear and cytoplasmic, with an area of intense perinuclear fluorescence which may represent the Golgi apparatus. There is relatively little nuclear drug fluorescence. The distribution of idarubicin in MGHU-1R cells is comparable, albeit at a slightly lower level of fluorescence (Duffy et al 1996). This effect was not explicable solely on the grounds of fluorescence quenching, and seems more likely to represent a different mechanism of intracellular drug handling for idarubicin, perhaps mediated at the nuclear:cytoplasmic interface.

These confocal and flow cytometric experiments with MGHU-1 and MGHU-1R cells confirm that both epirubicin and idarubicin enter and fluoresce within isolated intact nuclei. No significant difference in fluorescence of either drug was evident between nuclei from either cell type, suggesting that their intranuclear physicochemical environments are very similar. Alternatively it might suggest that drug fluorescence is similar even in differing physicochemical environments. A more direct assessment of such differences might be detectable using the fluorescent pH sensitive dyes SNARF and SNAFL but this was not pursued further here.

No consistent differences were seen in uptake of either drug at different pH values. Technical difficulties plagued the flow cytometry experiments, and it will be seen from tables 10 and 11 that standard deviations are large, suggesting the presence of

particulate debris and nuclear doublets/triplets. This difficulty might have been improved by more selective flow cytometric gating.

It is a quirk of the machine software that both parametric data and standard deviations are given. In view of the size of the standard deviations it was considered most appropriate to use median fluorescence values, as the total number of particles measured for each run (n=10,000) should be sufficient to allow their interpretation.

Confocal microscopy of pH-related uptake of epirubicin and idarubicin suggested inconsistencies with the flow cytometry results, although accurate quantitative comparisons were not possible. This phenomenon may represent slight technical differences in preparation (e.g. drug exposure times), may reflect the presence of more debris in the flow cytometry runs, or may simply represent the difference between single section analysis (confocal) and population analysis (flow cytometry) for these particles. In any event it seems likely that this phenomenon is worthy of further study.

Chromosome extracts also demonstrated binding and fluorescence with epirubicin and idarubicin using the confocal microscope, despite the likelihood of some degree of quenching. This adds further confirmatory evidence that idarubicin binds to DNA in its functional form. The use of metaphase chromosome spreads would shed further light on this, and is ongoing in our department.

Avian erythrocytes are considered dormant in terms of synthesis (Ringertz and Bolund 1974) and no ribosomal RNA synthesis is detectable (Zentgraf et al 1975) unless they are experimentally 'reactivated' in host cytoplasm (Ege et al 1975). They were chosen as a model of an intact nucleated cell with quiescent nucleoplasm, in order to establish whether idarubicin would be distributed in a typically anthracycline-like fashion to the nucleus, or whether it would be compartmentalised primarily in the cytosol and

excluded from the nucleus, as in mammalian MGHU-1 and MGHU-1R cells. As would be expected, fluorescence of both epirubicin and idarubicin was greatest at pH 8.0, which approximates to the pKa of epirubicin and is near to that of idarubicin. At all pH values the fluorescence associated with epirubicin was higher than that for idarubicin. This is likely to have two explanations - the pKa of idarubicin is slightly higher than that of epirubicin, and its DNA-quenching effect is reported as being greater (Duffy et al 1996).

Confocal microscopy confirmed that uptake of both epirubicin and idarubicin was primarily nuclear in avian erythrocytes and that epirubicin fluorescence exceeded that of idarubicin in these cells.

The technique of gel microelectrophoresis ('comet assay') is well established for quantifying DNA single-strand and double-strand breaks in individual mammalian cells (Olive et al 1992) often as a means of assessing apoptosis. It was chosen as a means of visualising anthracycline binding to DNA fragments from such cells (apoptosed rat thymocytes).

Alkaline lysis conditions result in the production of single-strand DNA breaks. Visible comets in such gels stained with epirubicin and idarubicin were absent, as would be expected - anthracyclines depend on interstrand intercalation for DNA binding, and such sites are absent in single-strand breaks. Propidium iodide does not depend on interstrand intercalation for DNA-binding and comets were consequently visible in alkaline gels stained with it.

Neutral lysis conditions, on the other hand, facilitate the production of double-strand DNA breaks. Consequently the presence of such fragments will allow anthracycline

intercalation - hence comets were detectable after staining with both epirubicin and idarubicin.

These experiments shed further light on the mechanisms by which anthracyclines are handled by cells. It seems that, at a subcellular level, idarubicin behaves as a 'typical' anthracycline (albeit with rather less striking fluorescence properties) - the drug is able to bind to isolated nuclei, 'vestigial' quiescent nuclei in avian erythrocytes, mammalian chromosomes and DNA. It has similar pH dependent characteristics to epirubicin. Yet, in MGHU-1 cells some mechanism is in operation which either prevents intranuclear idarubicin entry or facilitates its efflux from the intranuclear compartment. It is conceivable that such a mechanism may function at the nuclear:cytoplasmic interface, but its identity remains unknown.

## 7. SUMMARY - CLINICAL AND SCIENTIFIC IMPLICATIONS

Multidrug resistance must be viewed as a polymechanistic phenomenon, whereby several, perhaps many, features of cellular structure and function interact to protect the cell from a hostile external milieu. As described earlier, it is unusual for one single MDR component to be active in isolation mediating cellular protection from cytotoxic drugs - the converse is much more commonly the case. In addition, the mechanism combinations vary both for different drug molecules and different cell types. This intrinsic variability poses the main difficulty in studying MDR by investigating the molecular mechanisms which control it, whether in isolation or in combination.

The main aim of this work, therefore, was to establish a functional assay, rather than one based on mechanistic phenomena, for the identification of MDR in clinical biopsy material, that might be employable in the clinical arena. It seemed an attractive hypothesis that the patterns of epirubicin uptake that we had seen in bladder cancer cell lines, viewed by laser scanning confocal microscopy, and characteristic of either drug sensitivity or resistance (and therefore MDR), might be similarly identifiable in cells cultured from bladder cancer biopsies obtained from patients at cystoscopy. This proved to be so. As discussed in chapter 3, 70% of biopsies yielded successful explants for culture and MDR assay. Tumour grade, stage, progression and prior chemotherapeutic drug exposure did not, however, correlate to MDR phenotype score. The coincubation of tumour explants with MDR reversing agents prior to the assay resulted in either no change in phenotype or a trend towards increased drug sensitivity. The demonstration that few tumours seem phenotypically either entirely sensitive or resistant is an important one - it seems likely that drug resistant foci may be found in

most if not all tumours, and that this may represent a manifestation of the intrinsic genetic instability in neoplasms. This is broadly consistent with the findings of a number of other studies of MDR in clinical material conducted synchronously but independently in the 1990's, albeit using different methods and study designs.

Sufficient success was seen with this assay to justify its inclusion in a prospective randomised marker-lesion trial of pH-modified epirubicin intravesical chemotherapy, established in several Departments of Urology within Wessex NHS hospitals and co-ordinated by our research office. This was established as a result of the observation that epirubicin buffered to pH 8 was more cytotoxic in both wild type and MDR bladder cancer cell lines (personal communication Mr P M Duffy) than the standard preparation of epirubicin in saline or water.

Clearly, if further use and refinement of functional MDR assays based on this technique are encouraging, one could envisage several potential uses for it in more routine clinical practice. It might have a role in the prediction of response to intravesical chemotherapy, thereby potentially reducing morbidity in patients who might otherwise receive unnecessary or ineffective treatment, and reducing costs by limiting therapy to patients with drug-responsive tumours. A role in monitoring intravesical therapy could also be envisaged, perhaps facilitating surgical or other interventions by identifying treatment failure at an earlier stage, or allowing combination/alternative agent chemotherapy to be instituted in a more targetted fashion. It is also possible that the assay might potentially have prognostic value for patients with bladder cancer, a disease where good prognostic indicators are being avidly sought in an attempt to determine which patients are at risk of developing invasive disease, and therefore require earlier radical treatment. Finally a role might

also be found for the application of the assay in other tumour types where MDR is a significant impediment to the success of chemotherapy regimens.

As detailed in chapter 4 the induction of resistance to mitomycin C in the MGHU-1 cell line was associated with upregulation of the MDR-1 gene, coding for P-gp type 1 - cytotoxicity studies confirmed functional cross-resistance in these cells to both epirubicin and mitomycin C. These data provide further evidence, therefore, that mitomycin C should be considered an MDR-class drug. Currently many urologists and oncologists will substitute epirubicin for mitomycin C and *vice versa* in the face of patients with a need for intravesical chemotherapy who have failed treatment with one or other agent. This has evolved on an anecdotal basis, and there is little evidence in the literature to support this practice. Although some patients certainly respond, it seems likely that this represents another feature of the heterogeneity of bladder tumours rather than the ability of either drug to circumvent the cellular mechanisms mediating MDR.

The other cell line/cell fraction work documented in detail elsewhere in this thesis (chapters 5 and 6) has perhaps less immediate clinical significance, but sheds more light on some of the mechanisms mediating MDR.

MDR cells derived from MGHU-1 exhibited higher levels of plasma membrane P-gp and intracellular LRP, but not MRP. This result characterises in more detail the MDR phenotype in these cells and is a potential area for further study. In addition, other membrane labelling characteristics, such as annexin binding and fluorochrome labelling, were altered in these cells, confirming differences in membrane lipid composition and charge distribution which have previously been described. This feature of membranes in MDR cells should be borne in mind by investigators wishing,

for example, to track cells using such markers. Alterations in tissue factor binding in MDR MGHU-1 cells might in addition suggest potentially more significant implications for the investigation of angiogenesis and metastasis in tumours exhibiting MDR clones.

The binding of both epirubicin and idarubicin to fractionated nuclei, isolated chromosomes, avian erythrocytes and DNA comets was investigated in view of the controversy that continues about the role of quenching of anthracycline fluorescence by DNA molecules. This arose from our observation (Duffy et al 1996) that although epirubicin localises primarily to the nucleus in drug-sensitive cells, idarubicin does not. Very little nuclear idarubicin fluorescence is visible on confocal microscopy in such cells, where most drug is seen to be cytoplasmic and vesicular. This prompted our suggestion that intracellular idarubicin traffic differs from that of epirubicin, and that this was not simply a DNA quenching effect, confirming this by DNA spectrofluorimetry (Duffy et al 1996).

Binding of both epirubicin and idarubicin to fractionated nuclei, isolated chromosomes, avian erythrocytes and DNA comets was here seen to be comparable (chapter 6), suggesting that the mode of action of both drugs at the nuclear and subnuclear level may be similar, and adding further evidence that DNA quenching is insufficient explanation for the difference in cellular uptake characteristics of these two agents.

# 8. CONCLUSIONS

1. It is possible *in vitro* to culture explanted human transitional cell carcinomata from biopsy specimens. Anthracycline uptake patterns analogous to those seen in wild type and MDR bladder cancer cell lines may be seen using confocal microscopy after incubation with epirubicin in such explants. Intracellular epirubicin distribution can be manipulated in explants exhibiting MDR-type patterns in the presence of MDR reversing agents. These phenomena may be of use in the design of a functional assay for the identification of MDR in such tumours.

2. The induction of resistance to mitomycin C in the MGHU-1 cell line is associated with upregulation of the MDR-1 gene, coding for P-gp type 1 and associated with resistance to a variety of lipophilic drugs. Cytotoxicity studies confirm functional cross-resistance in these cells to epirubicin and mitomycin C, providing evidence that mitomycin C should be considered an MDR-class drug.

3. MDR cells derived from MGHU-1 exhibit higher levels of plasma membrane P-gp and intracellular LRP, but not MRP. In addition, other membrane labelling characteristics are altered in these cells suggesting differences in membrane lipid composition and charge distribution.

4. The binding of both epirubicin and idarubicin to fractionated nuclei, isolated chromosomes, avian erythrocytes and DNA comets was comparable, suggesting that the mode of action of both drugs at the nuclear and subnuclear level may be similar, and that DNA quenching is insufficient explanation for the difference in cellular uptake characteristics of these two agents.

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# 10. Appendices

## 10.1 Appendix 1

Principles of flow cytometry

Flow cytometry is essentially a technique for making single/multiple measurements on individual particles or cells (prestained by incubation with a fluorescent molecule/fluorescent-conjugated antibody) as they flow in a fluid stream, based on the detection of light scatter and fluorescence, after excitation by wavelength-specific laser light.

A complex laminar- and turbulent-boundary-flow fluidic system (which keeps a hydrodynamically focused core of sample in a surrounding fluid sheath) allows particles of the suspension to be delivered individually to a specific point intersected by the illuminating laser beam. User-selected filter sets are employed in order to ensure that only light of a specific wavelength is detected. Particle fluorescence is then measured at right-angles to the stream, with scatter measured both at right-angles ('side scatter') and in the forward direction ('forward scatter'). This light is detected by photodetectors which convert the photon pulses into electronic signals (see figure 32). Thereafter further processing allows graphic display and statistical analysis of the measurement.

Principles of confocal microscopy

In conventional light microscopy two dimensional images of the object (usually a section) are formed in the X and Y planes (parallel to the plane of sectioning) giving a single deep focal plane. This principle also applies in confocal microscopy, but with

this technology each image represents only a part of the thickness of the sample and it is possible to take serial sections through the object.

This is possible because the microscope optics exclude features outside the focal plane. If the focal plane is reassigned or the object moved it becomes possible to produce a series of X-Y images at different Z positions. Such an image series is a three dimensional representation of the object produced by optical rather than physical sectioning. This feature is afforded by the presence of a *confocal pinhole* in the microscope optics, which allows only light from the focal plane to reach the detector (see figure 31).

This confocal principle is combined with a *point-scanning system* using a laser light source. This constructs an image by scanning a point of incident laser light across the sample in the X and Y directions in a raster pattern, with photomultiplier tube detection. A pixel-based image is computer displayed for the purposes of intensity analysis, and presented on a high resolution video monitor for photography. In order to collect a series of images the computer shifts the focal plane and the object is rescanned at a different Z position. This image is stored and the process repeated to build a 3D data set.

The Leica TCS 4D Confocal Microscope System used in Southampton employs the Leitz DMR BE Research Microscope which is a high resolution light microscope with semi-automatic focusing controls and a galvanometric stage facilitating automatic specimen scanning in both X-Y and X-Z directions. A variety of objectives (from x10 to x63) of both water and oil immersion types allow wet preparations as well as permanently mounted specimens to be examined. Various microsope accessories allow the following illumination and optical systems to be used:

1. Transmitted light illumination, using a *halogen light* source for direct examination of stained preparations.

2. Transmitted *laser light* illumination using an air cooled, mixed gas (argon/krypton) 3μm fibre-optic, laser light source for non-confocal transmission and confocal reflectance imaging.

3. Transmitted light differential interference contrast (DIC) microscopy for assessment of overall specimen morphology.

4. Polarising microscopy for imaging birefringent/anisotropic material

5. Fluorescence microscopy using a *mercury vapour light* source for direct, microscopic examination of autofluorescent/fluorochrome labelled antibody preparations.

6. Fluorescence microscopy using the laser light source (with a choice of three distinct emission peaks of 488nm, 568nm and 647nm) for confocal imaging (optical slicing) of single or double fluorochrome stained or autofluorescent preparations.

Figure 31

The optical principles of confocal

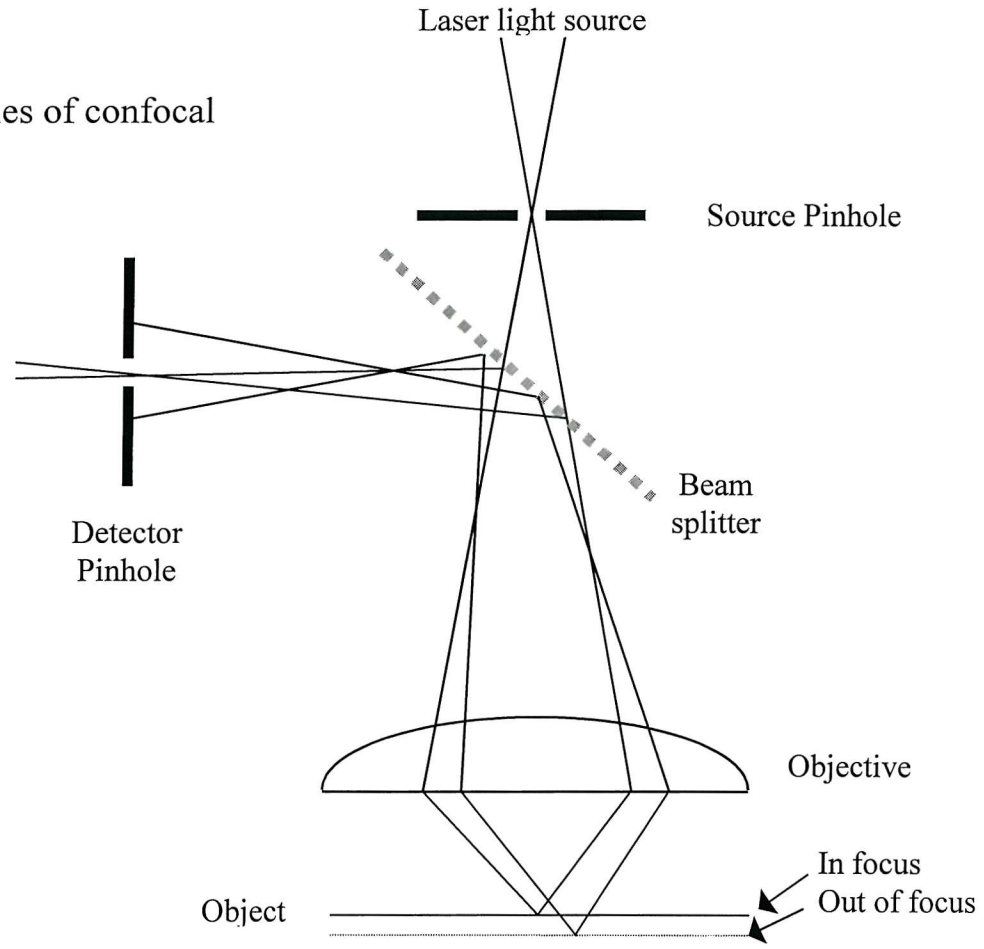
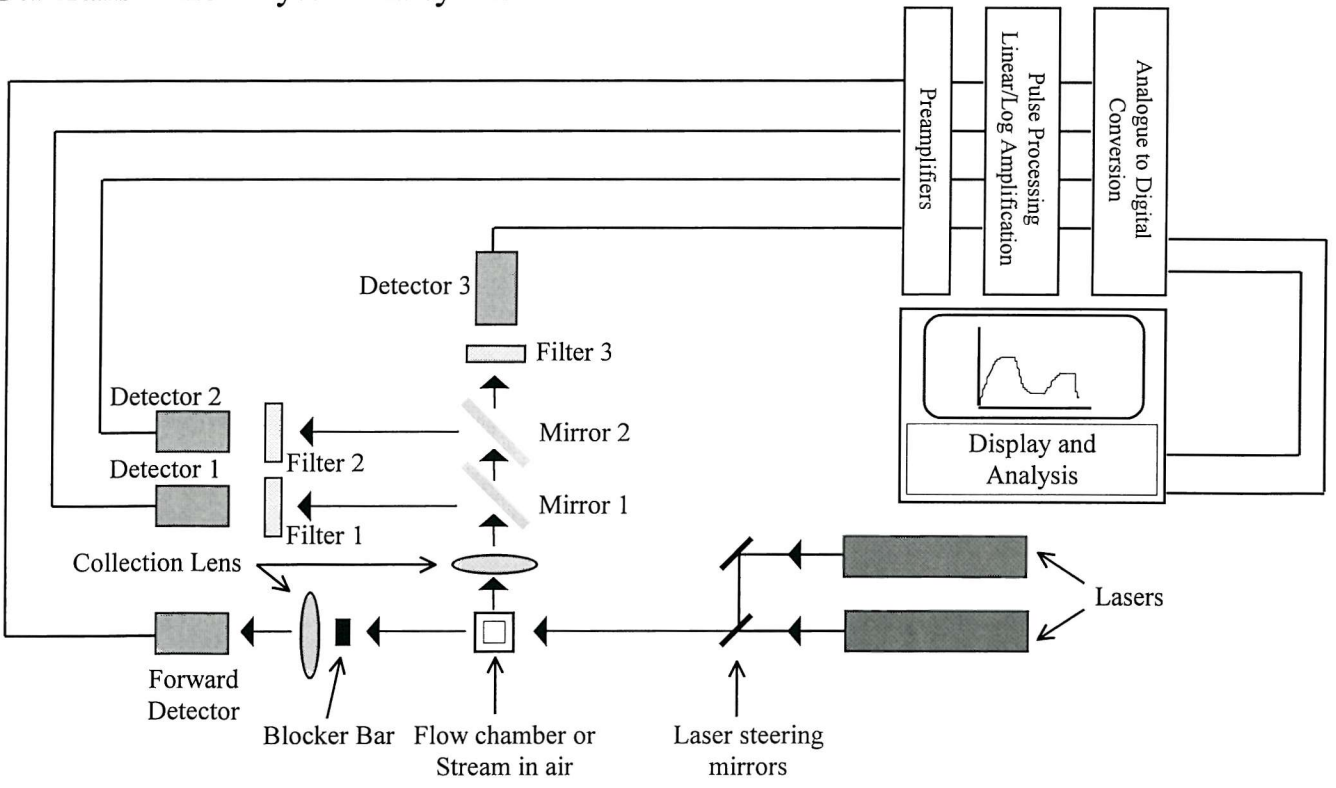



Figure 32

Generalised flow-cytometer system



## 10. 2 Appendix 2

The Polymerase Chain Reaction (PCR)

In 1987 Mullis and colleagues described a technique for the amplification of DNA sequences by enzymatic means which they termed a 'polymerase catalysed chain reaction'. This technique, now known the polymerase chain reaction (PCR), has revolutionised our understanding of molecular biology.

PCR is used for the selective amplification of defined DNA target sequences. Using information about the required DNA sequence, two oligonucleotide primers are designed which will specifially bind to their complimentary bases on opposite strands in denatured DNA so that they immediately flank the target region. The primers bind (anneal) in this way to opposing strands in the 5' to 3' direction. Thereafter new DNA strand synthesis is effected by the presence of a thermostable DNA polymerase and the four deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP).

Double stranded DNA is denatured to single strands for primer annealing by heating to 90-95°C. Primer annealing is allowed by lowering the temperature to between 50-75°C (primer dependent). Amplification of the primer products is then catalysed by DNA polymerase at 72°C. This completes only one cycle of the PCR. An exponential increase in product DNA is achieved by the chain reaction effect of each new DNA strand being available for further amplification, until a plateau is reached.

The products of these reactions may be visualised by means of polyacrylamide gel electrophoresis and ethidium bromide staining under ultraviolet illumination (see methods).

Reverse transcription-PCR (RT-PCR)

The discovery by Termin in 1972 of reverse transcriptase (RNA-directed DNA polymerase) allowed the development of a technique for creating complementary DNA (cDNA) strands from an RNA template.

mRNA is extracted from cells using a mild detergent in the presence of RNAse inactivators (e.g. β-mercaptoethanol). After extraction, mRNA is selectively 'primed' to allow cDNA formation on the desired RNA template by a reverse transcriptase enzyme. The cDNA is then amplified further by means of PCR and studied in the usual fashion. Oligo (dT) primers may be used for this purpose. These recognise and bind to the poly $(A)^+$ tail ($3'$) of mRNA, thus allowing it to be copied to cDNA in a selective fashion.

Application of this technique to the detection and analysis of RNA has increased the sensitivity of its detection by several orders of magnitude - only *in situ* hybridisation compares as favourably.

Minisatellite variant repeat (MVR) mapping

Throughout the genome, in both coding and non-coding sequences, may be found regions of repetitive DNA called *microsatellites* (dinucleotide or trinucleotide repeats). Variation in their sequence length may be due to induced replication errors. Longer sequence DNA repeats are termed *minisatellites*, where each repeat is 4-20 base pairs in length. In certain regions of the genome two types of minisatellite are found, and each of these regions may contain over 500 repeated minisatellites. These are *minisatellite variant repeat (MVR)* regions.

In these unique areas there is variation in both the repeat region length, and the interspersion pattern of two related but distinct 20bp satellites. This arrangement has

afforded the development of a PCR based methodology for the analysis of MVR interspersion patterns as a method for 'DNA fingerprinting' (Jeffreys et al 1991).

The detection of allele interspersion patterns by MVR-PCR necessitates two stages:

1. generation of MVR products of different lengths.
2. product amplification.

The technique employs two reactions per sample, each containing a primer complimentary to one of the minisatellite sub-types (31-TAG II AC or 31-TAG II GT) and a primer specific to a fixed point in the MVR flanking DNA (31-A), both at sufficiently low concentrations to prevent internal priming. This results in a stable product set of variable length extending from the flanking site to each MVR repeat unit sub-type. Further product amplification, for the purposes of detection, is achieved by the use of an additional driver primer (TAG) at relatively high concentration, which is complimentary to the repeat unit sub-type tail. Acrylamide gel electrophoresis then allows size separation of resulting products. This technique was employed to confirm/refute the common parental cell origin of the tumour cell lines used during the course of this work.

## 10.3 Appendix 3

Titration experiment to optimise primer conditions for MDR 1 and 3 expression

A condition titration table was constructed as shown below:

| **$MgCl_2$ (µl)** | **$MgCl_2$ (mM)** | | | | |
|---|---|---|---|---|---|
| **2** | **1** | Tube 1 | Tube 2 | Tube 3 | |
| **3** | **1.5** | Tube 4 | Tube 5 | Tube 6 | |
| **4** | **2** | Tube 7 | Tube 8 | Tube 9 | |
| **5** | **2.5** | Tube 10 | Tube 11 | Tube 12 | |
| | | **0** | **1** | **2** | **DMSO (µl)** |
| | | **0** | **2** | **4** | **DMSO (v/v)** |

Two mastermixes were made up according to the following protocol:

65µl Buffer (x10)
13µl dATP
13µl dCTP
13µl dGTP
13µl dTTP
26µl mixed RT-PCR sample
5.2µl MDR-1 primer **OR** 4µl MDR-3 primer
6.5µl MDR-U primer (constant)
0.5µl taq polymerase
462.8µl (for MDR-1) **OR** 464µl (for MDR-3) distilled water.

48µl from the MDR-1 mastermix were added to tubes 1-12, and 48µl from MDR-3 to tubes 13-24. Aliquots of magnesium chloride and DMSO were added variably

according to the above table, and samples covered by a drop of mineral oil. Tubes were then heated on the TECHNE Progene PCR machine as follows:

| | |
|---|---|
| One cycle of | 94°C for three minutes |
| Followed by 35 cycles of | 94°C for 30 seconds |
| | 55°C for 30 seconds |
| | 72°C for 30 seconds |
| Finally one cycle of | 72°C for two minutes. |

5μl from each of the resulting products were added to 3μl bromophenol blue, and run on an 8% polyacrylamide gel against ϕX Hae III DNA marker at 40mA for two hours, stained with ethidium bromide as above.

## 10.4 Appendix 4 - Data tables

Table 11 - Fluorescence of epirubicin in sensitive/resistant isolated nuclei

| Cell type | pH | Mean fluorescence | Median fluorescence | Standard deviation |
|---|---|---|---|---|
| Sensitive | 6.0 | 621 | 302.3 | 1223.5 |
| Sensitive | 7.4 | 755.7 | 313.4 | 1515.5 |
| Sensitive | 8.0 | 558.7 | 232.9 | 1268.9 |
| Resistant | 6.0 | 546.5 | 282.0 | 1041.5 |
| Resistant | 7.4 | 655.9 | 283.9 | 1412.9 |
| Resistant | 8.0 | 569.4 | 264.2 | 1248.8 |

Table 12 - Fluorescence of idarubicin in sensitive/resistant isolated nuclei

| Cell type | pH | Mean fluorescence | Median fluorescence | Standard deviation |
|---|---|---|---|---|
| Sensitive | 6.0 | 498.5 | 212.9 | 1163.4 |
| Sensitive | 7.4 | 877.8 | 349.1 | 1634.4 |
| Sensitive | 8.0 | 908.3 | 342.9 | 1786.2 |
| Resistant | 6.0 | 492.6 | 209.1 | 1133.6 |
| Resistant | 7.4 | 707.9 | 302.3 | 1428.3 |
| Resistant | 8.0 | 834.2 | 352.3 | 1616.2 |

Table 13 - Fluorescence of epirubicin in avian erythrocytes

| pH | Mean fluorescence | Median fluorescence | Standard deviation |
|---|---|---|---|
| 6.0 | 142.8 | 127.5 | 114.1 |
| 7.4 | 288.7 | 268.9 | 138.1 |
| 8.0 | 455.7 | 425.5 | 149.3 |

Table 14 - Fluorescence of idarubicin in avian erythrocytes

| pH | Mean fluorescence | Median fluorescence | Standard deviation |
|---|---|---|---|
| 6.0 | 72.3 | 66.7 | 25.3 |
| 7.4 | 92.3 | 83.5 | 41.8 |
| 8.0 | 101.3 | 88.2 | 72.3 |

Cytotoxicity experiments for mitomycin C and epirubicin in MGHU-MMC, MGHU-1 and MGHU-1R.

Table 15 - Mitomycin C and MGHU-MMC

| MMC concentration | 0 | 0.6 | 1.25 | 2.5 | 5 | 10 | 20 | 40 | 80 |
|---|---|---|---|---|---|---|---|---|---|
| | 188 | 234 | 161 | 179 | 184 | 133 | 95 | 100 | 116 |
| | 170 | 133 | 135 | 144 | 155 | 168 | 151 | 115 | 124 |
| | 185 | 136 | 150 | 126 | 160 | 160 | 135 | 141 | 130 |
| | 231 | 171 | 138 | 116 | 125 | 127 | 168 | 153 | 146 |
| | 172 | 151 | 217 | 234 | 146 | 149 | 81 | 152 | 111 |
| | 247 | 158 | 146 | 142 | 143 | 114 | 130 | 83 | 75 |
| mean O.D. | 193.5 | 168.5 | 146 | 141.25 | 156 | 147 | 137.25 | 127.25 | 129 |
| %mean | 100 | 87.1 | 75.4 | 73 | 80.6 | 76 | 70.9 | 65.8 | 66.7 |
| st dev | 29.5 | 33.9 | 27.8 | 39.7 | 18 | 18.9 | 30.2 | 26.6 | 21.8 |
| s err mean | 12 | 13.8 | 11.3 | 16.2 | 7.3 | 7.7 | 12.3 | 10.9 | 8.9 |
| % sem | 6.2 | 7.1 | 5.9 | 8.4 | 3.8 | 4 | 6.4 | 5.6 | 4.6 |
| 95%ci | 12.2 | 14 | 11.5 | 16.4 | 7.4 | 7.8 | 12.5 | 11 | 9. |

Table 16 - Epirubicin and MGHU-MMC

| EPI concentration | 0 | 0.6 | 1.25 | 2.5 | 5 | 10 | 20 | 40 | 80 |
|---|---|---|---|---|---|---|---|---|---|
| | 131 | 164 | 177 | 154 | 135 | 131 | 144 | 130 | 107 |
| | 166 | 149 | 136 | 186 | 167 | 156 | 151 | 127 | 115 |
| | 193 | 174 | 122 | 181 | 127 | 137 | 123 | 140 | 120 |
| | 170 | 179 | 172 | 156 | 151 | 145 | 148 | 145 | 137 |
| | 184 | 180 | 182 | 193 | 126 | 164 | 178 | 128 | 131 |
| | 167 | 195 | 182 | 168 | 135 | 125 | 100 | 133 | 112 |
| mean O.D. | 168.5 | 173.5 | 161.8 | 173 | 140.2 | 143 | 140.7 | 133.8 | 120.3 |
| %mean | 100 | 103 | 96 | 102.7 | 83.2 | 84.9 | 83.5 | 79.4 | 71.4 |
| st dev | 18.7 | 19.4 | 14.3 | 23.8 | 14.8 | 14.5 | 13.6 | 24.3 | 6.6 |
| s err mean | 7.6 | 7.9 | 5.8 | 9.7 | 6 | 5.9 | 5.6 | 9.9 | 2.7 |
| %sem | 5.2 | 4.7 | 3.5 | 5.8 | 3.6 | 3.5 | 3.3 | 5.9 | 1.6 |
| 95%CI | 10.2 | 9.2 | 6.8 | 11.3 | 7 | 6.9 | 6.5 | 11.5 | 3.1 |

Table 17 - Mitomycin C and MGHU-1

| MMC concentration | 0 | 0.6 | 1.25 | 2.5 | 5 | 10 | 20 | 40 | 80 |
|---|---|---|---|---|---|---|---|---|---|
| | 155 | 173 | 115 | 18 | 31 | 25 | 6 | 1 | 5 |
| | 156 | 62 | 53 | 37 | 45 | 12 | 7 | 22 | 7 |
| | 170 | 80 | 72 | 34 | 33 | 17 | 26 | 22 | 6 |
| | 164 | 54 | 50 | 39 | 51 | 23 | 23 | 9 | 3 |
| | 169 | 133 | 33 | 31 | 35 | 16 | 4 | 5 | 6 |
| | 164 | 69 | 34 | 37 | 36 | 11 | 5 | 9 | 26 |
| mean O.D. | 163 | 95.2 | 59.5 | 32.7 | 38.5 | 17.3 | 11.8 | 11.3 | 8.8 |
| %mean | 100 | 58.4 | 36.5 | 20 | 23.6 | 10.6 | 7.3 | 6.9 | 5.4 |
| st dev | 5.8 | 43.2 | 28 | 7 | 7.1 | 5.2 | 9 | 8 | 7.8 |
| s err mean | 2.4 | 17.6 | 11.4 | 2.9 | 2.9 | 2.1 | 3.7 | 3.3 | 3.2 |
| %sem | 1.4 | 10.8 | 7 | 1.8 | 1.8 | 1.3 | 2.3 | 2 | 1.9 |
| 95% CI | 2.8 | 21.2 | 13.8 | 3.4 | 3.5 | 2.5 | 4.4 | 3.9 | 3.8 |

Table 18 - Epirubicin and MGHU-1

| EPI concentration | 0 | 0.6 | 1.25 | 2.5 | 5 | 10 | 20 | 40 | 80 |
|---|---|---|---|---|---|---|---|---|---|
| | 127 | 128 | 112 | 65 | 30 | 48 | 16 | 14 | 13 |
| | 142 | 151 | 117 | 84 | 43 | 49 | 11 | 1 | 4 |
| | 162 | 114 | 110 | 83 | 39 | 45 | 28 | 9 | 12 |
| | 142 | 113 | 81 | 61 | 56 | 46 | 13 | 9 | 10 |
| | 150 | 128 | 111 | 47 | 47 | 47 | 19 | 13 | 15 |
| | 132 | 122 | 122 | 63 | 54 | 43 | 15 | 12 | 17 |
| mean O.D. | 142.5 | 126 | 108.8 | 67.2 | 44.8 | 46.3 | 17 | 9.7 | 11.8 |
| %mean | 100 | 88.4 | 76.4 | 47.1 | 31.5 | 32.5 | 11.9 | 6.8 | 8.3 |
| st dev | 11.4 | 12.7 | 13.1 | 12.9 | 8.9 | 2 | 5.5 | 4.3 | 4.1 |
| s err mean | 4.7 | 5.2 | 5.3 | 5.3 | 3.6 | 0.8 | 2.2 | 1.8 | 1.7 |
| %sem | 3.3 | 3.6 | 3.7 | 3.7 | 2.5 | 0.6 | 1.6 | 1.2 | 1.2 |
| 95% CI | 6.4 | 7.1 | 7.4 | 7.2 | 5 | 1.1 | 3 | 2.4 | 2.3 |

Table 19 - Mitomycin C and MGHU-1R

| MMC concentration | 0 | 0.6 | 1.25 | 2.5 | 5 | 10 | 20 | 40 | 80 |
|---|---|---|---|---|---|---|---|---|---|
| | 492 | 455 | 576 | 489 | 327 | 353 | 326 | 219 | 249 |
| | 327 | 574 | 539 | 373 | 272 | 298 | 378 | 210 | 204 |
| | 513 | 454 | 364 | 350 | 314 | 360 | 220 | 196 | 156 |
| | 571 | 404 | 494 | 495 | 338 | 322 | 290 | 227 | 218 |
| | 535 | 432 | 326 | 428 | 206 | 235 | 329 | 258 | 203 |
| mean O.D. | 487.6 | 463.8 | 459.8 | 427 | 291.4 | 313.6 | 308.6 | 222 | 206 |
| %mean | 100 | 95 | 94.3 | 87.6 | 59.8 | 64.3 | 63.3 | 45.5 | 42.2 |
| st dev | 84.4 | 58 | 98 | 58.8 | 48.2 | 45.2 | 52.4 | 20.7 | 30 |
| s err mean | 34.5 | 23.7 | 40 | 24 | 19.7 | 18.4 | 21.4 | 8.5 | 12.2 |
| %sem | 7.1 | 5.1 | 8.7 | 5.6 | 6.7 | 5.9 | 6.9 | 3.8 | 5.9 |
| 95%CI | 13.9 | 10 | 17 | 11 | 13.2 | 11.5 | 13.6 | 7.5 | 11.7 |

Table 20 - Epirubicin and MGHU-1R

| EPI concentration | 0 | 0.6 | 1.25 | 2.5 | 5 | 10 | 20 | 40 | 80 |
|---|---|---|---|---|---|---|---|---|---|
| | 469 | 359 | 342 | 456 | 474 | 437 | 332 | 311 | 258 |
| | 607 | 332 | 401 | 451 | 319 | 378 | 418 | 480 | 303 |
| | 522 | 382 | 424 | 522 | 377 | 285 | 369 | 332 | 260 |
| | 519 | 323 | 381 | 320 | 463 | 338 | 249 | 335 | 289 |
| | 350 | 314 | 418 | 442 | 404 | 320 | 300 | 230 | 196 |
| mean O.D. | 493.4 | 342 | 393.2 | 438.2 | 407.4 | 351.6 | 333.6 | 337.6 | 261.2 |
| %mean | 100 | 69.3 | 79.7 | 88.8 | 82.6 | 71.3 | 67.6 | 68.4 | 52.9 |
| st dev | 84.3 | 25. | 29.6 | 65.6 | 57 | 52.2 | 57.7 | 80.7 | 36.8 |
| s err mean | 34.4 | 10.2 | 12.1 | 26.8 | 23.3 | 21.3 | 23.6 | 33 | 15 |
| %sem | 7 | 2.1 | 2.4 | 5.4 | 4.7 | 4.3 | 4.8 | 6.7 | 3 |
| 95%ci | 13.7 | 4.1 | 4.8 | 10.6 | 9.2 | 8.5 | 9.4 | 13.1 | 6 |

Table 21 - Titration of PKH2-GL in sensitive & resistant MGHU-1 cells by flow cytometry

| **PKH2** Concentration | **Sensitive** - mean fluorescence intensity | **Resistant** - mean fluoresence intensity |
|---|---|---|
| Control (0 M) | 1.01 | 1.0 |
| $5x10^{-7}$ M | 17.15 | 1.04 |
| $1x10^{-6}$ M | 34.6 | 1.15 |
| $2x10^{-6}$ M | 92.2 | 1.9 |

Table 22 - PKH2-GL fluorescence in sensitive and resistant cells at 25°C and 0°C

| **Temperature** (°C) | **Sensitive** - mean fluorescence intensity | **Resistant** - mean fluorescence intensity |
|---|---|---|
| 25 (control) | 208.9 | 3.1 |
| 0 (on ice) | 41.8 | 2.8 |

Table 23 - PKH2-GL fluorescence at 25°C in MDR cells ± prior MDR reversal with verapamil

| **Preincubation with verapamil?** | **Mean fluorescence intensity** |
|---|---|
| **yes** | 3.2 |
| **no** | 3.5 |

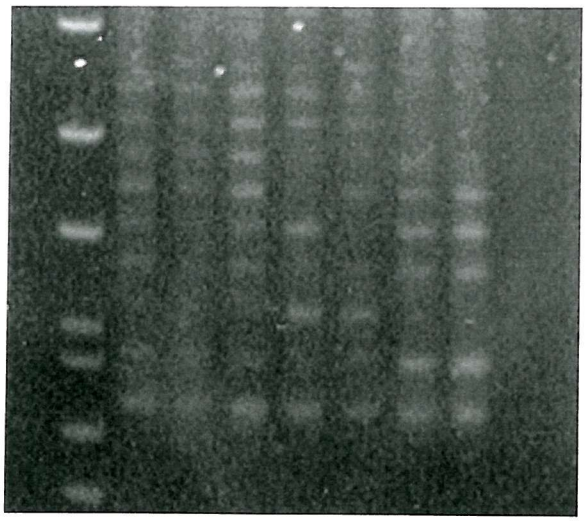
## 10.5 Appendix 5

Clonal characterisation of cell lines

The clonal characteristics of MGHU-1, MGHU-1R, MGHU-MMC, MCF7, MCF7R, HT-29 and HT-29R, characterised by (31) tagIIac MVR-PCR, are demonstrated on the following photograph *(figure 7)* of the 8% non-denaturing polyacrylamide gel which had been stained with 10 $ngml^{-1}$ ethidium bromide and viewed under ultraviolet illumination.

Lane 1 $\phi$X 174RF Hinf I DNA marker. Bands visible as indicated.

Lanes 2-8 show DNA bands for MGHU-1, MGHU-1R, MGHU-MMC, MCF7, MCF7R, HT-29 and HT-29R respectively.



The minisatellite band distributions (MVR product base pair lengths) for MGHU-1, MGHU-1R and MGHU-MMC are identical, as are those for HT-29 and HT-29R. This indicates that these cell lines are of clonal origin and that this relationship is unaffected by their MDR phenotype.

The minisatellite band distribution for MCF-7, in contrast, differs in at at least three minisatellite products, indicating that this cell line is polyclonal.