Investigation of the role of LRP in Multidrug Resistance

A thesis submitted for the degree of Ph.D.

by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

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Abstract

There have been many reports linking the overexpression of the lung resistance-related protein (LRP) with cross-resistance to chemotherapeutic drugs. However, no conclusive evidence existed to link LRP with a direct role in multidrug resistance (LRP). The OAW42SR is a cell line derived from a serous adenocarcinoma of the ovaries, and displays an increase in resistance to cytotoxic drugs concomitant with a moderate increase in LRP expression. Anti-LRP ribozyme and antisense expression plasmids were employed in this study in order to inhibit LRP expression in the OAW42SR cell line and examine any resulting effect on the drug resistance of the cells. Antisense oligonucleotides were also used to decrease LRP expression in the OAW42SR cell line in order to provide a clearer picture of whether LRP is involved in MDR.

A large number of clones were isolated after transfection of the OAW42SR cell line with anti-LRP ribozyme and antisense expression plasmids. These clones displayed varying levels of LRP at both the mRNA and protein level. Cells transfected with only a control vector also displayed decreases in LRP expression, highlighting the extent of clonal variation within the OAW42SR population. The anti-LRP ribozyme construct appeared to significantly reduce LRP expression at both the mRNA and protein level. The anti-LRP antisense RNA construct failed to reduce LRP mRNA expression levels, but dramatically reduced LRP at the protein level. This demonstrated that antisense RNA acts mainly through steric inhibition of mRNA processing rather than cleavage of the target RNA, as with ribozymes. Resistance to anthracyclines and *Vinca* alkaloids was reduced in many of the clones. However, the levels of LRP expression could not be correlated with the reduction in resistance to the tested drugs. The levels of expression of the MDR facilitators, multidrug resistance gene 1 (mdr-1) and multidrug resistance-associated protein (MRP), within the clones was largely invariant, and could not be directly correlated with the observed reductions in drug resistance. The drug resistance profiles of the OAW42SR clones were, however, strikingly similar to that of typical mdr-1 overexpressing cell lines. It cannot be ruled out, therefore, that variation in P-glycoprotein activity, due to post-translational modifications, may be the sole mechanism of drug resistance in these clones. Antisense oligonucleotides targeted to LRP, reduced expression at both the mRNA and protein level in the OAW42SR cells, but failed to induce a reduction in resistance to adriamycin. This thesis provides the first direct evidence that LRP is not involved in multidrug resistance, at last within the OAW42SR cell line.

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This thesis is dedicated to my beloved sister Fiona,
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Their love will never leave me.

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List of Abbreviations

2R120	SW1573-2R120 cell line
AAV	Adeno-associated viruses
ABC	ATP binding cassette
ALL	Acute lymphoblastic leukaemia
AML	Adult acute leukaemia
AMP	Adenosine-monophosphate
AS	Antisense
ATP	Adenosine-triphosphate
ATTC	American tissue culture collection
BABL/c mice	Immunodeficient mice
BSA	Bovine serum albumin
cAMP	Cyclic AMP
cDNA	Complementary DNA
CMV	Cytomegalovirus
CsCl	Caesium chloride
DAB	3,3-diaminobenzidine tetrahydrochloride
DEPC	Diethyl procarbonate
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate (N = A, C, T, G or U)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetracetic acid
FCS	Foetal calf serum
GAPDH	Glyceraldehyde-6-phosphate dehydrogenase
GnSCN	Guanidium thiocyanate
GSH	Glutathione
GST	Glutathione-S-transferase
GS-X pump	GSH S-conjugate export carrier

HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
HSV-tk	Herpes simplex virus thymidine kinase
IC_{50}/ID_{50}	50 percent inhibitory concentration
IMS	Industrial methylated spirits
Ip	Intraperotoneal
kD	Kilo Daltons
LRP	Lung resistance-related protein
LTR	Long terminal repeat
MAb	Monoclonal antibody
MDR	Multidrug resistance
MMLV-RT	Moloney murine luekaemia virus reverse transcriptase
MMTV	Mouse mammary tumor virus
mRNA	Messenger RNA
MRP	Multidrug resistance-related protein
MT	Metallothioneins
MVE	Minimal volume entrapment
MVP	Major vault protein
MWM	Molecular weight marker
NCI	National Cancer Institute (USA)
NPC	Nuclear pore complex
OD	Optical density
Oligos	Oligonucleotides
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pgp	P-glycoprotein
pHβ	pHβApr-1-neo expression vector
PKA	cAMP-dependent protein kinase
PKA-I	PKA type I
PKC	Protein kinase C
PO	Phosphodiester
PS	Phosphorothioate
RNA	Ribonucleic acid

RNase H	Ribonuclease H
RNasin	Ribonuclease inhibitor
RSV	Rous sarcoma virus
RT-PCR	Reverse transcription PCR
Rz	Ribozyme
SDS	Sodium dodecyl sulphate
TBE	Tris-boric acid-EDTA buffer
TBS	Tris-buffered saline
TE	Tris-Edta
Topo II	Topoisomerase II
UHP	Ultra high pure water
VP16	Etoposide

APPENDIX B

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1. INTRODUCTION

1.1 INTRODUCTION TO MULTIDRUG RESISTANCE (MDR).

The occurrence of cellular drug resistance remains a major problem in the chemotherapeutic treatment of cancer. For most metastatic cancers, the currently available anti-cancer drugs are merely used to prolong life and/or alleviate symptoms, rather than to cure the patient. Although many cancers, including breast and ovarian cancers, non-Hodgkin's lymphoma, adult acute leukaemia, and numerous childhood cancers initially respond well to chemotherapy, very often the tumors become resistant to cytotoxic drugs during or shortly after therapy (Nooter and Stoter, 1996). Other cancers, such as non-small cell lung cancer and cancers of the colon, stomach, kidney and pancreas, are inherently resistant to anti-cancer drugs even before the start of treatment.

A large variety of drug resistance mechanisms have been characterised, using *in vitro* cell lines made resistant against the different classes of anti-cancer agents. Alterations in target proteins, carrier mediated drug uptake, drug metabolism, cellular repair mechanisms and cellular drug efflux can mediate drug resistance *in vitro*. Most drug resistant cell lines have been obtained by step-wise increased exposure to a specific anti-cancer drug. This results in the cell line being resistant to the drug used for selection and drugs with similar chemical structure to the biologically active moiety, as expected. However, during selection of the cells for resistance to these so-called "naturally occurring" anti-cancer drugs, such as anthracyclines, *Vinca* alkaloids, epidophyllotoxins, actinomycin D and colchicine, the cells can develop cross-resistance to other apparently structurally and functionally unrelated natural compounds (Nielsen and Skovsgaard, 1992; Clynes, 1993; Hill 1993). This phenomenon is referred to as multidrug or multiple-drug resistance (MDR). These MDR-related naturally occurring drugs are derived from plants and micro-organisms, but are structurally dissimilar and have different intracellular targets. They are all, however, rather large (between 300-900 kD molecular weight), amphipatic, and enter the cell by passive diffusion, as they are soluble in lipids at physiological pH (Nooter and Stoter, 1996).

1.1.1 Multiple drug resistance 1 (mdr-1) gene and P-glycoprotein (Pgp)

The molecular basis for the phenomenon of broad-spectrum resistance to anti-cancer drugs, has been the subject of intensive research. Of the various mechanisms by which cancer cells evade the cytotoxic action of the anti-neoplastic drugs, the best characterised form of drug resistance has been ascribed to the expression of the multiple drug resistance 1(mdr-1) gene and its protein product phospho- or P-glycoprotein (Pgp) (Clynes, 1993; Kiehntopf *et al.*, 1994; Nooter and Stoter, 1996; Srivastava *et al.*, 1996). Pgp is a plasma membrane efflux pump with ATPase activity and a molecular weight of 170 kDa. It is composed of two similar halves, each containing six putative transmembrane domains and one nucleotide-binding site consensus sequence (Srivastava *et al.*, 1996). This unique structural characteristic of Pgp has been conserved in a large number of membrane associated transporters from bacteria to higher eukaryotes, forming a novel superfamily of proteins called the ATP binding cassettes (ABC) transporters (Higgins, 1992). Pgp acts to promote the efflux of a variety of drugs with resultant reduction in intracellular drug concentration (Clynes, 1993; Srivastava *et al.*, 1996). The physiological function of Pgp is not, however, completely understood. Detoxification of naturally occurring compounds and excretion of endogenous metabolites, e.g. steroid hormones, are possibilities (Gottesmann and Pastan, 1993), while there is evidence that it is an essential component of a volume-regulated chloride channel (Valverde *et al.*, 1992).

Due to the negative effects on chemotherapy of Pgp overexpression, and its prevalence (with some estimates that Pgp is increased in as much as 50% of all human tumours at some stage of treatment with natural drugs (Gottesmann, 1988)), there has been much interest and effort in reversing MDR. In the laboratory, the methods to reverse MDR caused by Pgp membrane transport system have focused mainly on two approaches: i) development of reversing agents that are recognised by Pgp but are less toxic and, in large concentrations block the efflux of chemotherapeutic drugs; and ii) development of analogs of chemotherapeutic agents which express a lower affinity for Pgp binding site, hence, less potential for resistance mediated by drug efflux (Srivastav *et al.*, 1996). To date, several classes of reversing agents have been studied including calcium-channel blockers (e.g. verapamil), immunosuppressants (e.g. cyclosporin A),

steroids (e.g. progesterone), steroid antagonists (e.g. tamoxifen), cardiac antiarrythmics (e.g. amiodarone and quinidine) and others (e.g. amphotericin, Tween 80, reserpine) (Srivastava *et al.*, 1996). Even cells with very high levels of P-glycoprotein can have active drug efflux blocked with the simultaneous addition of a P-glycoprotein antagonist. However, a lack of specificity or toxic side effects of these compounds has resulted in a limited clinical application for many of these chemo-modulators. Therefore, there is considerable need to find alternative ways of circumventing mdr-1 mediated drug resistance. To this end, the molecular biology tools of antisense and ribozymes, which can specifically target and disrupt the DNA or RNA of a gene, have been employed to great effect. These tools and their application to reversal of MDR are dealt with in detail later (see Sections 1.3 to 1.5).

1.1.2 Multidrug resistance-associated protein (MRP)

After the initial surge of interest and research into Pgp, it soon became evident that overexpression of mdr-1 was not the sole MDR mechanism, and that alternative mechanisms of multidrug resistance must exist. Some malignancies, such as lung cancer, frequently displayed either acquired or intrinsic multidrug resistance without elevated levels of mdr-1 (Lai *et al.*, 1989). A number of multidrug resistant tumour cell lines, such as the human small cell lung cancer cell line H69AR, have also been described which do not overexpress mdr-1 (Mirski *et al.*, 1987; Reeve *et al.*, 1990; Taylor *et al.*, 1991). H69AR cells display a cross-resistance profile very similar to cells which overexpress the mdr-1 gene, but express no more mdr-1 than parental NCI-H69 (H69) cells (Mirski *et al.*, 1987; Cole *et al.*, 1991). Additionally, multidrug resistance in these cells is poorly reversed by chemosensitisers that are effective in cells overexpressing mdr-1 (Cole *et al.*, 1989).

In a search for proteins responsible for the multidrug resistance of H69AR cells, cDNAs corresponding to an mRNA that is highly expressed in the resistant cells but not in drug-sensitive parental or revertant cells, were cloned, isolated and sequenced (Cole *et al.*, 1992). The mRNA coded for a protein of 1531 amino acids and was named the multidrug resistance-associated protein (MRP). The predicted primary sequence and secondary structure of MRP indicate that it is also a member of the

ATP-binding cassette superfamily of membrane transport proteins (Higgins, 1992), although it only shares 15% amino acid homology with Pgp (Lautier *et al.*, 1996). Increased concentrations of MRP mRNA have been detected in multidrug-resistant cell lines derived from a wide variety of tissues (Cole *et al.*, 1991; Krishnamachary and Center, 1993; Slovak *et al.*, 1993). Several of these cell lines were shown to contain multiple copies of the MRP gene as a result of amplification and translocation of a region of chromosome 16 spanning the MRP gene at band p13.1 (Slovak *et al.*, 1993). In H69AR cells, the MRP gene is amplified 40-50 fold.

In order to prove that the MRP gene itself could confer resistance, and that the gene was not simply co-amplified with some other gene that does, HeLa cells were transfected with MRP expression vectors (Grant *et al.*, 1994). The transfectants were found to display an increase in resistance to doxorubicin that is proportional to the levels of a 190 kDa integral membrane protein recognised by anti-MRP antibodies. However, the drug cross-resistance profiles of cells that overexpress MRP or Pgp are similar but not identical. For example, taxol is an efficient substrate for Pgp, but not for MRP (Zaman *et al.*, 1994). There also appear to be fundamental differences in the mechanisms by which the two proteins transport chemotherapeutic drugs. Pgp-enriched membrane vesicles have been shown to directly transport several chemotherapeutic drugs, whereas vincristine transport by MRP-enriched membrane vesicles is demonstrable only in the presence of reduced glutathione (Lautier *et al.*, 1996). Several potential physiologic substrates of MRP have been identified, such as leukotriene C_4 and 17β-estradiol-17-(β-D-glucuronide) (Loe *et al.*, 1996). In contrast, these conjugated organic anions are transported poorly, if at all, by P-glycoprotein. In addition, agents that reverse Pgp-associated resistance are usually much less effective in MRP-associated resistance (Lautier *et al.*, 1996). Due to lack of suitable circumvention agents, MRP represents an ideal target for antisense or ribozyme-mediated suppression for the reversal of MDR. Attempts to decrease MRP expression using these methods are dealt with later (see Section 1.3 to 1.5)

1.1.3 Alternative mechanisms of MDR

Alteration of Topoisomerase II (Topo II) activity is a frequent atypical mechanism of multidrug resistance (Eijdjems, 1985; Cole *et al.*, 1991). Topo II is a ubiquitous nuclear enzyme that is essential for many aspects of DNA function, including replication, recombination and transcription. This enzyme is the target of many clinically important antineoplastic drugs such as anthracyclines, ellipticines, amsacrines and epipodophyllotoxins (Zijlstra *et al.*, 1990). These drugs stabilise the cleavable complex formed between topo II and DNA, resulting in increased DNA excision, detectable as DNA single-strand or double-strand breaks, and DNA-protein cross-links (Mattern and Volm, 1995). Drug induced cell destruction is proportional to the level of topo II, the more the enzyme the greater the toxicity. Therefore, a reduction in topo II could be a major mechanism of resistance to many antineoplastic drugs. Characteristically, there is cross-resistance to the full range of drugs that interact with the enzyme, including those mentioned above, but not the *Vinca* alkaloids (Nooter and Stoter, 1996). Although atypical MDR is potentially of clinical importance, the question whether the phenomenon contributes to clinical drug resistance cannot yet be answered, because only very limited data are currently available on the expression of topoisomerase II in human tumour specimens (Gekeler *et al.*, 1992; McKenna *et al.*, 1993; Kaufmann *et al.*, 1994; Van der Zee *et al.*, 1994; Nooter and Stoter, 1996).

Another form of drug resistance that can affect several classes of drugs is associated with increased cellular levels of glutathione (GSH) and/or glutathione S-transferase (GST) (Kramer *et al.*, 1988; Puchalski and Fahl 1990; Godwin *et al.*, 1992). Although it is firmly established by transfection experiments that increased levels of GST cause resistance to some alkylating agents, it has been more difficult to prove that GSH and GST are directly involved in other forms of resistance, e.g. resistance to cisplatin and anthracyclines (Kramerv *et al.*, 1988; Puchalski and Fahl, 1990; Godwin *et al.*, 1992). It has been shown that this type of resistance may be complex, because it involves two steps; i) formation of GSH S-conjugate and ii) removal of toxic conjugate from the cell by a GSH S-conjugate export carrier (GS-X pump) (Ishikawa, 1992). Conjugation of cisplatin and GSH can occur non-enzymatically under physiological conditions, but export from the cell requires the GS-X pump. The GS-X pump is also known as the

multispecific organic anion transporter (MOAT) or the leukotriene C_4 (LTC_4) transporter, and is present in many mammalian cells such as hepatocytes, erythrocytes, cardiac cells, leukaemic cells, mast cells, and lung cells (Muller *et al.*, 1994). The GS-X pump has a relatively broad substrate specificity. It transports substrates containing a hydrophobic section and at least two negative charges (Ishikawa, 1992). Transport can be inhibited by orthovanadate and by competing anionic organic substrates, but not by many of the basic or neutral amphiphillic compounds that act as substrates for Pgp (Gottesmann and Pastan, 1993).

Cyclic-AMP (cAMP)-dependent protein kinase (PKA) and protein kinase C (PKC) are recent additions to the list of MDR facilitators. The number of studies involving PKA is limited, but it has been shown that phosphorylation of mouse Pgp is enhanced by cAMP and that Pgp is phosphorylated by the catalytic subunit of PKA (Mellado and Horwitz, 1987). PKA may also phosphorylate human Pgp (Chambers *et al.*, 1994). mdr-1 expression can be modulated by PKA type I (PKA-I), opening up the possibility of modulating MDR by selectively down regulating the activity of PKA-dependent transcription factors which up-regulate MDR expression (Rohlff *et al.*, 1993). High levels of PKA-I occur in primary breast carcinomas and patients exhibiting this phenotype show decreased survival (Miller *et al.*, 1993). Cells containing a mutated RI cAMP-binding subunit of PKA-I do not have an active PKA and exhibit down-regulation of mdr-1 expression and increased sensitivity to MDR related drugs (Chin *et al.*, 1992). It has been shown that analogs of cAMP down-regulate PKA-dependent MDR-associated transcription factors, and that a selective inhibitor of PKA decreased mdr-1 gene transcription and the activity of the mdr-1 promoter (Srivastava *et al.*, 1996). These results indicate that PKA-I plays an imortant role in drug resistance and site selective cAMP analogs are novel modulators of multidrug resistance.

PKC, an enzyme that is activated by diacylglycerol resulting from the receptor-mediated hydrolysis of inositol phospholipids, relays information of a variety of extracellular signals across the cell membrane to regulate many intracellular processes (Nishizuka, 1988). There is evidence to support the idea that the MDR phenotype is associated with changes in PKC activity and its isozyme content (to date, 11 PKC isozymes have been identified(Nishizuka, 1995)). This conclusion was based on: i)

Drug resistant lines have higher levels of PKC, calcium and a PKC activator than their parental lines (Tsuruo *et al.*, 1984; O'Brian *et al.*, 1989). MDR cell lines also contain more PKC in the membrane fraction than parental cell lines suggesting intrinsic activation of PKC (Aquino *et al.*, 1988); ii) Inhibitors of PKC such as H7, staurosporine, calphostin C, calcium channel blockers, phenothiazines, antiarrythmics, antiestrogens and synthetic peptide inhibitors can partially reverse MDR and inhibit Pgp phosphorylation (Srivastava *et al.*, 1996); iii) PKC activators (including phorbol esters, OAG and deoxycholate) can induce the MDR phenotype in non-MDR cells and enhance the phenotype of cells already expressing MDR (Srivastava *et al.*, 1996); iv) PKC has been shown to phosphorylate Pgp on similar sites both *in vitro* and *in vivo* (Chambers *et al.*, 1990). Inhibition of PKC in MDR tumor cells is associated with decreased Pgp phosphorylation and enhanced intracellular drug retention (Bates *et al.*, 1993); v) Over expression of PKCα in cells expressing Pgp can enhance the MDR phenotype of those cells and the overexpression of PKCβ1 can induce MDR by a Pgp independent manner (Fan *et al.*, 1992). All these studies define a specific role for PKCα in modulating the MDR phenotype.

Metallothioneins (MT) are intracellular proteins of low molecular weight (6-7 kDa) that are present in a wide variety of eukaryotes (Mattern and Volm, 1995). The synthesis of MT by tumour cells has been proposed as a possible mechanism for the intracellular inactivation of metal-containing chemotherapeutic agents such as cisplatin. MT content and MT mRNA levels correlate well with the sensitivity of small cell lung carcinoma cell lines to cisplatin (Kasahara *et al.*, 1991). A transfected cell line that overexpresses MT proved not only resistant to cisplatin but also to chlorambucil, melphalan and doxorubicin (Kelley *et al.*, 1988). However, cells of various origins selected for cisplatin resistance often, but not always, show increased MT expression, suggesting that increased MT expression alone may not be the sole mediator of cisplatin resistance. There are other mechanisms of MDR, on which only limited work has been carried out , O6-alkylguanine-DNA alkyltransferase, thymidylate synthase and certain cell cycle related proteins (Muller and Volm, 1995).

From the above review of currently known MDR mechanisms, it can be seen that the MDR phenomenon is a complicated and widespread problem in the treatment of almost every class of tumour. The methods of circumvention are, so far, of quite limited, which leaves the way open for the use of specific genetic techniques which may prove to be the most effective means available for tackling this hindrance to effective chemotherapy.

1.2 LUNG RESISTANCE-RELATED PROTEIN (LRP)

1.2.1 Discovery and Characterisation of LRP

While investigating alternative mechanisms of MDR, Scheper *et al.* (1993), selected the Pgp negative MDR cell line 2R120 by stepwise doxorubicin exposure (to 120 nM) of SW1573 non-small cell lung carcinoma cell line. The SW1573/2R120 MDR cell line was characterised by energy-dependent reduction of drug accumulation and exhibited cross-resistance to vincristine, gramicidin D and etoposide. No mdr-1 gene overexpression or Pgp was detectable. In contrast, SW1573/2R160 subline, obtained by exposure of 2R50 cells to slightly higher doxorubicin concentration (160 nM) displayed strong mdr-1 overexpression. BALB/c mice were immunized with 2R120 cells and a monoclonal antibody (LRP-56) was selected for strong immunoreactivity with 2R120 cells compared to parental SW1573 cells (Scheper *et al.*, 1993). The LRP-56 monoclonal antibody (MAb) displayed a characteristic cytoplasmic punctate staining pattern in the 2R120 cells, which has subsequently been found in all other LRP-56-positive MDR cell lines tested (Izquierdo *et al.*, 1995, Izquierdo *et al.*, 1996a, 1996b, Scheffer *et al.*, 1995). Immunoprecipitation studies showed that the LRP-56 antibody specifically reacted with a protein of approximately 110kDa. This protein was given the name Lung Resistance-related Protein (LRP) (Scheper *et al.*, 1993). It was found to be overexpressed in various Pgp-negative MDR cell lines, including a fibrosarcoma, small cell lung cancer and myeloma cell lines (Scheper *et al.*, 1993).

The cDNA coding for the *LRP* gene product was isolated by screening a cDNA library generated from a MDR human fibrosarcoma cell line with mouse MOP8 cells and the LRP-56 antibody (Scheffer *et al.*, 1995). The LRP sequence was found to display a single open reading frame of 2688 basepairs coding for an 896-amino-acid protein with a calculated Mr of 100 kDa. From the sequence, several potential phosphorylation motifs for protein kinase C, casein kinase II and tyrosine-protein kinase, as well as a phosphopantetheine attachment site and an amidation site were identified (Scheffer *et al.*, 1995). It did not appear, however, that transmembrane fragments or the ATP binding 'active transport' signature that is characteristic for the transmembrane transporter proteins Pgp and MRP were present. The LRP gene was localised, using

fluorescence *in situ* hybridisation, to the short arm of chromosome 16, within the 16p11.2-16p13.1 chromosomal region, close to the MRP gene site (Scheffer *et al.*, 1995, Slovak *et al.*, 1995). However, it appeared that MRP and LRP were rarely co-amplified and are not normally located within the same amplicon (Slovak *et al.*, 1995). Interestingly, the protein kinase C-β gene, involved in MDR by activating the mdr-1 gene, and possibly the LRP gene also, has been mapped to this same region (Komarov *et al.*, 1997). Comparison of the elucidated LRP sequence with known gene sequences on databases revealed that LRP showed strong homology with the major vault protein (MVP) from *Dictyostelium discodeum* and *Rattus norvegicus* (Kickhoefer *et al.*, 1994, Scheffer *et al.*, 1995, Vasu *et al.*, 1995). Alignment of the protein sequences of human LRP and rat MVP showed that 87.7% of the amino acids are identical, indicating that LRP is the human MVP (Scheffer *et al.*, 1995).

1.2.2 LRP and Vaults

In 1986, a hitherto unknown cell organelle was described and given the name "vault", chosen to describe the morphology of the particles consisting of multiple arches reminiscent of those from cathedral vaults (Kedersha and Rome,1986). They were first discovered (by negative staining and transmission electron microscopy) as contaminant particles of clathrin-coated vesicle preparations derived from rat liver (Kedersha and Rome, 1986, Rome *et al.*, 1991). They are ribonucleoprotein particles which are composed of a major vault protein of 104 kDa, which accounts for over 70% of the mass of the particle, three minor proteins of 210, 192 and 54 kDa, and a small RNA molecule. The vault components are assembled in a barrel-like structure with a molecular mass of around 13 MDa, approximately three times the size of a ribosome, and as such compose the largest ribonucleoprotein complex reported to date (Izquierdo *et al.*, 1998). The vaults have two-fold symmetry and each half can be opened into a flower-like structure which contains eight petals surrounding a central ring (Kedersha *et al.*, 1991). It is thought that these dynamic structural variations are likely to play a role in vault function (Izquierdo *et al.*, 1998).

Vaults have been isolated from a wide variety of species including lower eukaryotes, amphibians, avians and mammals (Kedersha *et al.*, 1990; Rome *et al.*, 1991). The amino acid composition of the MVP is highly conserved through evolution, with the identity between the mammalian MVPs being approximately 90% (Scheffer *et al.*, 1995). The potential phosphorylation sites are evolutionarily conserved in all MVPs (Scheffer *et al.*, 1995). Also antibodies raised against rat vaults recognise the MVP in all eukaryotic species tested. This data supports the notion that vault function is essential to eukaryotic cells (Kedersha *et al.*, 1990; Kedersha *et al.*, 1991). The majority of vaults are present in the cytoplasm and most cells contain thousands of vaults (Izquierdo *et al.*, 1998). A small fraction of vaults are localised to the nuclear membrane and the nuclear pore complex (NPC) (Chugani, *et al.*, 1993; Rome *et al.*, 1991). Structural similarities support the hypothesis that vaults constitute the central plugs of the NPC (Izquierdo *et al.*, 1998). However, as yet, the precise function of vaults is unknown. The location of vaults at the NPC along with the data suggesting that vaults are the transporter units of the NPC raises the possibility that vaults mediate the bidirectional transport of a variety of substrates between the nucleus and the cytoplasm (Chugani, *et al.*, 1993; Rome *et al.*, 1991). There is also evidence to suggest that vaults are involved in vesicular transport processes (Herrmann, *et al.*, 1996; Kedersha and Rome, 1986), cell motility (Rome *et al.*, 1991), cell growth (Vasu *et al.*, 1995) and play a central role in cell homeostasis (Herrmann *et al.*, 1996; Rome *et al.*, 1991).

1.2.3 LRP and drug resistance

Overexpression of LRP has been found in a large number of drug-selected MDR cell lines of various histogenic origins and selected by different drugs (Moran *et al.*, 1997; Scheper *et al.*, 1993; Verovski *et al.*, 1996). This indicates that diverse cancer cells react by up-regulating the expression of the LRP gene after exposure to anti-cancer agents. Overexpression is seen in both highly resistant MDR cell lines and at the early steps of resistance selection (Moran *et al.*, 1997; Verovski *et al.*, 1996; Wyler *et al.*, 1997). LRP is also expressed in cell lines not selected with drugs, which may be more clinically relevant. LRP was found to be expressed at various levels in 78% of 61 human cancer cell lines of different histogenic origins used at the National Cancer

Institute (NCI; USA) for screening of new anticancer drugs, highlighting the widespread nature of LRP-associated mechanisms of resistance in human malignancies (Izquierdo *et al.*, 1996b).

In drug-selected MDR cell lines, expression of LRP and Pgp appears to be mutually exclusive (Moran *et al.*, 1997; Scheper *et al.*, 1993). Up-regulation of LRP at low levels of resistance and a switching to up-regulation of Pgp at high levels of resistance has been frequently observed (Moran *et al.*, 1997; Scheper *et al.*, 1993, Versantvoort *et al.*, 1995). However, concomitant expression of LRP and Pgp has been seen in some unselected MDR cell lines (Moran *et al.*, 1997; Izquierdo *et al.*, 1996b). In contrast to Pgp, most LRP overexpressing cell lines display increased levels of MRP as well (Flens *et al.*, 1994; Scheper *et al.*, 1993). The concomitant expression of several drug resistance mechanisms may be necessary to cause the phenotype of drug resistance observed in LRP and MRP positive drug-selected MDR cell lines. Although MRP gene amplification has been shown to be the cause of overexpression and resistance in a number of cells lines (Slovak *et al.*, 1995), amplification of the LRP gene has not been widely reported. As mentioned earlier, despite the chromosomal proximity of MRP and LRP, they are very rarely co-amplified, and indeed, to date there is only one reported finding of LRP gene amplification (Laurencot *et al.*, 1997). Interestingly, tumor necrosis factor-α has been shown to reduce LRP gene expression at both mRNA and protein level in colon carcinoma cell lines, similar to its effect on the mdr-1 gene (Stein *et al.*, 1997). However, it had the opposite effect on MRP expression. Additional data indicates that the genes coding for Pgp, MRP and LRP are differentially regulated by 12-O-tetradecanolphorbol-13-acetate and cytotoxic drugs (Komarov *et al.*, 1997). In drug unselected cancer cell lines and clinical tumor specimens, expression of only one of these three proteins is not uncommon (Izquierdo *et al.*, 1995, 1996b).

The range of drugs which have been used to select LRP overexpressing drug resistant cell lines is broad, including doxorubicin, mitoxantrone, etoposide, vincristine, cytarabine, methotrexate and cisplatin (Scheper *et al.*, 1993; Ikeda *et al.*, 1997; Komarov *et al.*, 1997; Moran *et al.*, 1997; Parker *et al.*, 1997; Wyler *et al.*, 1997). Thus, the overexpression of LRP is not only associated with classical MDR drugs, but

also with drugs not included in the classical MDR phenotype. Most MDR cell lines with LRP overexpression have been selected with doxorubicin, but display a cross-resistance to etoposide, vincristine and other MDR-related drugs (Scheper *et al.*, 1993; Moran *et al.*, 1997). This broad spectrum of drug resistance associated with LRP overexpression is also seen in drug-unselected cell lines. From a panel of 61 human cancer cell lines from the NCI, LRP was found to show the greatest individual value as a marker of *in vitro* resistance to both classical-MDR related drugs (i.e. doxorubicin, vincristine) and also non-classical MDR drugs (i.e. cisplatin, carboplatin and melphalan) (Izquierdo *et al.*, 1996b). Interestingly, LRP mRNA expression was a somewhat better indicator of drug sensitivity than LRP protein expression (Laurencot *et al.*, 1997). Although LRP overexpression and increased drug resistant generally go hand in hand, it is not always the case. In an ovarian carcinoma cell line (OAW42-S), an increase in LRP expression in later passages of the cells did not result in an increase in drug resistance, which points towards a possible non-functional form of LRP (Moran *et al.*, 1997). It has also been shown that the up-regulation of LRP gene expression is accompanied by up to a 15-fold increase in the synthesis of whole vault particles (Kickhoefer *et al.*, 1997). It appears that the formation of vaults is limited by the expression of the major vault protein LRP, or possibly the other minor vault proteins, but not by the synthesis of vault RNA which is in excess to LRP (Izquierdo *et al.*, 1998). The fact that the cancer cells response to cytotoxic anticancer agents is the formation of such large complex particles as vaults, lends support for their role in drug resistance.

1.2.4 LRP expression in Tissues/Tumours and clinical value as prognostic indictor

Proteins related to *in vitro* drug resistance have been found expressed in various normal human tissues, where they are proposed to play a protective role against toxic compounds. LRP has been shown to be widely distributed in human normal tissues and tumours (Izquierdo *et al.*, 1996a; Sugawara *et al.*, 1997). High expression levels were seen in tissues chronically exposed to xenobiotics (i.e. epithelia of the bronchus, digestive tract, and keratinocytes), in metabolically active tissues (i.e. adrenal cortex),

and in macrophages, with varying levels found in other organs (Izquierdo *et al.*, 1996a). This distribution pattern resembles that of Pgp and MRP, suggesting a common role in the defence against xenobiotics (van der Valk *et al.*, 1990; Flens *et al.*, 1996). In support of this theory, it has been observed that in normal lung tissue, LRP expression is higher in those who had smoked more than 10 pack years (1 cigarette pack per day/10 years) compared with those who had never smoked.

In order to investigate whether the expression of LRP in clinical specimens is predictive of response to chemotherapy, a number of studies have been undertaken on various tumor types. The monoclonal antibody LRP-56 has been used extensively, for immunocytochemical, flow cytometry and immunohistochemical studies (Izquierdo *et al.*, 1998). More recently the LMR-5, rat monoclonal antibody, which can also detect LRP, has also been used in these studies (Flens *et al.*, 1997). In a series of ten melanoma cell lines, MDR-1, *MRP* and *LRP* gene expression (as measured at the mRNA level by RT-PCR) was detected in 2, 4 and 10 cell lines respectively (Schadendorf *et al.*, 1995). The same group studied 21 primary and 37 metastatic malignant melanoma specimens using immunohistochemistry, and found the expression of Pgp, MRP and LRP to be 2, 43 and 62% respectively (Schadendorf *et al.*, 1995). The number of metastatic melanomas that expressed high levels of LRP was significantly greater among those that had previously been exposed to chemotherapy. These results pointed to a role for LRP, and also MRP, in malignant melanoma drug resistance.

In two separate studies, non-small cell lung cancer (NSCLC) cell lines were found to express LRP at a rate of between 80 to 86%, while a more sensitive subtype, small cell lung carcinoma (SCLC) showed no expression of LRP (Dingemans *et al.*, 1996; Izquierdo *et al.*, 1996a). However, further studies are required to elucidate the prognostic significance of LRP in lung cancer. The expression of LRP in multiple myeloma has been reported to be between 13 and 48%, according to two separate studies (Izquierdo *et al.*, 1998). No difference in LRP expression was found between myelomas with or without prior chemotherapy. The expression levels of LRP in neuroblastoma, a relatively chemosensitive tumor type, was found to be low in untreated patients (16%), similar to other chemosensitive tumour types (Ramani and

Dewchand, 1995; Izquierdo *et al.,* 1996a). However, after treatment, the expression rate rose to between 78 to 82% (Ramani and Dewchand, 1995). This data suggests an association between exposure to anticancer agents and induction of expression of LRP.

The molecular basis of drug resistance in childhood acute lymphoblastic leukaemia (ALL) is largely unknown (Pieters *et al.,* 1997). In one study of 30 patients with relapsed childhood ALL, the expression of LRP, but not Pgp, was significantly associated with an increased *in vitro* resistance of fresh leukaemic cells to daunorubicin (Klumper *et al.,* 1995). More recently, the expression of LRP was found to be significantly higher in relapsed versus initial samples and correlated weakly with *in vitro* resistance to daunorubicin and etoposide (Veerman *et al.,* 1997). These preliminary studies, suggest that expression of LRP may result in low intracellular concentrations of daunorubicin and point to LRP as a relevant resistance protein in childhood ALL.

In adult acute leukemia (AML), only 1 out of 6 leukaemia cell lines from the NCI panel expressed LRP (Izquierdo *et al.,* 1996b). However, the studies performed to date show that the expression of LRP is consistently associated with poor response to induction chemotherapy, as well as with shorter progression-free and overall survival (List *et al.,* 1996; Izquierdo *et al.,* 1998). Hart *et al.* (1995), showed that LRP mRNA expression, but not MDR-1 *or MRP*, was significantly increased in patients failing to respond to intensive chemotherapy compared with those achieving complete response, while List *et al.,* (1996), showed that the prognostic value of LRP was superior to that of Pgp. These data show that expression of LRP is a poor prognostic factor in AML and point to LRP as a clinically relevant drug resistance gene in AML. A study of 57 women with ovarian cancer indicated that LRP positive tumors had a significantly inferior response to platinum and alkylating agent based chemotherapy (Izquierdo *et al.*, 1995). Furthermore, the expression of LRP was significantly associated with a shorter interval until tumor progression and shorter overall survival. This clinical data correlated with *in vitro* studies on the 61 cell lines of the NCI panel, which showed an association between LRP expression and intrinsic resistance to cisplatin (Izquierdo *et al.*, 1996b).

The results of the studies mentioned above, while limited in number, seem to support a strong role for LRP as a useful prognostic indicator in the clinic. It is interesting that LRP was found to be a much stronger prognostic indicator in these particular studies than the original MDR marker Pgp, and also MRP. This tends to indicate a direct role for vaults in drug resistance to both classical and non-classical MDR drugs. Alternatively, it may be that LRP is simply co-expressed with other resistance mechanisms.

1.2.5 Postulated mechanism of LRP-related drug resistance

From the observations carried out into drug re-distribution in MDR cancer cell lines and leukaemic blast cells and from data on structure and cellular localisation of vaults, a plausible hypothesis regarding the functional role of vaults in drug resistance has been put forward (Izquierdo *et al.*, 1996c, Izquierdo *et al.*, 1998). It has been shown that LRP overexpressing MDR cancer cells, in a similar fashion to most MDR cells, distribute daunorubicin into the perinuclear region and subsequently redistribute drug away from the nucleus into a punctate cytoplasmic pattern, whereas parental cells localise daunorubicin in a diffuse nuclear and cytoplasmic pattern (Schuurhuis *et al.*, 1989; Dietel *et al.*, 1990). Micheili *et al.*, (1997) reported that leukaemic blast cells which only expressed LRP, but not Pgp or MRP, showed an impaired intracellular accumulation of daunorubicin and suggested that vaults could be implicated in this phenomenon. Vaults are good candidates to be the perinuclear and cytoplasmic structures mediating daunorubicin re-distribution within MDR cells and leukaemic blast cells. Vaults are located at the nuclear pore complex (NPC), and could be, in fact, the transporter units (Chugani *et al.*, 1993). With this information, Scheper *et al.* hypothesised that vaults can mediate drug resistance by regulating both the nucleocytoplasmic transport of drugs and their cytoplasmic redistribution within vesicles, keeping cytotoxic agents away from their cellular targets (Izquierdo *et al.*, 1996c). Transfection of the LRP gene alone has failed to confer MDR, an expected finding considering that the complete vault particle will be required for functional

activity (Scheffer *et al.*, 1995). It is therefore apparent that the optimal method for assessing LRPs role in MDR is to perform knock out assays, where LRP expression is eliminated or decreased and examine the resulting effect on drug resistance. Anti-LRP ribozymes and antisense molecules allow such experiments to be carried out in a similar fashion to those investigating the role of Pgp and MRP.

1.3 RIBOZYMES

The word "ribozyme" is derived from the words ribonucleic acid (RNA) and enzyme, and it denotes an RNA molecule with catalytic properties (Kashani-Sabet and Scanlon, 1995; Ohkawa *et al.*, 1995). The first ribozyme was described by Cech and colleagues, which was the 413-nucleotide Group I intervening sequence in the pre-rRNA of *Tetrahymena Termophila* (Cech *et al.*, 1981). The intervening RNA sequence catalyzes its own excision, called self-splicing. The first truly catalytic ribozyme that could cleave other molecules with multiple turnover, was reported by Altman and collaborators (Guerrier-Takada *et al.*, 1983). It was the 400-nucleotide RNA component of bacterial RNase P. To date, a number of naturally occurring ribozymes have been identified and can be classified into 6 groups :

1) Ribozymes derived from self-splicing tetrahymena group I introns (Cech *et. al,*. 1981; Kruger *et al.*, 1982);
2) RNA components of RNase P (Guerrier-Takada *et al.*, 1983);
3) Hammerhead ribozymes (Uhlenbech, 1987)
4) Hairpin ribozymes (Buzayan *et al.*, 1986);
5) Genomic and anti-genomic RNase of hepatitis δ virus (Perotta and Been, 1992);
6) RNA transcripts of mitochondrial DNA plasmid of Neurospora (Symons, 1994).

1.3.1 Hammerhead ribozymes

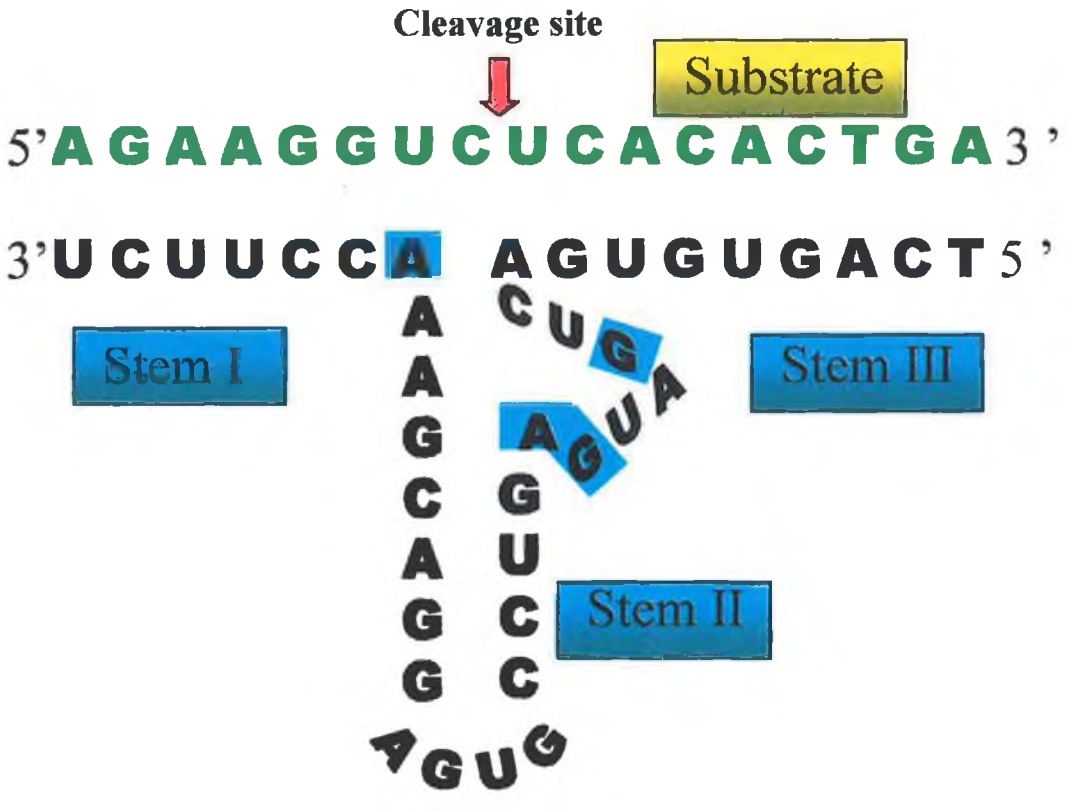
Among these catalytic RNAs, the hammerhead ribozyme is the smallest (Kashani-Sabet and Scanlon, 1995; Ohkawa *et al.*, 1995). The name "hammerhead" derives from the predicted shape of the ribozymes secondary structure. Naturally occurring hammerhead ribozymes were found within RNA viruses and they act in *cis* during viral replication by the rolling circle mechanism (Symons, 1992; Bratty *et al.*, 1993). Through genetic engineering, the hammerhead was manipulated to enable it to cleave its target in *trans* and act in a truly catalytic manner (Uhlenbeck, 1987). Using *in vitro* mutagenesis studies of the plus strand of satellite RNA of tobacco ringspot virus

(sTobRV0), the consensus sequences required to maintain catalytic cleavage by the ribozyme were defined (Haseloff and Gerlach, 1987). It is this information that allows for the design of ribozymes to target any gene of interest once the sequence is known.

In terms of secondary structure, the *trans*-acting hammerhead ribozyme developed by Haseloff and Gerlach, is composed of the catalytic core (or hammerhead domain) region and three hybridising helices or stems (Figure 1.3.1): stems I and III hybridise to the flanking sequences of the cleavage site and act as an antisense, and the stem loop II is usually composed of eight complementary ribonucleotides in the loop structure (Kashani-Sabet and Scanlon, 1995; Ohkawa *et al.*, 1995). In terms of the substrate, the mutational analysis revealed the requirement of XUN sequences, with X being any nucleotide and N being A, C or U (Haseloff and Gerlach, 1988; Ruffner *et al.*, 1990). Gerlach and co-workers have expanded the substrate sequences cleaved by the hammerhead ribozyme (Perriman *et al.*, 1992). In general, targets containing GUC, GUA, GUU, CUC and UUC sequences are well cleaved and targets containing, GUG, AUC, XAC, and XCC sequences are either not cleaved or cleaved with a substantially reduced rate. It is the likelihood of finding an appropriate target within a given gene sequence that makes hammerhead ribozymes such a potentially useful tool.

The basic reaction scheme of a ribozyme cleaving its target is as follows (Ohkawa *et al.*, 1995): First the substrate (together with Mg^{2+} ions) binds to the ribozyme via the formation of base pairs with stems I and III. Then, a specific phosphodiester bond in the bound substrate is cleaved by the action of the Mg^{2+} ions (the ribozyme functions as a metalloenzyme as it requires the presence of magnesium). This cleavage generates products with 2', 3'-cyclic phosphate and 5'-hydroxyl groups. Finally the cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events. With respect to the ribozyme itself, several requirements must be met for the development of an effective catalytic RNA. Several groups have probed the actual requirements within the catalytic core, which are almost exclusively composed of RNA (Perreault *et al.*, 1990, 1991; Yang *et al.*, 1992). An all DNA ribozyme was shown to be devoid of catalytic activity (Perreault *et al.*, 1990). Further studies using DNA at specific sites established that the minimum

Figure 1.3.1 Diagram of *Trans*-acting hammerhead ribozyme



ribonucleotide requirement is that at bases highlighted in Figure 1.3 (Perreault *et al.*, 1990, 1991; Yang *et al.*, 1992).

Several investigators have examined the effects of changes in the flanking sequences of the ribozyme on catalytic activity (Fedor and Uhlenbeck, 1990; Herschlag, 1991; Bertrand *et al.*, 1994). Fedor and Uhlenbeck (1990) demonstrated that ribozymes differing in the number as well as in the sequence of RNA helices I and III differed drastically in cleavage kinetics. Herschlag (1991) observed that while increasing the recognition sequence increased ribozyme specificity, the dissociation step between ribozyme and substrate was too slow to increase overall cleavage. Recent studies have suggested that 12 bases may represent the optimal length of flanking sequence (Bertrand *et al.*, 1994). In addition, substrate sequences flanking the cleavage site rich in A or U were favoured over GC rich sequences to enhance discrimination (Herschlag, 1991). In particular, U-rich sequences are preferred in the substrate and A-rich sequences in the ribozyme. A further study identified that stem II in the hammerhead ribozyme, may not be essential to the cleavage reaction, as stems with two base pairs rather than four retained catalytic activity, while however, further elimination's were not tolerated (Tuschl and Eckstein, 1993).

1.3.2 Hairpin ribzoymes

The hairpin ribozyme is derived from the minus strand of sTobRV RNA, and site-specifically cleaves RNA in *trans* (Hampel and Tritz, 1989; Feldstein *et al.*, 1989). The original hairpin ribozyme consisted of 50 bases and cleaves corresponding 14 base RNA substrates, in the presence of Mg^{2+}. The proposed secondary structure from which this ribozyme derives its name, was devised by mutational analysis, computer modelling and phylogenetic studies (Hampel *et al.*, 1990). This consists of four helical regions separated by two internal loop sequences. The substrate binds to the ribozyme through two helices (helix 1 and 2). Cleavage occurs to the 5' side of a guanosine within the internal loop (loop A) of the substrate separating helices 1 and 2. A second internal loop (loop B) separates the two helices (helix 3 and helix 4) of the ribozyme. As with the hammerhead ribozyme, studies have been carried out to determine the nucleotide sequences essential for catalytic activity. Within loop A, there are four

essential bases and one in the substrate (Berzal-Herranz *et al.*, 1993). Within loop B, nine of 11 bases are essential sequences, while only one base within the four helices is important for catalytic activity. It therefore appears that both loop A and loop B play an important role in catalysis.

1.3.3 Ribozyme delivery

For the ribozyme to be effective in a cellular environment, it needs to be delivered to the intracellular milieu. Researchers have used either exogenous delivery (using naked ribozymes complexed with cationic liposomes) or vector-based systems to promote endogenous ribozyme expression. In the case of exogenous delivery, the susceptibility of RNA oligonucleotides (including ribozymes) to ribonuclease attack intracellularly or in the serum, required the search for modifications to enhance ribozyme stability while maintaining cleavage capability. To this end, a number of chemical modifications of the nucleotides have been made. These include the incorporation of 2'-fluoro, 2'-amino, 2'-O-allyl and 2'-O-methyl nucleotides into hammerhead ribozymes (Pieken *et al.*, 1991; Paolella *et al.*, 1992). A second class of modifications involves the substitution of phosphorothioate molecules in the phosphate moiety. One study examined the combination of 2'-pyrimidine modifications as well as four terminal phosphorothioate linkages and demonstrated that the resultant ribozyme had a cleavage efficiency similar to that of the unmodified ribozyme.

A third and most successful approach, yielding ribozyme stability without sacrificing cleavage activity, concerns the introduction of deoxynucleotides outside of the catalytic core. Rossi and co-workers showed that chimeric DNA/RNA ribozymes, with DNA in helices I and III had a 6-fold greater catalytic activity than an analogous all-RNA ribozyme (Taylor *et al.*, 1992). This difference could be attributable to the differences in dissociation of the DNA-RNA complex versus that of the RNA-RNA complex. One group combined the use of DNA in helices I and III of the ribozyme with phosphorothioate linkages in stems I, II and III and demonstrated a 7-fold higher cleavage activity, as well as resistance to degradation in human serum (Shimayama *et al.*, 1993).

In contrast to exogenous delivery, many studies utilise the cellular machinery to express the ribozyme. Here, the ribozyme gene is cloned into an available vector (expression plasmid or retroviral vector) and delivered to the cells by transfection of the plasmid or by retroviral infection. Other delivery systems, such as cationic liposomes, adenoviruses and adeno-associated viruses (AAV), are being studied in order to optimize ribozyme activity (Kashani-Sabet and Scanlon, 1995). The choice of delivery system can depend heavily on the disease type. Adenoviruses, due to their tissue tropism, could be suitable for the therapy of respiratory diseases, such as lung cancer and cystic fibrosis. AAV systems, however, may be useful for ribozyme delivery, as their small genome size would accommodate ribozyme genes, but not necessarily larger ones. In addition, as AAV may infect nonreplicating cells, this system could be used to transduce bone marrow cells (Kashani-Sabet and Scanlon 1995).

Another critical element in ensuring optimal ribozyme activity is the choice of promoter. The first reported intracellular expression of a ribozyme (anti-CAT) used the SV40 early promoter, with the ribozyme embedded in the 3' untranslated region of the firefly luciferase gene (Cameron and Jennings, 1989). Other viral promoters tested so far include the herpes simplex virus thymidine kinase (HSV-tk) promoter, the HIV and Rous sarcoma virus (RSV) long terminal repeats (LTRs), and the cytomegalovirus (CMV) promoter. The use of cellular promoters is also common, such as the β-actin promoter or by incorporating the ribozyme in to tRNA genes to take advantage of RNA polymerase III-mediated transcription (Kashani-Sabet and Scanlon 1995). Tighter control over ribozyme expression is achievable with inducible promoters such as the mouse mammary tumor virus (MMTV) dexamethasone-inducible promoter and the bacteriophage T7 promoter induced by isopropyl-β-D-thiogalactoside.

Once the ribozyme has been successfully introduced intracellularly, demonstration of ribozyme activity is required. Expression of the ribozyme itself can be detected by reverse-transcriptase-polymerase chain reaction (RT-PCR) or Northern blot analysis. Proof of efficacy of the ribozyme is reliant on a demonstration of inhibition of the target gene expression (at RNA and/or protein level) and any downstream phenotypic effects such as decreased tumor growth, viral replication or drug resistance. Since, the

ribozyme has the potential to act as antisense, it is desirable to demonstrate that the ribozyme retains the ability to cleave. Saxena and Ackerman, (1990), demonstrated that a ribozyme cleaved its target when both elements were injected into oocytes. Others have shown that cellular extracts of ribozyme expressing cells cleave target RNA *in vitro* (Chang *et al.*, 1990; Scanlon *et al.*, 1991; Kashani-Sabet *et al.*, 1992), suggesting that the ribozyme expressed intracellularly retains the ability to cleave its target. However, detection of the cleavage products has been elusive in many studies, due, most likely, to the rapid degradation of short RNAs. Some studies have detected such products by PCR analysis (Sarver *et al.*, 1990; Kashani-Sabet *et al.*, 1992; Cantor *et al.*, 1993). More recently, detection of the cleaved fragment of mdr-1 RNA was reported by Northern analysis in human ovarian carcinoma cells (Scanlon *et al.*, 1994). But of all the ribozyme studies carried out to date, these remain the few cases where cleavage products have been readily detectable.

1.3.4 Ribozyme in the study of MDR

Several groups have demonstrated ribozyme-mediated modulation of MDR in human cancer cells (for review see Byrne *et al.*, *in press*). Scanlon and co-workers report the reversal of the MDR phenotype in human ovarian carcinoma cells using either a mdr-1 ribozyme or a *fos* ribozyme (Scanlon *et al.*, 1994). Using an anti-mdr-1 ribozyme designed to cleave the CUC sequence of codon 880 (a target site between 2 ATP binding sites which may play a role in the Pgp pump), resistance in a human ovarian carcinoma cell line (16.6 fold resistant to actinomycin D and over-expressing mdr-1) was completely reversed to the sensitive level. Mdr-1 expression was reduced and actinomycin D intracellular transport level was increased. The anti-fos ribozyme (which targets the GUC sequence of codon 309) reversed actinomycin D resistance more quickly than did the mdr-1 ribozyme. This may suggest that c-fos may modulate the expression of genes other than mdr-1 which also contribute to the MDR phenotype. In this cell line the anti-fos ribozyme down-regulated not only c-*fos* but also the expression of mdr-1 and topoisomerase I (the mdr-1 promoter has an AP-1 binding site (Teeter *et al.*, 1991)).

The mdr-1 ribozyme has been shown to be effective in a number of other MDR cell types. Holm and co-workers report the reversal of daunorubicin resistance in resistant (1600 fold) human pancreatic carcinoma cells (Holm *et al.*, 1994). The ribozyme-containing cells were only 5.3-fold more resistant to daunorubicin than the parent sensitive cells. In two lung cell lines the ribozyme has a similar effect; mdr-1 ribozyme transfectants of two MDR lung cell lines (DLKP-A and SKMES-1ADR) were found to be more sensitive to adriamycin, vincristine and VP-16 (Daly *et al.*, 1996). The anti-mdr-1 ribozyme does not result in complete reversal of resistance to the level of the sensitive parent. Possibly the level of mdr-1 mRNA remaining in the mdr-1 ribozyme transfectants is capable of mediating drug resistance and/or other mechanism of resistance may be present in these lines.

Kobayashi and colleagues have designed ribozymes directed at other areas of the mdr-1 mRNA transcript (Kobayashi *et al.*, 1994). They designed two hammer-head ribozymes; one targeted at codon 179 and the other at codon 196. In cell free studies the ribozyme targeted at the 196 codon proved most effective. This ribozyme was then used in transfections to target mdr-1 mRNA in an acute leukemia cell line. The ribozyme down-regulated mdr-1 mRNA as well as Pgp expression. Vincristine resistance was decreased from 700- to 20-fold. Once again complete reversal of drug resistance did not occur. This leukemic cell line was highly drug resistant and as such possibly not enough ribozyme was expressed to down-regulate the over-expressed mdr-1 mRNA or the resistance mechanism in these cells could be multi-factorial.

Bertram and co-workers have studied the ribozyme-mediated reversal of drug resistance in a human colorectal carcinoma (LoVo/Dx) cell line resistant to doxorubicin (LoVo/Dx^R) (using a ribozyme directed against mdr-1 mRNA) (Bertram *et al.*, 1995). These authors designed two ribozymes against mdr-1, ribozyme 1 (targeting the CUC sequence at position 2429) and ribozyme 2 (targeting the GUC sequence at position 2440). *In vitro* these ribozymes reduced the mdr-1 mRNA from the LoVo/Dx^R cells by up to 80%. Modified ribozymes (containing fluoro and allyl substituted bases to increase stability against ribonucleocytic attack) reduced chemo-resistance of these cells by up to 50%. Using three mdr-1 ribozymes (ribozyme 1 targeting position 2429, ribozyme 2 targeting position 2440 and ribozyme 3 targeting

2408) in *in vitro* assays, Palfner and co-workers (1995) assayed conditions such as ribozyme-to-target ratio, pH, $MgCl_2$ concentration and incubation time on cleavage efficiency (Palfner *et al.*, 1995). Their most efficient ribozyme cleaved 91% of an *in vitro* transcribed mdr-1 mRNA transcript.

Ribozyme studies can also be used in functional assays of multi-drug resistance. Eijdems and co-workers report the down-regulation of mdr-1 mRNA using an mdr-1 ribozyme targeted at codon 196 (Kobayashi *et al.*, 1994; Eijdems *et al.*, 1995) in a human non-small cell lung cancer cell line SW-1573 selected in a low concentration of doxorubicin. In a clone having reduced mdr-1 mRNA level there was no detectable change in sensitivity to drug which suggests that mdr-1 does not contribute to drug-resistance in these cells. These authors have concluded that resistance in this cell line is associated with the presence of an altered form of MRP.

Increased expression of c-fos is often found associated with drug-resistance and several of these drugs (e.g. cisplatin) are not substrates for mdr-1 or MRP (Hollander and Fornace, 1989; Scanlon *et al.*, 1994). *fos* is believed to mediate its effects through transcriptional activation, after interaction with the Jun protein, to form the AP-1 complex. This complex affects proliferation, apoptosis and drug resistance through transcriptional activation of genes via AP-1 elements in their regulatory regions. The A2780 ovarian carcinoma cell line resistant to cisplatin has been shown to exhibit c-fos overexpression as well as the over-expression of c-*myc*, H-*ras*, thymidylate synthesis, DNA polymerase B and Topoisomerase I (Scanlon *et al.*, 1990, 1991; Kashani-Sabet *et al.*, 1990). This may suggest that c-fos is involved in cisplatin resistance by directing expression of enzymes carrying out DNA synthesis and repair processes. Tumour tissue from a patient with colon carcinoma failing cisplatin/5-fluorouracil treatment revealed a similar pattern of gene expression to the resistant A2780 subclone (Kashani-Sabet *et al.*, 1990). These data suggest that the c-fos gene regulates downstream enzymes associated with DNA synthesis and repair and may play a central role in cisplatin resistance.

It has already been discussed how anti-fos ribozymes are effective in lowering resistance in classic MDR cell lines. *fos*-ribozymes have also been shown to be

effective in altering resistance in non-classic MDR e.g. resistance to cisplatin. Cisplatin is one of the most widely used anti-cancer agents and its multifactorial mechanisms of resistance pose serious clinical problems in cancer chemotherapy (Ishida *et al.*, 1995). Studies in cisplatin-resistant cell lines support the importance of the c-fos oncogene in maintaining the drug-resistant phenotype (Scanlon *et al.*, 1989). A hammer-head ribozyme against the c-fos gene has been investigated in a cisplatin resistant line (Scanlon *et al.*, 1991; Funato *et al.*, 1992). The cisplatin resistant human ovarian carcinoma A2780 subclone (A2780DDP, 10-fold resistant to cisplatin) was transfected with an anti-fos ribozyme and was rendered sensitive to the antineoplastic effects of cisplatin (as well as camptothecin, 5-fluorouracil and azidothymidine to which A2780DDP cells are cross-resistant). The ribozyme transfectant was found to have down-regulation of c-fos gene expression as well as down-regulation of c-*fos* responsive genes such as DNA polymerase B, Topoisomerase I and metallothionein IIA. Down-regulation of c-*fos* may reverse drug-resistance by several mechanisms involving DNA synthesis as well as Pgp.

Funato and co-workers (1997) prepared a hammer-head ribozyme to selectively cleave fos mRNA. The ribozyme was transfected into implanted human colon cancer cells SW480DDP and SW620DDP (which over-express the fos gene and are resistant to cisplatin treatment) and it reduced the expression of the fos gene *in vivo* and also reversed cell sensitivity to cisplatin.

1.4 THE USE OF ANTISENSE RNA TO INHIBIT GENE EXPRESSION

The use of antisense RNA to inhibit gene function within cells is very similar to the use of ribozymes. The main difference is that the antisense sequences don't have any catalytic activity. However, in general, plasmid derived-antisense RNA tends to be much longer than ribozyme sequences. Whole or partial cDNA fragments are usually cloned into a plasmid in a reverse (antisense) orientation, giving rise to antisense RNA sequences, usually 0.6 to 2 kb in size. The very size of many of these antisense RNA molecules can have significant effect on the potency of these molecules. For instance, annealing rates must be considered, as they have been shown to be related to the extent of inhibition (Rittner *et al.*, 1993). In addition, secondary structure formation (such as stem-loops, internal loops, bulges) can play an important role the accessibility of the RNA and its stability. However, the antisense RNA sequence used in this thesis comprises of only 14 bases, and differs from the LRP-ribozyme construct only in its lack of catalytic domain.

It is also important to understand that antisense RNA differs from the more common antisense DNA oligonucleotides in its mode of action. Antisense RNA molecules bind to the target mRNA, forming RNA-RNA duplexes. These duplexes are not susceptible to attack by RNase H, which is the main method of action for most DNA based antisense oligonucleotides (Branch, 1996). They act mainly through steric hindrance of the ribosomal machinery along the mRNA molecule, preventing the formation of proteins. However, there are a number of cellular enzymes that can interfere with RNA duplexes, as these duplexes play a role in cell functioning. The RNA duplexes 1) guide ribosomal maturation; 2) serve as substrates for RNase III, an enzyme that cleaves ribosomal RNA precursors and dsRNA; 3) activate enzymes of the interferon-associated antiviral pathway; and 4) serve as substrates for a deaminase (Branch, 1996). As a result of these interactions, antisense RNA may not reduce the expression of target RNA to the same levels as a ribozyme or antisense oligonucleotides directed at the same target, but may be just as effective at decreasing expression of the protein product.

Antisense RNAs have been used widely in the study of oncogene function. Targets have included DNA polymerase B (Horton *et al.*, 1995), phosphoprotein p18 (Jeha *et al.*, 1996), cyclin D1 (Zhou *et al.*, 1995), K-*ras* (Zhang *et al.*, 1993), glial fibrillary acidic protein (GFAP) (Rutka *et al.*, 1994), vascular endothelial growth factor (VEGF) (Saleh *et al.*, 1996), epidermal growth factor receptor (EGF-R) (Giovanni *et al.*, 1996), insulin-like growth factor I (IGF-I) (Trojan *et al.*, 1992), insulin-like growth factor I receptor (IGF-IR) (Resnicoff *et al.*, 1994), c-*myb* (Raschella *et al.*, 1992), p53 (Velasco *et al.*, 1995), fibroblast growth factor 2 (FGF-2) (Maret *et al.*, 1995), and transforming growth factor α (TGF-α) (Laird *et al.*, 1994).

There has only been one report of the use of antisense RNA for the downregulation of mdr-1 expression. Hanchett and colleagues used a 963 bp fragment of the mdr-1 cDNA cloned into an expression vector, which used a β-actin promoter, in a reverse orientation (Hanchett *et al.*, 1994). They transfected this construct into MDR variants of a human nasopharyngeal epidermoid carcinoma cell line (KB 3-1) and analysed the cells for a reduction in drug resistance, mdr-1 mRNA and Pgp expression. However, out of 10 isolated clones that expressed the antisense construct, only one actually exhibited a decrease in drug resistance. Studies using rhodamine-123, a fluorescent substrate for Pgp, revealed that dye retention in individual cells was highly variable within this antisense-expressing clone. Sub-populations were established from this original clone, based on rhodamine-123 retention. Despite the fact that all the subclones expressed similar amounts of the antisense, the levels of mdr-1 mRNA varied dramatically. Nuclear run-on analysis indicated that the mdr-1 gene was transcribed at the same rate in all populations, which suggested that the reduction in mdr-1 mRNA was mediated post-transcriptionally. Cells with the greatest reduction in mdr-1 mRNA accumulated distinct antisense RNA transcripts in the nuclear RNA fraction, suggesting that antisense effectiveness in this system was associated with a nuclear event or process.

These results reveal that antisense RNA activity is not necessarily distributed evenly within a clonal population. The results also highlight potential problems with the transfection of antisense RNA. Out of 16 that were initially transfected, only 10

actually expressed the antisense RNA. Of these 10, only one exhibited a decrease in mdr-1 mRNA levels (50%) and Pgp (50%) as measured by Northern and western blot respectively. Some studies have suggested that a large excess of antisense RNA is necessary to drive hybrid RNA duplex formation and establish an antisense effect (Yokoyama and Imamoto, 1987; Krystal *et al.*, 1990). There have also been descriptions of antisense effects in situations where a large excess of antisense RNA has not been detected (Nishikura and Murray, 1987; Kasid *et al.*, 1989). Hanchett and co-workers stated that there was an 80-fold excess of antisense to mdr-1 mRNA in their original clone (Hanchett *et al.*, 1994). However, in another study with the same cell line, Wang and Dolnick have shown that an antisense RNA excess of 200-fold was insufficient to achieve an antisense effect targeting di-hydrofolate reductase mRNA (Wang and Dolnick, 1993).

Why antisense RNA works well in some systems but is ineffective in others is an important, but as yet largely unanswered. Different mRNA targets can be expected to have unique characteristics with respect to structure, stability, processing, and post-transcriptional control, which may affect the susceptibility of a particular mRNA to antisense RNA. The results of Hanchett *et al.*, (1994), illustrate a dynamic aspect of antisense action at the cellular level, and demonstrate the extent of variability in effectiveness that can be observed with antisense RNA.

The anti-LRP antisense RNA expression plasmid used in this thesis targets exactly the same region in the LRP sequence, bases 1147 to 1160 as the LRP ribozyme. This allows direct comparison of the efficacy of the antisense RNA and ribozyme, as both should have equal accessibility to the target site. It would be expected that the ribozyme would be more efficient, as it has the added benefit of catalytic cleavage in addition to its basic antisense effect.

1.5 USE OF ANTISENSE OLIGODEOXYNUCLEOTIDES TO STUDY DRUG RESISTANCE

1.5.1 Antisense Technology

The notion of using specific oligonucleotides for the modulation of gene expression surfaced two decades ago when Zamenick and Stephenson inhibited the replication of the Rous Sarcoma virus (Zamenick and Stephenson 1978). They added a synthetic piece of DNA to the medium of chick fibroblasts in tissue culture to block the circularisation step by hybridising specifically with the 3' end of the viral RNA in a competitive way. It inhibited the formation of new virus, and also prevented the transformation of chick fibroblasts into sarcoma cells.

The term 'antisense' ascribed to a nucleic acid sequence, refers to the fact that it forms complementary base pairs to a part or all of a genetic coding sequence which is termed the 'sense' strand. Thus once all or part of the sequence of a target gene is known, an antisense molecule can be designed which specifically binds to this gene alone. In addition to this hydrogen bonding, the affinity of the antisense molecules to their targets is affected by base-stacking in the double helix of the oligonucleotides and also the ionic strength of the system (Crooke and Bennett, 1996). Affinity between the antisense oligonucleotide and target polynucleotide increases as the length of the antisense molecule increases, due to the increased hydrogen bonding between bases and stacked pairs (Crooke and Bennett, 1996). Theoretically, at least, this allows for the design of drugs to attack any unwanted or mutated form of a gene, and leave the normal copy of the gene untouched, even if the two forms differ by only a single base pair or nucleotide (Crooke and Bennett, 1996, Milligan *et al., 1994*, Giles *et al., 1995*).

Antisense molecules can consist of relatively short synthetic oligonucleotides introduced into cellular systems by various means (Stein *et al., 1993*, Brysch and Schilingensiepen, 1994, Helene, 1994, Scanlon *et al., 1995*, Zon, 1995, Crooke and Bennett, 1996, Wagner and Flanagan, 1997). This form is currently the most widely used and entails the use of DNA and RNA based oligonucleotides or combinations of both. Alternatively, antisense molecules can consist of a whole gene, or a specific

fragment of a gene, cloned into an expression vector in a reverse orientation and transfected into a cell, where it is expressed as antisense RNA either endogenously or upon stimulation (Scanlon *et al., 1995*, Branch, 1996, Tolume *et al., 1996*, Zhang, 1996, Sczakiel, 1997).

1.5.2 Mechanisms of antisense action

The mechanisms by which interactions of antisense oligonucleotides with nucleic acids may induce biological effects are quite complex. The most basic mode of action of antisense is an occupancy-only one (Crooke and Bennett, 1996). The antisense acts as a classic competitive antagonist by binding to specific sequences, inhibiting the interaction of the RNA or DNA with proteins, other nucleic acids or factors required for the essential steps in the intermediary metabolism of the RNA or its utilisation by the cell. Another mechanism is the inhibition of excision of introns or 'splicing', which is a key step in the intermediary metabolism of most mRNA molecules (Crooke and Bennett, 1996, Sharma and Narayanan, 1995, Neckers *et al.*, 1992). The mechanism for which the majority of oligonucleotides have been designed to date is to cause translational arrest by binding to the translation initiation codon or alternatively to bind to areas in the coding region to attempt steric hindrance of ribosome progression along the mRNA (Brysch and Schilingensiepen, 1994, Crooke and Bennett, 1996, Ohkawa *et al.*, 1996, Bouffard *et al., 1996*, Probst and Skutella, 1996). Although the ribosomal machinery is quite powerful and tends to sweep away most obstacles in its path, targeting the AUG initiation codon where the ribosomes first begin translation has been shown to be a very effective target (Vasanthakumar and Ahmed, 1989, Jaroszewski *et al., 1990*, Rivoltini *et al., 1990*, Clynes *et al., 1992*, Corrias *et al., 1992*, Efferth *et al., 1993*, Thierry *et al., 1993*, Quattrone *et al., 1994* (b), Bertram *et al., 1995*, Nakashima *et al., 1995*, Alahari *et al., 1996*, Cucco and Calabretta, 1996 , Liu *et al., 1996*, Sola and Colombani, 1996, Stewart *et al., 1996*, Hirtake *et al., 1997*, Li *et al., 1997*)

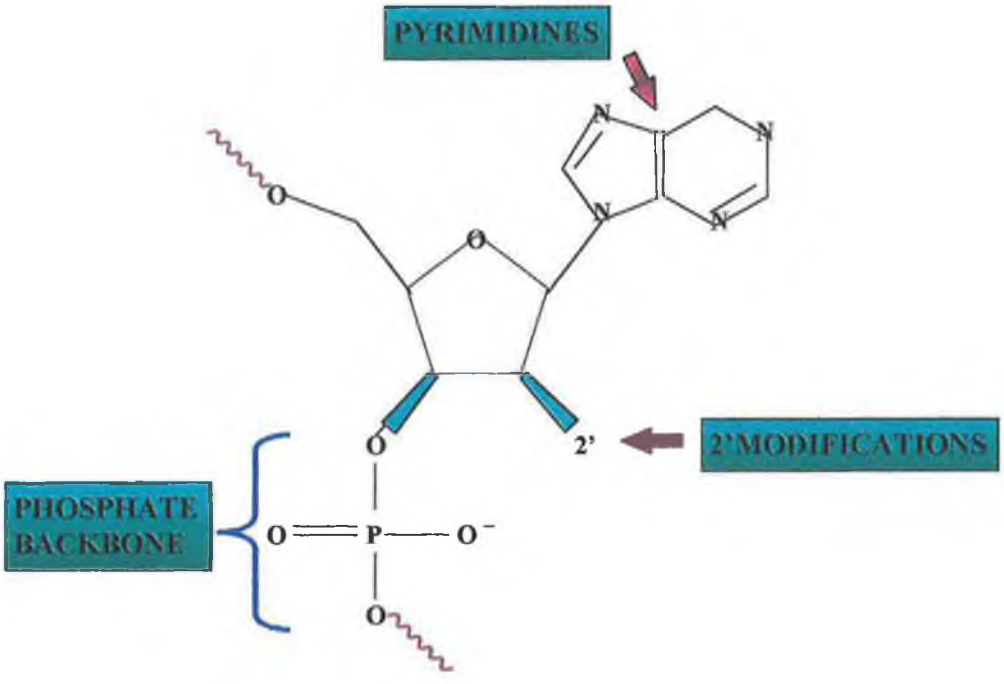
One of the most important mechanisms of action of DNA based-antisense targeted to RNA is the activation of ribonuclease H (RNase H) (Brysch and Schlingensiepen,

1994, Crooke and Bennett, 1996, Helene, 1994, Bouffard *et al.*, 1996, Ohkawa *et al.*, 1996, Sharma and Nayaran, 1995, Wagner and Flanagan, 1997, Giles *et al., 1995*, Branch, 1996). RNase H is an ubiquitous enzyme that selectively cleaves the RNA component of RNA-DNA duplexes. Other mechanisms of inhibition of translation include interference with secondary structures, such as stem loops, (Vickers *et al., 199*1, Ecker *et al., 199*2, Thierry *et al.*, 1993, Crooke and Bennett, 1996, Liu *et al.*, 1996, Tolume *et al.*, 1996), inhibition of 5' capping (Baker, 1993, Alahari *et al.*, 1996, Stewart *et al.*, 1996, Crooke and Bennett, 1996) and interference with 3' polyadenylation (Chiang *et al., 199*1, Alahari *et al.*, 1996, Stewart *et al.*, 1996). Oligonucleotides conjugated to alkylating and photoactivable alkylating species have been synthesised. These can then inhibit the target DNA by covalently modifying them, rendering them non-functional (Webb and Mateucci, 1986, Crooke and Bennett, 1996). Activation of mRNA breakdown is not universal, however; Probst & Skatella (1996) found elevation of specific mRNAs by antisense, but not by sense treatments.

1.5.3 Antisense modifications

Numerous chemical modifications have been made to the oligonucleotide backbones and sugar bases to render them more nuclease resistant and give them greater affinity to their targets. The earliest modifications involved substituting the non-bridging oxygen atoms in the internucleotide bonds (see Figure 1.5.3) with either a methyl or a sulphur group to give methylphosphonate and phosphorothioate oligodeoxynucleotides respectively (Marcus-Sekura *et al.*, 1987, Matsukura *et al.*, 1987). This made the oligonucleotides more resistant to nuclease degradation, which was a problem for natural phosphodiester oligonucleotides (Wickstrom, 1986), thus extending the half-life of the oligonucleotides and improving their efficacy. In the methylphosphonate substitution, the negative charge of the oligonucleotide is eliminated, and they exhibit low toxicity and high stability, while, however, being unable to elicit the action of RNase H (Wickstrom *et al., 1992*). Phosphorothioates keep the negative charge and retain the ability to activate RNase H (Gao *et al., 1992*). As a result, phosphorothioate oligos remain the most widely used base analogue, and are currently being tested in a

Figure 1.5.3 Basic diagram of phosphodiester oligonucleotide structure



number of clinical trials (Roth and Cristiano, 1997, Wagner and Flanagan, 1997).

Second generation oligonucleotides include: substituting pyrimidines at the C-5 position with 5-methyl, 5-bromo and 5-propynyluracil (Lonnberg and Vuorio, 1996) replacement of the sugar and phosphate residues with alkylamide or carbamate linkage (Crooke and Bennett, 1996, Stirchak *et al.*, 1989); 2'-O-propyl, 2'-methoxyethoxy, 2'-O-methyl, 2'-O-allyl and 2'-fluoro ribose modifications (Monia *et al.*, 1993, Wagner, 1995, Crooke and Bennett, 1996); covalent linkage of functional groups, such as cholesterol, to alter physical properties, provide ligands or provide resistance (Krieg *et al.*, 1993, Manoharan *et al.*, 1995, Crooke and Bennett, 1996); replacement of the central phosphorus with a methylene group creating a formacetal linkage (Milligan *et al.*, 1994). All of these modifications display either enhanced affinity or resistance or both, but many of them do not elicit RNase H activity.

As a result, many researchers are looking towards the use of chimeric oligonucleotides, which combine segments of RNase H activating oligonucleotide, flanked by sequences which enhance nuclease resistance and target affinity (Giles and Tidd, 1992, Monia *et al., 1993*, Giles *et al., 1993*, Kandamilla *et al., 1997*).

A very important consideration when designing antisense experiments is the possibility of the oligonucleotide causing sequence specific and non-sequence specific side effects of a non-antisense nature. This can be due to the down-regulation of non-targeted genes and the binding to cellular proteins and nucleic acids, thereby inhibiting their function. These effects can lead to misinterpretation of results that might otherwise be ascribed to an expected antisense effect. Although these unforeseen side effects are sometimes even beneficial, leading to new potential therapies, they should be distinguished from a true antisense effect. The only way to efficiently design new and improved antisense therapies is through a proper understanding of the antisense mechanisms. Most of the work examining non-specific side effects has been performed on phosphorothioate oligonucleotides, as these are the most commonly used oligonucleotide analogue and are the only form currently in use in clinical trials.

The ubiquitous enzyme RNase H can cause unspecific side effects. It can cleave DNA-RNA duplexes which are as short as 4 bp *in vitro* and 10bp *in vivo* (Donis Keller, 1979; Wolf *et al.*, 1992). As a result, it is probably not possible to obtain cleavage of an intended RNA target without causing at least partial degradation of many nontargeted RNAs. It is therefore prudent to screen potential antisense oligonucleotides against gene databases to identify and select those expected to knock out the fewest essential genes. A fact often ignored, is that charged oligonucleotides, such as phosphorothioates are actually polyanions (Stein, 1994). Polyanions, such as the naturally occurring glycosaminoglycans heparin and heparan, play extremely important physiological roles (Stein, 1995). Therefore, the introduction of PS oligonucleotides can interfere with normal cellular functioning, thereby mimicking an antisense effect. G-quartets are well known as causing sequence-specific non-antisense effects (Higgins *et al.*, 1993; Maltese *et al.*, 1995; Stein, 1995) as are CG dinucleotides (Krieg *et al.*, 1995).

It is therefore clear that there is a requirement, when designing antisense experiments, for strict and rigorous control measures, to allow the clear and unambiguous demonstration of an antisense effect. There are now a number of generally accepted guidelines for the design and evaluation of antisense experiments (Stein, 1994; Wagner, 1994; Branch, 1996).

1. There should be a clear demonstration of a decrease in the levels of the target protein. Showing a decrease in the mRNA levels of a target gene is not a prerequisite, as it requires that the oligonucleotide in question being able to activate RNase H, and implies that blockade of the ribosomal readthrough is irrelevant. If, however, the measurement of target protein levels is omitted for whatever reason, additional controls should be included that demonstrate a lack of effect on cell lines that do not have the target sequence.

2. The choice of target sequence must be made carefully. Many investigators have chosen to target the translation initiation site of an mRNA on the assumption that this region is important and accessible. However, most regions are now though to be accessible (Dean *et al.* 1994), with the relative efficacy of different sites depending on secondary structures and the chemistry of the oligo modification (Fenster *et al.* 1994). To avoid biasing the outcome of an experiment by the choice of target sequence selection, it is important to show that the same effect is produced by more than one antisense sequence.

3. The choice of control sequences is a critical element in the design of any antisense experiment. There are four types of control oligos that should be considered.

(a) Sense control: This type maintains structural features (e.g. palindromes, stem loops, class), but does not maintain composition. G-quartet effects will not be picked up.

(b) Scrambled control: This type of control does not maintain structural features, but does maintain composition. However, this type will also not highlight G-quartet effects.

(c) Mismatched control: This type of control, with only one or two mismatches in the central section of the oligo, demonstrates target hybridisation selectivity, and can maintain composition if two mismatches are made. Depending on where the mismatches are made, it may or may not be able to maintain structural features.

(d) Mismatched target control (i.e. using cells with a mutant or deleted gene): This control may demonstrate a lack of non-sequence specificity, but the control cells may thus be significantly different from the target cells with regard to other critical parameters, such as oligo internalization and compartmentalization.

Ideally, the control should differ from the antisense sequence no more than is necessary to prevent specific hybridisation. There is no scientifically correct number of controls to employ in a certain experiment. The more control oligos that are used, however, the more likely that the observed end point has resulted from a true antisense mechanism.

1.5.4 Use of Antisense Oligonucleotides to Inhibit MDR-1 Expression

Over the last number of years, there have been several studies that have looked at the effect of using antisense oligonucleotides to down-regulate expression of the mdr-1 gene (See Table 1.5.4. For review see Byrne *et al.*, *in press*). These will be discussed in some detail, since they illustrate well some of the different approaches and achievements, as well as the limitations and problems which may be expected when applying antisense technology to investigate drug resistance. The first such study came in, 1989, carried out by Vasanthakumar and Ahmed (1989). They used a 15 base pair (bp) methylphosphonate oligonucleotide, targeted to a region containing the initiation codon of mouse mdr-1 (see Table 1.5.4), on a human erythroleukemic cell line K562/III which had been selected for Daunorubicin resistance (119-fold compared to parent K562/S). This cell line was cross-resistant to vincristine, doxorubicin and etoposide. It also exhibited mdr-1 amplification and increased expression of its transcripts (30-fold increase). The oligo was used at a 30μM concentration and was added free in the cell culture medium (which contained 10% heat-inactivated serum). After 72 hour incubation time at this concentration PGP expression was totally eliminated in the K562/III cell line. A complementary sense oligo targeted to the same region had no effect on PGP levels. Both the antisense and sense oligos were shown to have no apparent biological effect on the parental sensitive cell line K562/S. The IC_{50}

values of Daunorubicin in the K562/III cell line fell from 119-fold greater than the parent to 85-fold. There was no change in the IC_{50} values of Daunorubicin in the antisense treated parent cell line, while the sense oligos had no effect on either cell line. These results indicated the potential of using antisense against mdr-1 to cause a down-regulation in PGP expression and a concomitant decrease in drug-resistance. However, the antisense sequence chosen was compatible with the mouse mdr-1 sequence and not human mdr-1. There were in fact three base-pair mismatches as regards the human sequence, and this should be taken into account when considering the results.

Jaroszewski *et al.* (1990), designed five different phosphorothioate oligos which were used to down regulate mdr-1 in the human breast cancer cell line MCF-7 ADR, which was 192-fold more resistant to adriamycin than its parent cell line MCF7. One was targeted to the initiation codon region, one just 3' to the initiation codon, and three spread out within the coding region (Table 1.5.4). They demonstrated the uptake and distribution of the phosphorothioate oligos in MCF-7 cells by using ^{35}S-labelling, showing that only 2% of the oligos were taken up from the media and, of that, 64% remained in the cytoplasm. Of the five oligos tested the most effective was the one targeted at nucleotide +18 to +32 in relation to the first base in the mRNA sequence (taken as +1). It caused a 4-fold increase in Adriamycin toxicity. However they did not present evidence of decreases in the mdr-1 levels or give actual IC_{50} values. They also speculated on the reasons behind the variation in effect of the five oligos. They postulated that this was due to differences in the mdr-1 mRNA secondary structure at the different sites targeted, as the mRNA is extensively folded, 62% being paired.

Rivoltini *et al.* (1990) used a 12 bp phosphodiester oligo targeted to bases -6 to +6 of mdr-1 mRNA (Table 1.5.4). Human colorectal adenocarcinoma cells LoVo/Dx, which are resistant to Doxorubicin and show increased PGP expression, were cultured for 3 days in the presence of the oligos, resulting in a reduction of PGP to a level equal to or lower than that of the sensitive parent cell line LoVo/H. The ID_{50} of the LoVo/Dx cells

Table 1.5.4 Types and target sites of antisense directed against mdr-1 mRNA

Target site*	Oligo Type	Cells used	Reference
-457 to -474	Phosphorothioate(PS)	LoVo/Dx,S180Dx,KBCh85	Bertram *et al.*
-20 to -1	PS oligo	CEM 60VCR	Li *et al.*
-20 to -1	2'-O-methyl modified	CEM 60VCR	Li *et al.*
-20 to +1	Phosphodiester	LoVo/Dx	Corrias *et al.*
-14 to +4	PS oligo	NIH 3T3	Alahari *et al.* **
-9 to +6	PO with PS 3' & 5'ends	Kidney Primary cultures	Efferth *et al.*
-9 to +6	PS oligo	MCF-7/ADR	Jaroszewski *et al.*
-9 to +6	PS oligo	$K562/VCR_{100}$	Sola *et al.*
-9 to +6	PS oligo	LoVo/Dx,S180Dx,KbCh85	Bertram *et al.*
-9 to +6	Methylphosphonate	K562/III	Vasanthakumar *et al.*
-9 to +9	PO oligo	LoVo/Dx	Quattrone *et al.*
-6 to +6	PO oligo	LoVo/Dx	Rivoltini *et al.*
-6 to +9	PO with PS 3' & 5'ends	SKVLB	Thierry *et al.*
-6 to +10	PS oligo	K562/ADM	Liu *et al.*
-1 to +24	PS oligo	P388/Adr	Nakashima *et al.*
+1 to +18	PS oligo	HL-60/Vinc	Cucco & Calbretta.
+1 to +18	PO oligo	CHrC5	Clynes *et al.*
+1 TO + 20	PS oligo	P388/Doxorrubicin	Hirtake *et al.*
+1 to +20	PO oligo	LoVo/Dx	Corrias *et al.*
+12 to +36	PS oligo	CEM 60VCR	Li *et al.*
+18 to +32	PS oligo	MCF-7/ADR	Jaroszewski *et al.*
+21 to +40	PO oligo	LoVo/Dx	Corrias *et al.*
+156 to +1118	cDNA	KB 8-5	Hanchett *et al.*
+336 to +354	PS oligo	MCF-7/ADR	Jaroszewski *et al.*
+336 to +359	PS oligo	MCF-7/ADR	Jaroszewski *et al.*
+991 to +1007	PS oligo	K562/ADM	Liu *et al.*
+993 to +1008	PO with PS 3' & 5'ends	SKVLB	Thierry *et al.*
+1152 to +1176	PS oligo	MCF-7/ADR	Jaroszewski *et al.*
+2420 to +2434	PS oligo	LoVo/Dx,S180Dx,KbCh85	Bertram *et al.*
+2990 to +3007	PS oligo	LoVo/Dx,S180Dx,KbCh85	Bertram *et al.*
+4026 to +4045	PO oligo	LoVo/Dx	Corrias *et al.*

* The first base in the coding sequence (i.e. the A in the ATG initiation codon) is given a position of +1

** Alahari *et al.* tested almost 40 different antisense sequences to various regions of the mdr-1 transcript with different backbones and modifications. Only the most effective antisense sequence is given above.

was reduced 100-fold when cultured in the presence of the antisense oligo, giving a similar effect to that obtained from the MDR modulator, verapamil.

Clynes *et al.* (1992) found that antisense, but not sense, oligodeoxynucleotides corresponding to the first 18 bases of the human mdr-1 sequence caused an increase in adriamycin sensitivity in the human lung squamous cell PGP over expressing MDR line DLKP-A, and also, perhaps surprisingly in view of some sequence difference between the species, in the hamster MDR line CHrC5.

Corrias and Tonini (1992) targeted five 20bp phosphodiester oligonucleotides (Table 1.5.4) to the human mdr-1 gene in the LoVo/Dx doxorubicin resistant cell line. They analysed stability and cellular uptake of the oligos. One of the oligos, targeted immediately 5' to the initiation codon, stood out from the others in terms of effectiveness, causing 60% of the cells to lose their resistance to doxorubicin, preventing them from forming colonies in the presence of the drug. They determined the half-life of the mRNA of mdr-1 to be approximately 4 h, and demonstrated that alteration of the mRNA occurred after treatment with the antisense. However, no actual IC_{50} data was given after the antisense treatment. They suggested that the other antisense oligonucleotides were ineffective due to these sequences being highly conserved among pgp and many other proteins.

In, 1993, Efferth and Volm used 2x15 bp oligonucleotide targeted to bases -9 to +6 of the mdr-1 mRNA sequence (Table 1.5.4), taking in the AUG initiation codon and a Shine-Dalgarno like sequence (AGGUGG), which recognises the ribosome binding site (Efferth and Volm, 1993). One was a simple phosphodiester oligonucleotide, the other with 3 phosphorothioate bases at the 3' end. The corresponding sense sequence was used as a control. The oligos were incubated with primary cultures of kidney tumor and normal kidney from 3 different patients, for 3 days at concentrations of 1, 5 and 10μM. They found that the 10 μM concentration of antisense significantly decreased PGP levels in cells which expressed high levels of PGP initially, while there was only a slight decrease observed for the patient with low-PGP expressing cells. The latter patients cells also showed no inhibition of growth when the antisense was

combined with vincristine or doxorubicin in a 7-day growth assay. The cells for the patients with high PGP expression were inhibited by up to 70% by the combination of antisense and drug. The inhibitory effect was more pronounced for the 3' phosphorothioate capped oligo. This gave an early illustration of the increased efficacy of using nuclease-resistant phosphorothioate oligos when targeting mdr-1 mRNA.

Thierry *et al.*, (1993), used an unique approach to delivering the two 15 bp antisense and one 15bp sense oligonucleotides they designed against mdr-1 (Table 1.5.4). They delivered the oligos to the human ovarian carcinoma cells SKOV3 and the multidrug resistance variant SKVLB, either free to the cells or by Minimal Volume Entrapment (MVE). Small unilamellar vesicles were made from a mixture of different lipids, which were dried by evaporation, rehydrated with phosphate-buffered saline (PBS) containing 10 μg/ml of the oligonucleotide, vortexed vigorously and then sonicated, giving a final concentration of between 60 and 70 μg oligo/ml lipid. 5μM final concentration of the oligos, which had two phosphorothioate bases at each end of a phosphodiester backbone, was used. The two antisense oligos were directed towards the AUG initiator codon-containing region (ASin) and a loop forming site located at bases +993 to +1008 from the first AUG codon (ASlp). Using a 5 μM concentration of the ASlp oligo added free to the cell culture media, they caused a 40% reduction in PGP expression, as measured by flow cytometry and doxorubicin resistance. However, using MVE to deliver the oligos, they demonstrated a nearly complete inhibition of PGP expression and a four-fold decrease in doxorubicin resistance. The lack of complete reversal of doxorubicin resistance accompanying the complete inhibition of PGP indicated that there were other mechanisms of resistance involved in these cells. Adding free ASin oligo caused very little effect on PGP levels or resistance, as did the freely added corresponding sense oligo. ASin added by the MVE method, however, gave a two-fold decrease in PGP levels and a four-fold decrease in the doxorubicin resistance. This down-regulation of the mdr-1 gene was shown to be sequence non-specific, due to the fact that the corresponding sense oligo delivered by MVE also demonstrated a significant, if not as large, decrease in PGP expression and resistance to doxorubicin. This sequence non-specific effect was dependent on the sequence used, though, as the sense to the loop-forming region had no effect whether free or

liposomally encapsulated. Thierry *et al.* postulated that bases at the 5' end of mdr-1 coding region might be pairing intramolecularly and the sense oligo to this region might be forming a triple helix structure, thereby inhibiting mRNA processing. They proposed loop-forming regions as a more effective site for antisense targeting, and they backed this claim up by citing that another loop-targeting antisense (bases +568 to +583) was equally as effective as the one above, but they did not present data to support this. The results, therefore, demonstrated the importance of the choice of target site and also the benefits of encapsulating the oligonucleotides in liposomes. It was shown, during their work, that oligos were effectively protected from environmental degradation when encapsulated by MVE, showing no degradation after incubation for a week in 10% serum containing medium, while the free oligonucleotide was mostly degraded after 30 min.

In, 1994, Quattrone *et al.* demonstrated the use of a mixture of antisense oligos for down-regulating mdr-1 mRNA in the 75-fold doxorubicin resistant subline (LoVo/Dx) of the human adenocarcinoma cell line LoVo (Quattrone *et al., 1994* (b)). They designed three contiguous 18 bp phosphodiester oligos to attack the region from -9 to +45 from the first AUG codon (Table 1.5.4), which had previously been shown to be effective (Uhlmann and Peyman, 1992). They used an equimolar mixture of the three at a final concentration of 10 μM in conjunction with 5 μg/ml of the liposomal transfection reagent DOTAP. They incubated the cells in the presence of the antisense mixture for 15 days, changing the media and adding fresh oligos every 72 h. At the end of the culture, the cells were exposed to an IC_{50} value of doxorubicin (relative to the resistant and sensitive lines respectively) while maintaining the presence of the oligos. They used the three oligos together in a mixture because of a postulated synergistic inhibitory effect of contiguously targeted oligos (Maher and Dolnick, 1998). They demonstrated that the DOTAP used for delivery of the oligos increased their cellular uptake 25-fold and afforded protection from cytoplasmic nuclease cleavage. The 15 day incubation was employed due to the relatively long half-life of PGP (72 h), and the fact that previous studies had only used 3 or 4 day studies, which were not deemed sufficiently long. The combination of 10μM oligos with 5μg/ml of DOTAP was shown to a moderate inhibitory effect on cell growth, 26% and 38% for a scrambled control

oligo and the antisense oligos respectively. The decrease in cell growth when the doxorubicin was added was double the expected additive effect of growth inhibition of the antisense oligos/DOTAP mixture and the doxorubicin separately for the antisense treatment. The inhibitory effect was simply an additive for the combination of the sense oligo/DOTAP and the drug. After 5 days of the incubation, the mdr-1 mRNA levels were shown to be significantly decreased by the antisense mixture, with no effect observed on untreated or sense treated cells. After the full 15 day incubation, the mRNA levels had decreased down to the level of the drug-sensitive parent line. As regards PGP expression, there was no observed change after 5 days treatment with the antisense oligos, while at 15 days, there was a reduction in PGP levels in comparison to the untreated and sense-treated cells, but not to the level of the drug-sensitive parent. They showed, however, that there was a good correlation between the mdr-1 mRNA/PGP levels and resistance to doxorubicin induced cytotoxicity, and postulated that the greater cytotoxic effect of the antisense/DOTAP mixture compared to the sense/DOTAP mixture, without the addition of drug, indicated that a marked and prolonged decrease in mdr-1 gene expression could be intrinsically toxic for cells. This is a relevant consideration for possible future *in vivo* administration of mdr-1 targeted antisense.

Hanchett and his colleagues tried a different approach by using antisense RNA transcribed from an expression vector (Hanchett *et al., 1994*). They used a 963bp fragment from the mdr-1 cDNA (bases +156 to +1118) cloned into an expression vector pHβAPr-3-neo (Table 1.5.4), driven by the β-actin promoter, in both the normal and reverse orientation, to give the sense and antisense molecules. They transfected the mdr variant, KB 8-5 (4-fold resistant to colchicine), of the human nasopharyngeal epidermoid carcinoma cell line with HeLa markers, KB 3-1, with the antisense containing plasmid, as well as the sense containing vector and the vector with no insert as controls. They isolated 16 clones from each transfection. Of the 16 selected from the antisense transfection, only 10 actually expressed the antisense RNA, and of these only 1 exhibited a decrease in mdr-1 mRNA levels (50%) and PGP levels (50%) as detected by Northern Blot/ RNase protection assay and Western Blots respectively. This clone also displayed a corresponding decrease in colchicine

resistance (50%) but was still almost 3-fold more resistant than the sensitive parent KB 3-1. Despite the large amount of effort involved in producing one successfully transfected clone, this work showed the potential of using antisense RNA as an alternative to DNA-based oligonucleotides. However, despite the fact that all cells in this population were isolated from one clone, the authors pointed out that there was a high degree of heterogeneity, with highly varying levels of PGP expression, as demonstrated by Rhodamine 123 retention assays. Even subclones of the initial clone retained this heterogeneous phenotype, showing that it was an inherited trait of this clone. The authors went as far as performing Restriction-Fragment-Length-Polymorphism assays to prove that all phenotypically distinct subpopulations descended from the same transfected clones. This level of heterogeneity in transfected cells could be a possible deterrent to the future use of antisense RNA.

Bertram *et al.*, (1995), attempted reversal of the MDR phenotype in two PGP overexpressing variant human cell lines (LoVo/Dx^R and $KBCh^R$8-5) and one murine resistant cell line (S180Dx^R). They used antisense designed to four different regions in the mdr-1 mRNA, the 5' promoter region, S-ODN1, the ATG initiation region (bases -9 to +6), S-ODN2, and two within the coding region (bases +2420 to +2434 and +2990 to +3007), S-ODN3 and S-ODN4 (Table 1.5.4). They were all phosphodiester oligonucleotides with a G-C content of between 40 and 60%. They were used at a concentration of either 0.2, 2 or 5μM, with 2μM being the most efficient. Only a single dose of the antisense was applied and incubated with the cells for 12 h before the addition of 10% fetal-calf serum (FCS). Cells were then further incubated for a total of 72h before various assays were carried out. A 2μM concentration of S-ODN2 and 3 applied to LoVo/Dx^R cells caused a decrease in [^{3}H]thymidine incorporation of 10 and 50% respectively, with corresponding sense oligos exerting no effect. Western blots using the mdr-1 MAb C-219 showed that S-ODN2 and 4 were unable to reduce PGP expression in LoVo/Dx^R compared to untreated cells, while S-ODN 3 reduced the PGP down to the level of the sensitive cells. S-ODN1 showed a less pronounced effect than S-ODN3, but still caused around a 75% reduction. Analysis of the time scale of the reduction showed that after 24h the levels of PGP remained unchanged, after 48h a reduction was apparent, while after 72 h the levels of PGP were down to the those in

the sensitive parent cells. S-ODN3 was found to be most effective in the LoVo/Dx^R cell line with a 62% reduction in the ID_{50} in the presence of doxorubicin, with the S-ODN1 only giving a reduction of 20%. In $KBCh^R8$-5 cells, S-ODN3 was once again more effective, decreasing the ID_{50} by 30%, with S-ODN1 having no effect. In the murine sarcoma cell line $S180Dx^R$, however, S-ODN3 was ineffective, while S-ODN1 reduced the ID_{50} in the presence of doxorubicin by 60%. The differences in effectiveness of the same oligonucleotides in different cell lines highlighted the importance of the accessibility of the target region for the antisense, which could be altered between various species and cells due to slight variations in sequence and in the processing of the mRNA. Tests comparing the effectiveness of the S-ODN3 antisense on LoVo/Dx^R cells as compared to the classic chemomodulators verapamil and tamoxifen on PGP function, demonstrated that in this cell line at least, the antisense molecule was at least equally effective in decreasing the ID_{50} values.

Nakashima *et al.* (1995) tested a 25bp phosphodiester targeted to the AUG initiation codon and loop forming region (Table 1.5.4) on mouse leukaemia adriamycin sensitive (P388/S) and resistant (P388/ADR) cells. The resistant cells were 100-fold and 300-fold more resistant than the sensitive cells to Vincristine and adriamycin respectively. Concentrations of oligo below 30μM were used, due to toxicity of higher concentrations. They showed, by flow cytometry, that after 72h the level of PGP in the P388/ADR cells was slightly, but significantly, reduced as compared to untreated P388/ADR cells, while the sense oligo was ineffective. 15μM of the antisense oligo caused 2-fold more vinblastine accumulation in P388/ADR cells than the sense oligo. Correspondingly, the antisense potentiated the growth-inhibitory effect of the vinblastine, decreasing the IC_{50} value significantly (approx. 2-fold). This reversal of resistance by the antisense was slightly more effective than verapamil on its own, and when the antisense was combined with 1μM of verapamil, the IC_{50} value decreased approximately 4-fold.

Alahari *et al.* (1996) conducted an extensive study, analysing 32 different phosphorothioate oligonucleotides spanning almost every region of the mdr-1 mRNA including the 5' Untranslated, AUG codon, Coding (splice junction), open reading

frame (ORF), and stop codon, 3' untranslated and 5' Cap (Table 1.5.4). The cells used were mouse NIH3T3 fibroblasts, which had been transfected with an expression plasmid containing the human mdr-1 cDNA (pSK1 MDR-1). The antisense oligos were used at a concentration of 1μM in the presence of 20μg/ml of Lipofectin, and incubated with the cells for 24 h. One oligonucleotide which stood out from the others was one overlapping the AUG start codon (AS 5995), as it caused a substantial reduction in the mdr-1 message levels as measured by Northern blots. The other sequences tested were largely ineffective, including two other oligonucleotides that also overlapped the AUG codon. This result emphasises that slight alterations in the positioning of an antisense target can be of utmost importance in mediating antisense effects. Maximum specific reduction on mdr-1 mRNA was observed after 24h treatment of the cells with AS 5995, but reduction occurred only with the use of serum-free media and cationic liposomes. Multiple treatments with the AS 5995 did not cause any greater specific reduction in the mdr-1 messenger levels than a single treatment, whereas greater cytotoxicity was observed. The reduction in the mRNA expression was found to be readily reversible after the 24h exposure to AS 5995, with normal levels returning after 24 h if the cells were returned to complete culture medium. The treatment of the MDR 3T3 cells was concentration dependent with a slight reduction observable at 100nM and maximum reduction (60%) at 1μM. Higher concentrations of oligos (5-10μM) lead to greater non-specific effects with reduced control gene β-actin message levels and cytotoxicity observed. The PGP levels of the MDR 3T3 cells were also reduced, with the decrease being minimal after 24 h, readily detectable after 48 h and maximal after 72 h exposure to the antisense. By using a 2'-O-methyl analog of AS5995, Alahari *et al.* gave evidence for the role of RNase H in mediating antisense inhibition of PGP expression. 2'-O-methyl oligonucleotides do not illicit the action of RNase H, and, as expected, this analog did not cause any reduction in PGP levels of the MDR 3T3 cells when incubated under the same conditions as the AS 5995 oligo. Conjugation of oligonucleotides with lipophilic substituents had been reported to enhance oligonucleotide accumulation in cells and result in improved biological effects (Krieg *et al., 1993*). They therefore synthesised a 5' cholesterol derivative of AS 5995. This analog used alone was shown to be as effective as the phosphorothioate molecule in combination with Lipofectin, causing a minimum

decrease in PGP expression of 60%. In addition, the cholesterol oligonucleotides showed less experiment-to-experiment variation than the standard phosphorothioate oligos administered with cationic lipids. By conjugating these two oligonucleotides to FITC and using flow cytometry, the authors showed that over a 2 h incubation period, the cholesterol conjugated oligo was rapidly accumulated by cells, whereas both free AS 5995 and AS 5995 complexed with Lipofectin was accumulated to a far lesser degree. After an overnight incubation, free AS 5995 still showed considerably less accumulation than the cholesterol analog, while the Lipofectin complexed AS 5995 displayed substantial but very heterogeneous cell uptake. Using confocal microscopy, these results were confirmed. In addition, while only a sub-population of the Lipofectin/antisense treated cells showed nuclear accumulation of oligo, cells treated with the cholesterol conjugate displayed uniformly extensive flourescence in both the cytoplasm and the nucleus. These results suggested that the cholesterol conjugation enhanced the rapidity, amount and uniformity of cellular uptake of the oligonucleotide. Therefore there appears to be significant advantages in using relatively low-molecular weight cholesterol oligonucleotides compared with extremely large oligonucleotide/cationic lipid complexes.

Cucco and Calabretta (1996) used just a single 18bp phosphorothioate antisense oligonucleotide along with sense and antisense controls (Table 1.5.4). The oligos were added to sensitive HL-60 human promyelohcytic cell line and a vincristine selected resistant variant, HL-60/Vinc, at a concentration of 200μg/ml over 4 days (80 μg/ml on the first day, and 40 μg/ml each subsequent day) or 360μg/ml over 7 days. The oligos alone had no effect on cellular proliferation. Treatment with vincristine alone caused only a 48% inhibition at the highest concentration used (1 μg/ml). Antisense oligo in combination with vincristine treatment caused significant inhibition, 58% with 0.01μg/ml vincristine and 92% with 1 μg/ml vincristine: mdr-1 mRNA and protein levels were also reduced. There was no difference in any of these parameters with vincristine alone or vincristine with sense oligo or vincristine with scrambled oligo for sense or scrambled oligo treatment. In order to test the efficacy of this antisense oligonucleotide *in vivo*, Cucco and Calabretta, used SCID mice which were given injections of HL-60/Vinc leukaemia cells, and were subsequently treated by tail

injection of antisense (1mg/mouse/day) for 10 days in combination with vincristine (20μg/mouse/day). Survival of the mice was not prolonged with vincristine alone, or when treated with sense or scrambled oligos either alone or with vincristine. Treatment with the antisense oligo alone was ineffective, with a median survival time of 57 days post leukaemia implant. However, the antisense and vincristine combination gave a median survival time of over 300 days. The mice were sacrificed at this time point and no trace of c-myb transcripts (a leukaemia cell load marker) was found by RT-PCR, indicating a lack of tumor cell presence. This evidence supports the specific mechanism of action of antisense to mdr-1 *in vivo*, leading to possibilities of future use in cancer patients.

Liu *et al.* designed two 17 bp phosphorothioate oligonucleotides complementary to ATG initiator codon region (bases -6 to + 10), AS-1, and a loop forming site within the coding region (bases +991 to +1007), AS-2 (Table 1.5.4) (Liu *et al., 1996*) . They tested these two antisense molecules on the human leukaemic cell line K562 and its Adriamycin resistant subline K562/Adm, which was 155-fold and 74-fold more resistant than the parent to adriamycin and vincristine respectively. Both PGP and mdr-1 mRNA were shown to be overexpressed in the resistant subline as compared to the sensitive parent. In the presence of adriamycin, both AS-1 and AS-2 at 10μM concentration caused significant inhibition of K562/Adm cell proliferation after 24, 48 and 72 h incubation times. The inhibition was most prominent after 48 h, 66% for AS-1 and 72.8% for AS-2. The antisense oligos showed no inhibitory effects in the absence of adriamycin, indicating a sequence specific action of the antisense and a lack of unwanted toxicity. A control sense oligo showed no effect with or without adriamycin. Both AS-1 and AS-2, after a 48h incubation, caused a pronounced increase in daunorubicin accumulation from 18.2 % to 67.3% and 75.2% respectively, as measured by flow cytometry. The MDR modulator Verapamil caused an increase from 18.2% to just 27.2%, indicating the much greater efficacy of the antisense oligos. Correspondingly the number of PGP positively staining cells decreased from 71.62% for the control to 45.44% for a 10μM concentration of AS-1 incubated for 48 h, while the ratio of mdr-1 mRNA to control β-actin expression decreased from 1.56 to 0.97.

Sola and Colombani (1996) chose to target the initiation codon of mdr-1 using a 15bp phosphorothioate oligonucleotide (Table 1.5.4), with the corresponding sense as a control They also used the human erythroleukemic K562 cell line and a vincristine-selected resistant subline K562/VCR_{100}, which had a daunorubicin IC_{50} 40-fold higher than the parent and exhibited higher expression of PGP (95.9% of cells) as compared to K562 cells (22.1%). The cells were incubated with 10μM of the antisense or sense oligos for 48 h. The antisense, but not the sense, oligo enhanced daunorubicin toxicity and rhodamine uptake in the resistant line.

Hirtake *et al.* (1997) also used just a single phosphorothioate oligonucleotide, once again targeted to the initiation codon, but this time, of murine mdr-1 (Table 1.5.4). The oligo was tested on murine multidrug resistant P388/ADR lymphoid leukaemia cell line and the parental drug sensitive P388/p. The cells were cultured for 2 days in the presence of up to 500 μg/ml (6.6 μM) of either the 20bp antisense oligo or the corresponding sense. This resulted in a dose-dependent decrease in PGP expression in the P388/ADR cell, which was reversed after a further 4 day incubation in the absence of antisense. Mdr-1 mRNA expression was also inhibited in a dose-dependent fashion. However, no internal housekeeping gene, such as β-actin, was used as a control for these experiments, with the authors relying an equal input of total RNA amounts for RT-PCR and an extraction of equal amounts of 18S rRNA from the AS treated cells. The adriamycin IC_{50} on the P388/ADR cells was reduced by approximately 2 log (100-fold), giving a similar effect to that obtained with the immnosuppressant FK506, used as a positive control. No change in resistance was observed after treatment with the sense oligo. In an effort to demonstrate the *in vivo* effect of the antisense, they injected B6D2F1 mice with $1x10^6$ P388/ADR leukaemia cells which causes cancerous ascites and death within 2 weeks (mean survival time 13.4 days). These mice were then treated with the antisense or sense oligos via ip injection of 1, 3, 6 or 12 μm of oligo/g of body weight, followed by ip injection of ADR 48h later. With a single injection of 12μm antisense oligo/g body weight the mean survival time of the mice increased to approximately 24 days. When the antisense was administered twice daily for three days, the survival increased further to a mean of over 35 days. No toxicity of the oligos was observed in the mice as measured by changes in behaviour, weight gain and

peripheral blood count. Although none of the antisense treated mice were actually cured, the authors emphasise that the treatment duration was short during these studies. They state that since the responses observed were dose- and time- dependent, it was reasonable to assume that longer periods of infusions and larger amount of antisense oligo would be more effective in prolonging survival or leading to a cure. However, they did not actually recover any P388/ADR cells from the mice to check for decreases in PGP or mdr-1 mRNA levels, and stated simply that the results suggest that this was the case. They also said that although phosphorothioate oligonucleotides are reported to be nuclease resistant *in vitro*, some results indicate that injection of these type of oligonucleotide is followed by significant degradation (90% in 24 h) (Agrawal *et al., 1991*).

Li and his co-workers designed three antisense phosphorothioate oligonucleotides to mdr-1 (Li *et al., 1997*). One (1729) was just inside the coding region from bases +12 to +36, another (474) was targeted to the last 20 bases of the 5' untranslated region (-20 to -1), while the last oligo (1795) had the same sequence as 474 but with four 2'-O-methyl modified sugar linked bases at both ends (Table 1.5.4). The cells which were treated were a drug resistant subline (CEM60VCR) of CCRF-CEM leukaemia cells. The oligos were added to the cells at a concentration of 1μM combined with 2.5μg/ml of Lipofectin, and incubated for four days, with the addition of 1ml of fresh medium containing oligonucleotide after three days. Each of the oligonucleotides decreased mdr-1 expression by 30 to 45% as measured by antibody staining and mean relative fluorescence, while the 1795 oligo, with the DNA-RNA hybrid backbone, being significantly more effective than the other two purely phosphorothioate oligos. 2'-O-methylribonucleotides at the 3' and 5' ends of the 474 antisense gives this oligo greater *in vivo* stability and allows for increased binding affinity at the ends of the sequence, while retaining the ability to activate RNase H in the middle segment. The persistence of PGP suppression was measured at 0, 3 and 6 days after the end of treatment. It ranged from 28-46% immediately after treatment, 20-34% 3 days after treatment and by 6 days, levels had returned to pretreatment levels. Rhodamine 123 retention was significantly increased by as little as 0.2μM of antisense oligo, while being unaffected by control oligonucleotides. Sensitivity to vincristine was also greatly increased by

antisense treatment. The LD_{50} in antisense-treated CEM60VCR cells was almost 3-fold less than in untreated cells. The induction of mdr-1 expression through daunorubicin exposure for 24 h was examined, and was shown to be inhibited by as much as 50% by pretreating the cells for antisense oligonucleotides for 4 days. Cyclosporin induction of PGP expression was abolished by similar pretreatment with antisense. These results show the potential of using chimeric oligonucleotide hybrids to increase the potency of antisense sequences, as opposed to using purely phosphorothioate or phosphodiester backbones. The authors also stated that, as the level of resistance in CEM60VCR cells was comparable to levels of drug resistance in clinical samples, the degree of sensitisation observed (almost 3-fold) might expect to be observed in the clinic. They postulated that this level of sensitisation would be sufficient to show an improvement of the therapeutic index, and proposed a use for the antisense oligonucleotides in the clinical setting both as sensitizing agents (alone or in combination with a functional PGP inhibitor) and as prophylactic agents during initial chemotherapy to prevent the emergence of mdr-1 expressing clones.

1.5.5 Use of Antisense Oligonucleotides to Inhibit MRP Expression

Due to the relatively recent discovery of the multidrug resistance protein (MRP) as a mediator of the MDR phenotype, only a limited amount of work on the antisense modulation of MRP gene expression has so far been carried out. The first report of the use of antisense oligonucleotides for the reduction of MRP expression came from Stewart *et al.* (1996). They designed sixteen oligonucleotides (15 phosphorothioate and one 2'-O-methyl derivative) complementary to different regions along the entire length of the MRP mRNA along with one sense control oligonucleotide (see Table 1.5.5). They tested these antisense sequences on a multidrug resistant MRP-overexpressing small cell lung cancer cell line, H69AR, and T5 (MRP cDNA transfected HeLa cells). Lipofectin was used to deliver the oligonucleotides to the cells at a concentration of 5 μg/ml for HeLa/T5 cells and 10 μg/ml for H69AR cells. Oligonucleotide concentrations were between 0.1 and 0.5 μM as higher concentrations were found to be mildly toxic in combination with Lipofectin. The cells were incubated

Table 1.5.5 Types and target sites of antisense targeted to MRP mRNA.

Target site	Oligo type	Cells used	Reference
-194 to -176	Phosphorothioate	H69AR, HeLa/T5	Stewart *et. al.* (1996)
-106 to - 87	"	"	"
-20 to -2	"	"	"
+1 to +16	"	"	"
+19 to + 38	"	"	"
+106 to +125	"	"	"
+1911 to +1930	"	"	"
+1911 to +1930	2'-O-propyl/PS chimera	HeLa/T5	Canitrot *et. al.* (1996)
+1911 to +1930	2'-O-Fluoro/PS chimera	"	"
+2307 to +2326	Phosphorothioate	H69AR, HeLa/T5	Stewart *et. al.* (1996)
+3328 to +3347	"	"	"
+4526 to +4545	"	"	"
+4579 to +4590	"	"	"
+4616 to + 4635	"	"	"
+4639 to + 4658	"	"	"
+4742 to + 4761	"	"	"
+4789 to + 4808	"	"	"

with the Lipofectin/oligonucleotide for 4 h, then washed and incubated in fresh serum containing medium until harvested. In some cases they were retreated with the same concentrations of Lipofectin/oligonucleotide for another 4 h after a 48 h interval. After two 4 h treatments, MRP protein levels in the H69AR cells were found to vary considerably, depending on the antisense sequence used. Two oligonucleotides were found to be reproducibly the most effective. These were termed ISIS 7597 and 7598 and were complementary to nucleotides +2107 to +2126 and +2503 to +2522 of MRP mRNA respectively. All the oligos were also screened for ability to reduce MRP mRNA levels, and after a single treatment only one was found to have no effect. This was the 2'-O-methyl oligoribonucleotide. The authors stated that as 2'-O-methyl oligonucleotides are not substrates for RNase H, a decrease in the MRP mRNA levels was not necessarily expected in the 4 h time frame of the experiment. All subsequent experiments focused on the ISIS 7597 oligonucleotide because of its efficacy in decreasing both MRP protein and mRNA levels. The other most efficacious oligo,

ISIS 7598, was not chosen for further study because of its complementarity to a region highly conserved among the ABC transporter superfamily, and, as such, could potentially affect the expression of other proteins, making it less specific. Also, Stewart *et al.* decided to carry out all subsequent tests on the HeLa/T5 cells, as resistance in this cell line was known to be solely attributable to overexpression of MRP, while the resistance in H69AR was previously shown to be multifactorial (Cole, 1992, Almquist *et al., 1995*).

After a single treatment of T5 cells with ISIS 7597 a concentration dependent decrease in MRP mRNA was observed, with a significant decrease at 0.1μM and virtually complete elimination of detectable MRP mRNA with 0.3 and 0.5 μM of oligo. The corresponding sense had no effect at concentration up to 0.5 μM. This reduction in MRP mRNA was transient, with maximal inhibition reached at 4 h and maintained for 24 h after treatment, but returning to 70 and 100% of those in untreated controls by 48 and 72 h respectively. 0.5 μM of ISIS 7597 decreased MRP protein levels transiently and maximally to 30% of control levels after 48 h. This is consistent with the previously determined half life of MRP protein and the kinetics with which MRP mRNA levels are depleted and restored. When a double treatment with 0.5 μM of ISIS 7597 was used, the levels of MRP mRNA 24 h after the second treatment were approximately 10% of those in control cells. 24 h later again, the mRNA levels had returned to 30% of the controls. A 0.3 μM concentration of the oligonucleotide caused equal but more transient reduction. MRP protein levels were also decreased by 90% 24 h after the second treatment, and this reduction was maintained for an additional 24 h. Thus, a double treatment of HeLa/T5 cells with ISIS 7597 was able to extend significantly the response at the mRNA level to at least 3 days and to decrease MRP protein levels by 90%. The authors also demonstrated the role of RNase H in the reduction of MRP mRNA levels. By using two DNA probes for Northern blots corresponding to regions in the 5' and 3' coding ends of the mRNA, they were able to detect the oligonucleotide induced cleavage fragments of MRP mRNA in whole cells.

Stewart *et al.* proposed that variations in secondary structure at different sites within the MRP mRNA may have been the cause of differences in efficacy of the various

oligonucleotides tested. The AUG start site, as seen above, has been targeted in many studies because of the proposed accessibility of this sequence. However, this group found that oligos complementary to the coding region to be the most effective. These results indicate the importance of evaluating the activity of a number of oligonucleotides complementary to different regions of a given mRNA target rather than testing oligonucleotides directed against a single site. They also postulated that, variations in the doubling time of cells had an effect on the transiency of the antisense effect in these cells. They observed that, of the two cell lines they tested, inhibition was more prolonged in H69AR cells, which have a doubling time significantly longer than HeLa/T5 cells. They suggested that the rapidity with which MRP mRNA returns to normal levels is influenced by the decrease in intracellular concentration of oligodeoxynucleotide during cell division.

Many of the same group were involved in the second report of MRP directed antisense by Canitrot *et al.* (1996). The work presented was a continuation of that described above by Stewart *et al.* (1996). They used the same ISIS 7597 sequence with proven efficacy and made 2'-modifications in an attempt to improve this efficacy further. There were two oligonucleotides with different numbers of 2'-O-Propyl modified bases at both ends, while there were three oligos with 2'-O-F modification, either at both or just at single ends (Table 1.5.5). They were attempting to determine whether or not the 2'-O-modified chimeric analogs containing various 2'-O-deoxy gaps were more effective at reducing MRP mRNA expression than the 2'-O-deoxyphosphorothioate ISIS 7597. The cells used in the assays were once again the HeLa/T5 cells derived by stable transfection of the MRP cDNA expression vector, pRc/CMV-MRP1. In the first set of experiments the effect of the modified chimeric oligos on viability of the cells was examined. The results indicated that the 2'-O-Fluoro/2'-O-deoxy oligos were considerably more toxic than the 2'-O-propyl/2'-O-deoxy oligos and the basic ISIS 7597. The Fluoro modified oligos were toxic at concentrations greater than 0.1 μM whereas the propyl modified oligos were not toxic up to 0.5 μM. The T5 cells were then exposed to 0.5 μM of the oligos for 4 h and RNA and protein samples were isolated at various times thereafter. All the modified oligos markedly but transiently reduced both the MRP protein and mRNA levels, by between 60 and 70% for both.

There was complete restoration of the mRNA levels after 24 h. Once again, the role of RNase H in the decrease in MRP mRNA levels was demonstrated by using 3' and 5' Northern Blot probes to identify mRNA cleavage products. The bands obtained were of size 2.3 and 3.2 kb, which would be expected from the 5.5 kb MRP mRNA. Similar results were obtained for both fluoro and propyl modified oligos. To assess whether the reduction in MRP protein and mRNA levels was associated with enhanced chemosensitivity, cells were treated with 0.5 μM of the 2'-O-propyl modified oligos for 4 h and then exposed to various concentrations of doxorubicin. The results indicated that, as with ISIS 7597, these oligos caused a significant but only partial reversal of MRP-mediated resistance. The 2'-O-fluoro modified oligos were not tested because of their inherent toxicity. So, in summary the 2'-O-modified chimeric oligonucleotides were equally, but not more, effective as the 2'-O-deoxyphosphorothioate oligonucleotides, with the Fluoro modification being increasingly cytotoxic.

1.5.6 Antisense approaches in the Study of MDR: Conclusion

The results from the various authors cited above, demonstrate clearly the potential usefulness of using antisense oligonucleotides, whether modified or chimeric, to effectively down-regulate the expression of MDR-related genes. It seems evident at this point that the use of liposomal carriers for the transfer of the antisense oligos into the cell can greatly enhance the efficacy and prolonged activity of the oligos, through efficient delivery to the intracytoplasmic and nuclear regions and affording protection from cellular nucleases. The conjugation of oligonucleotides to lipophilic substituents also appears to be a very effective means of delivering the antisense directly to the nucleus. However, it is also apparent that a large amount of work remains to be carried out in this area. In almost all of the studies carried out above, full reversion of multiple drug resistance was not achieved. In many cases, this is due to the MDR phenomenon being multifactorial, with a combination of proteins causing increased cytotoxic drug resistance, so that reducing the expression of any one individual gene will not eliminate the MDR phenotype.

AIM OF THESIS

There have many reports proposing LRP as a novel mechanism of multidrug resistance. However, the evidence presented is mainly inconclusive and presumptive. There has been no direct evidence which can conclusively link LRP to drug resistance. This thesis aims to use ribozyme and antisense technology to provide a clear picture of the function of LRP in relation to drug resistance. Ribozyme and antisense technology has been widely used in the study of oncogenes and other mediators of drug resistance. This previous work sets a precedent for the use of ribozymes and antisense in investigating LRP function. The ribozymes and antisense will allow an examination of the cytotoxic drug resistance of cells exhibiting reduced levels of LRP expression. The comparison of resistance levels in these reduced-LRP cells with untreated parental cells will allow a determination of whether LRP can mediate MDR. To this end, there are three main questions to answer:

a) is LRP expression clearly reduced at the mRNA and/or protein level in any of the cells;
b) is there a reduction in the resistance to cytotoxic drugs in these cells; and
c) can the levels of LRP expression be correlated with the levels of drug resistance.

By answering these questions, it is hoped to determine conclusively whether LRP plays any role in MDR.

2. MATERIALS AND METHODS

2.1 Preparation for Cell Culture

2.1.1 Water

Water used in the preparation of media and solutions was purified by passing it through a Millipore milli-RO Plus system with an Elga Elgastat UHP. A pre-treatment step involving activated carbon, pre-filtration and anti-scaling, was carried out followed by a reverse osmosis step. Organic adsorption, ion exchange, ultra-microfiltration, photo-oxidation and ultra-filtration completed the process. The quality of water was monitored on-line and a measure of 16MΩ/cm at 25°C was considered acceptable.

2.1.2 Glassware

All glassware and bottle-caps used were soaked, for 1-2 hours in a 2%(v/v) solution of RBS (AGB Scientific; RBS-25) in warm water. The bottles were then scrubbed and both bottles and caps were rinsed in warm water and machine washed using Neodiser detergent, followed by rinsing twice in double-distilled water and once in ultra-pure water. The bottles were then prepared for autoclaving. Waste bottles containing spent medium from cells were autoclaved, rinsed in tap water and treated as above.

2.1.3 Sterilisation

Water, glassware and solutions containing thermostable compounds were sterilised by autoclaving at 120°C for 20min. at 15 p.s.i. pressure. Temperature labile compounds were filtered through a 0.22μm sterile filter (Millipore; millex-gv).

2.1.4 Medium Preparation

Growth media for cell culture was prepared as indicated in Table 2.1.4.1. The pH was adjusted to 7.45 - 7.55 by the addition of sterile 1.5M NaOH, and the volume adjusted to 5 litres and filtered through a sterile 0.22μm bell filter (Gelman; G.1423S) into sterile 500ml bottles. Sterility checks were performed on each bottle by placing :

(a) 3ml in a sterile universal to check for turbidity

(b) 1ml streaked onto a Columbia (Oxoid; CM331) blood agar plate

(c) 1ml in a 5ml sample of sterile Sabauraud (Oxoid; CM421) dextrose

(d) 1ml in a 5ml sample of sterile Thioglycollate (Oxoid; CM173) broth.

Sterility checks were incubated at 37°C and 4°C for 1 month and checked every 24hrs. Blood agar plates were kept for 7 days. The media bottles were labelled, dated and stored at 4°C until required. ATCC media was prepared by mixing equal volumes of DMEM and Hams F12.

Table 2.1.4.1 Preparation of Growth Media.

Components	DMEM (Gibco;042-02501M)	Hams F12 (Gibco;074-01700N)	MEM (Gibco;21430-020)
10XMedium	500ml	Powder	500ml
Ultra-pure H_2O	4300ml	4700ml	4300ml
1MHepes (pH7.5)*	100ml	100ml	100ml
7.5% $NaHCO_3$	45ml	45ml	100ml

* The weight equivalent of 1M N-(2-Hydroxyethyl)piperazine -N'-(2-ethanesulfonic acid) (Hepes) was dissolved in an 80% volume of ultra-pure water and autoclaved. The pH was then adjusted to 7.5 with 5M NaOH.

2.2 Routine Management of Cells in Culture

All routine management of cells in culture, including cell feeding, sub-culturing, freezing and thawing, were performed aseptically in a down-flow re-circulating laminar flow cabinet (Holton or Gelman Cytoguard) which had been swabbed with 70% IMS (industrial methylated spirits). To maintain a sterile atmosphere inside of the laminar flow all articles entering the cabinet were also swabbed with 70% IMS. Gloves were worn at all times during these procedures.

2.2.1 Cell lines

All cell lines used throughout this thesis are outlined in Table 2.1.4.1. All cell lines are

anchorage dependent. Cells were routinely grown in 25, 75 or 175cm^2 flasks (Costar; 3050, 3075: Nunc; 1-56502A, respectively). Cells were grown at 37°C and fed every 2-3 days or when a medium pH change was observed (colour change in medium due to the presence of a phenol red indicator). Waste media was removed from the cells at this stage and replaced with fresh media, as indicated in Table 2.2.1 Separate waste and medium-containing bottles were kept exclusive to each cell line to prevent cross-contamination. When feeding more than one cell line a minimum of 15min should be left before introducing a new cell line in to the laminar flow to further ensure against cross-contamination.

Table 2.2.7.1 Cell lines used throughout the course of this Thesis.

Cell Line	Growth Medium	Cell Type	Source
SW1573	ATCC[1]	Non-small cell lung cancer	Scheper[2]
SW1573-2R120	ATCC[1]	Drug selected resistant variant of SW1573	Scheper[2]
A2780	ATCC[1]	Ovarian carcinoma	Scheper[2]
A2780-AC16	ATCC[1]	Clone of A2780 transfected with LRP cDNA	Scheper[2]
DLKP-A	ATCC[1]	Adriamycin-selected MDR variant of DLKP	NCTCC
OAW42	ATCC[1]	Human serous adenocarcinoma of the ovaries	ECACC[3]
OAW42-S	ATCC[1]	Drug sensitive clone of OAW42SR	NCTCC
OAW42-SR	ATCC[1]	Spontaneously resistant population of OAW42	NCTCC

[1] 50:50 mixture of Hams F12 medium and Dulbecco's Modified Eagle's medium supplemented with 5% foetal calf serum and 2mM L-Glutamine (Gibco; 25030-024) prior to use.
[2] Rik Scheper, Dept. Pathology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, the Netherlands.
[3] European Culture Collection

2.2.2 Sub-Culture of Cell Lines

Monolayer cells grow attached to the bottom of flasks and upon reaching confluency (or when required for further studies) the cells were enzymatically detached from the flask base and sub-cultured. This involved removing waste medium from the flask of cells, rinsing the cells with 1ml of trypsin/EDTA (0.25% trypsin (Gibco; 043-05090), 0.01% EDTA (Sigma; EDS) solution in PBS (Oxoid; BR14a)) and then incubating with a further 4ml of the trypsin/EDTA solution for 5-10min (or until a single cell suspension had been obtained) at 37°C. An equal volume of complete medium was then added to the flask and the total cell suspension was transferred to a 30ml sterile universal (Sterilin; 128a) and

centrifuged at 120g. for 5min. The medium was poured off the cell pellet which was then resuspended in an appropriate volume of complete medium and re-seeded into fresh flasks at the cell density required (estimated by a cell count ; see section 2.2.3).
Suspension cells were sub-cultured simply by removing the cell suspension from the flask and pelleting the cells by centrifugation as above. Re-seeding and counting was carried out as for adherent cells.

2.2.3 Cell Counting

A sample of a single cell suspension was mixed in a ratio of 4:1 with trypan blue (Gibco; 525) and incubated for 2min. after which 10µl of the cell mixture was applied to a haemocytometer in the area under the cover-slip. Cells in the 16 squares of the four outer corner grids were counted, and the average of the four squares was multiplied by 10^4 and the initial dilution factor to determine the number of cells per ml of cell suspension. Cells that stained blue were considered non-viable while those unstained were accepted as viable cells.

2.2.4 Large-Scale Cell Culture

Cells required in large numbers were cultivated in roller bottles. Approximately 100ml of growth medium was allowed to equilibrate in a roller bottle at 37°C after which a single cell suspension of approximately $2x10^7$ cells was added. The roller bottle was incubated at 0.25rpm overnight and then the rotor speed was increased to 0.50rpm. The cells were allowed to grow to 80% confluency and were fed when determined necessary.

2.2.5 Freezing Cells in Culture

Stocks of all cells used in this study were frozen to allow their long-term storage and adequate supply within a given passage number range.
A single-cell pellet suspension was prepared (Section 2.2.2) from a sub-confluent large-scale culture of cells (Section 2.2.4). The cell pellet was resuspended in foetal calf serum (FCS) and an equal volume of 10% (v/v) DMSO (Sigma; D5879) in FCS was added drop-wise, with constant agitation, to result in a final concentration of 10^7 (viable) cells/ml. 1.5ml aliquots of the resulting cell suspension were placed in cryovials (Greiner; 122 278)

(labelled with the cell line, passage number, date and operators initials) and stored in the vapour phase of liquid nitrogen for 2.5h. and then stored in the liquid phase until required. A vial of cells was thawed 2-5 days after freezing to determine the sterility and viability of the stock.

2.2.6 Cell Thawing

The required vial of cells was removed from its liquid nitrogen store and thawed in a 37°C water-bath. The thawed suspension was quickly transferred to a universal containing 5ml of medium and was centrifuged at 120g for 5min. The medium was poured off and the cell pellet was resuspended in 5ml of complete medium, transferred to a 25cm^2 flask and incubated at 37°C. Following cell attachment, the cells were re-fed with fresh medium.

2.2.7 *Mycoplasma* Detection

All cell lines used in this study were routinely checked to ensure that *Mycoplasma* contamination had not occurred. These procedures were performed in isolation from the routine cell culture designated areas (by Dr. Mary Heenan and Mr. William Nugent) to avoid possible contamination of clean cell stocks. Two methods were used during analysis, namely the Hoechst 33258 indirect staining method and *Mycoplasma* culture methods.
The cell lines to be tested were grown in drug-free medium for a minimum of three passages following thawing. A 5ml aliquot of conditioned medium i.e. medium in which near-confluent cells had been grown for 2-3 days, was removed and analysed for the presence of *Mycoplasma*.

2.2.7.1 Hoechst 33258 Indirect Staining

Indicator cells (NRK) were grown (2x10^3) overnight on sterile coverslips in 1ml DMEM medium supplemented with 5% FCS and 2mM L-Glutamine, in individual 35mm sterile petri-dishes. 1ml aliquots of the conditioned media (Section 2.2.7), from each cell line to be tested, were added to duplicate cover-slips of NRK cells and incubated for 5 days (to approximately 50% confluency). The cover-slips were then washed of media twice with PBS, once with a 1:1 solution of ice-cold PBS:Carnoy's fixative (a freshly prepared 1:3 solution of glacial acetic acid (Sigma; A0808) with methanol (BDH; 101584W) which had

been stored at -20°C for 30min prior to use), and fixed for 10min in Carnoy's fixative. The cover-slips were then allowed to air dry. 2ml of Hoechst 33258 stain (Sigma; B2883), at a concentration of 50ng/ml in PBS, was added to each cover-slip and incubated in darkness for 10min. The coverslips were then washed in water and mounted on a glass slide using 50% glycerol (BDH; 101184K) in 0.1M citric acid (Sigma; C2916), 0.2M disodium phosphate (Sigma; S9390), pH 5.5 as the mounting solution. The slides were examined for *Mycoplasma* contamination under oil immersion using a mercury fluorescent lamp. Hoechst 33258 stains nucleic acids and therefore staining in the NRK cell nuclei was observed, any extra-nuclear staining was an indication of the presence of *Mycoplasma* contamination of the cell line under analysis. Both positive (a sample of medium known to be contaminated) and negative (medium not exposed to cells) controls were included in this procedure.

2.2.7.2 *Mycoplasma* Culture Method

The substrate used for the *Mycoplasma* culture method of detection consisted of 90ml of *Mycoplasma* agar (Oxoid; CM401) and *Mycoplasma* broth (Oxoid; CM403) bases, which were supplemented with 16.33% FCS, 0.002% DNA (BDH; 42026), 2μg/ml fungizone (Gibco; 05290), $2x10^3$ U penicillin (Sigma; Pen-3) and 10ml of a 25% (w/v) yeast extract solution (which had been boiled for 10min. and filtered through a 0.2μm filter). A 0.5ml aliquot of sample medium from the cell line being tested was incubated with 3ml of the broth for 48h. at 37°C in a 5% CO_2 environment. An aliquot of the broth was then streaked onto a 10ml agar plate, which was incubated for up to 3 weeks at 37°C in 5% CO_2, and frequently monitored microscopically for colony formation. The presence of "fried egg"-type colonies was indicative of *Mycoplasma* contamination of the cell line.

2.2.8 Serum Batch Testing

One of the main problems associated with the use of FCS in cell culture is its batch to batch variation. In extreme cases this variation may result in a lack of cell growth, whereas in more moderate cases growth may be retarded. To avoid the effects of the above variation, a range of FCS batches were screened for growth of each cell line. A suitable FCS was then purchased in bulk for a block of work with each particular cell line

in use. Screening involved growing cells in 96 well plates and growth was recorded as a percentage of growth of a serum with known acceptable growth rate.

Logarithmically growing cells were seeded into a 96 well plate (Costar; 3599) from a single cell suspension at a density of 10^3 cells/well in 100µl of medium without FCS. 100µl volumes of medium containing 10%, 20% or higher (if required by the particular cell line under analysis) (v/v) FCS was added to respective wells on the 96 well plate, resulting in final dilutions of the FCS to 5% and 10%, respectively. The first column of each plate was maintained as a control where FCS resulting in a known acceptable growth rate was used. Plates were placed at 37°C in 5% CO_2, for 5 days, after which growth was assessed by a crystal violet dye elution method or acid phosphatase (Martin and Clynes, 1991). Crystal dye elution method involved removing the media from the wells and rinsing twice in PBS. The wells were then fixed in 10% formalin (Sigma; F1635) for 10min, after which the formalin was removed and the plates allowed to dry. When the plates had dried 100µl of 0.25% crystal violet dye (Sigma; C3886) was added to each well of the plate for 10min; the dye was then removed and the plates were washed under running tap water 4 or 5 times and allowed to dry. The dye was eluted with a 33% solution of glacial acetic acid (Sigma; A6283) 100µl/well. The plates were then read in a dual beam plate reader at 570nm (reference wavelength 620nm) (Titertek; Multiskan). When growth was assessed by the acid phosphatase method, the plates were washed twice in PBS and incubated with 100µl of acid phosphatase buffer (consisting of 10mM p-nitrophenyl phosphate (Sigma; C104) in 0.1M Na-acetate, pH5.5 and 0.1% Triton X-100 (Sigma; X-100) for 2 hours at 37°C. Following incubation, 50µl of 1.0M NaOH was added to the buffer and the plates read in a dual beam plate reader at 405nm (reference wavelength 620nm) (Titertek; Multiskan).

2.3 Miniaturised Toxicity Assay

Logarithmically growing cells were used in all miniaturised toxicity assays. The day prior to setting up the assay the cells were fed with fresh, complete medium. On the first day of the assay the cells were sub-cultured and a single cell suspension was obtained (as described in Section 2.2.2).

2.3.1 Toxicity Assay - 96 well plate

Cells were seeded, from a single cell suspension, into a 96 well plate (Costar; 3599) at a cell density of $1x10^3$cells/well in 100µl medium; the first column of the plate was not seeded with cell suspension and was used as a control containing only medium. The cells were allowed to attach overnight at 37°C in a 5% CO_2 environment. Drug concentrations used in each assay ranged from concentrations which would result in no kill (i.e. no drug) to approximately 100% kill. The required drug dilutions range was prepared (at twice the final concentration to be assayed) and 100µl of each drug dilution was added to each well, in replicas of eight. The plates were covered in aluminium foil (most chemotherapeutic drugs are light sensitive) and incubated at 37°C in a 5% CO_2 environment for a further 6 days or until control wells (where no drug was added) reached 80-90% confluency. Drug toxicity was then determined by the acid phosphatase method (see Section 2.2.8).

2.3.2 Pulsing of cells with Drugs

Cells were seeded at a concentration of $1x10^4$ cells/ml into 25cm² flasks, 24h prior to addition of drug, and incubated at 37°C. Cells were washed once with PBS. The required drug concentration was added to the flasks in a total volume of 2ml. The cells were incubated with the drug for 2 hours at 37°C. Cells were then washed twice with PBS. Finally fresh ATCC media, supplemented with 10% FCS, was added to the cells, and the flasks incubated for a further 7 days. Drug toxicity was assayed using the acid phosphatatse method (see Section 2.2.8). In this case, 2ml of the acid phosphatase buffer was added to the flasks and incubated for 2h. Then 1ml of 1M NaOH was added to the flasks to stop the reaction. 150 µL of each sample was placed in wells in a 96-well plate for reading in a dual beam plate reader (see Section 2.2.8).

2.3.3 Calculation of IC_{50} values

The values from the dual beam plate reader for each drug concentration were calculated as a percentage of the control wells, which conatined no drug. This gave a percentage kill value for each drug concentration. The results were subsequently plotted on a graph of

drug concentration versus percentage kill. The drug concentration which gave a 50% kill was read from the graph. This represented the IC_{50} value for the given drug. When a number of repeats of a given toxicity assay were carried out, the IC_{50} values were averaged and the standard deviation calculated.

2.4 Safe Handling of Drugs

There are many potential safety risks when using cytotoxic drugs and in order to minimise such dangers extreme care was exercised in handling and disposing of cytotoxic agents. All work with such drugs was performed in a Gelman "Cytoguard" laminar air flow cabinet (CG Series), face masks and double gloves were worn when dealing with concentrated stocks and all drug waste (pure or diluted in medium or in contaminated plastics) was disposed of as recommended by the manufacturers (outlined in Table 2.4.1).

Table 2.4.1 Storage and Disposal of Cytotoxic Drugs

Cytotoxic Agent	Stock concentration	Storage	Disposal
Adriamycin[1] (Doxoxrubicin)	2 mg/ml	4°C in darkness	Inactivate with 1%hyperchlorite Autoclave
Vincristine[2]	1 mg/ml	4°C in darkness	Autoclave Dispose with excess water
VP-16[3] (Etoposide)	20mg/ml	R.T. in dark	Incinerate
Carboplatin[2]	10mg/ml	R.T. in dark	Incinerate
Methotrexate[4]	5mg/ml	-20°C in dark	Autoclave Dispose with excess water
5-Fluorouracil[2]	25mg/ml	R.T. in dark	Neutralise with 5M NaOH Incinerate
Epiubicin[1]	5mg/ml	4°C in darkness	Incinerate
Daunorubicin	5mg/ml	4°C in darkness	Incinerate
Taxotere	5mg/ml	4°C in darkness	Incinerate
Taxol[3]	6mg/ml	4°C in darkness	Incinerate
Melphalan	5mg/ml	4°C in darkness	Incinerate

Cytotoxic drugs used were supplied by [1] Farmatalia; [2] David Bull Laboratories, Ltd.; [3] Bristol Myers Pharmaceuticals; [4] Sigma-Aldrich;

2.5 Dilution Cloning

To propagate a clonal population from a mixed parent population, individual cells were plated into wells of a 96 well plate and allowed to grow as an individual clonal sub-population of the parental line. To achieve this a single cell suspension (see Section 2.2.2) was prepared at a density of approximately 1 cell per 300µl of media. The cell suspension was then plated out by placing 100µl into each well of a 96 well plate. The plates were then incubated at 37°C and 5% CO_2 and monitored after 2 days for cell attachment; wells that were seen to have only one cell adhered after 2 days were chosen for expansion as clonal populations. When each individual well of the 96 well plate containing a clonal population had reached 80% confluency the cells were sub-cultured in to a well of a 24 well plate (Greiner; 662160) and grown again to confluency after which time they were transferred to a 25cm^2 flask. Frozen stocks of all clonal populations were made (Section 2.2.5) as soon as possible after propagation.

2.6 Preparation for RNA Analysis

Due to the labile nature of RNA and the high abundance of RNase enzymes in the environment a number of precautionary steps were followed when analysing RNA throughout the course of these studies.

- General laboratory glassware and plasticware are often contaminated by RNases. To reduce this risk, glassware used in these studies was baked at 180°C (autoclaving at 121°C does not destroy RNase enzymes) for at least 8h. Sterile, disposable plasticware is essentially free of RNases and was therefore used for the preparation and storage of RNA without pre-treatment. Polyallomer ultracentrifuge tubes, eppendorf tubes, pipette tips, etc. were all autoclaved prior to use. All spatulas which came in contact with any of the solution components were baked, chemicals were weighed out onto baked aluminium-foil and a stock of chemicals for "RNA analysis only" was kept separate from all other laboratory reagents.

- All solutions (which could be autoclaved) that came into contact with RNA were all prepared from sterile ultra-pure water and treated with 0.1% diethyl pyrocarbonate* (DEPC) (Sigma; D5758) before autoclaving (autoclaving inactivates DEPC), with the exception of Tris-containing solutions (DEPC reacts with amines and so is inactivated by Tris). The Tris-containing solutions were made with previously DEPC-treated ultra-pure water.

- Disposable gloves were worn at all times to protect both the operator and the experiment (hands are an abundant source of RNase enzymes). This prevented the introduction of RNases and foreign RNA/DNA into the reactions. Gloves were changed frequently.

- All procedures were carried out under sterile conditions when feasible.

* DEPC is a strong, but not absolute inhibitor of RNases. It is also a suspected carcinogen.

2.7 Total RNA Isolation, Preparation and Analysis by RT-PCR

Total RNA was extracted from cultured cell lines and human tumour specimens throughout the course of these studies and analysed by the reverse transcriptase polymerase chain reaction.

2.7.1 Total RNA Extraction from Cultured Cell Lines

The following procedure was carried out in a laminar flow cabinet to maintain sterile conditions. Adherent cells were grown in 175cm^2 tissue culture flasks until approximately 80% confluent. The medium was removed and the cells in two replica flasks were rinsed twice with DEPC-treated PBS. Non-adherent cells were pelleted then resuspended and pelleted twice in DEPC-PBS. Cells prepared by either method were then lysed directly in a 4M guanidium thiocyanate (GnSCN) solution (Appendix K) (25ml per cell sample). The pooled cell lysate was centrifuged at 120g. for 5min and layered on 5.5ml of a 5.7M caesium chloride (Appendix K) cushion in a polyallomer ultracentrifuge tube. The mixture was spun at 100,000g. at 15°C for 21-24h in a swinging bucket centrifuge. This resulted in the separation of protein (at the top of the GnSCN layer) and DNA (at the GnSCN:CsCl interface) from the RNA pellet (at the bottom of the tube). Care was taken to prevent disturbing the RNA pellet or contaminating the RNA with DNA. The GnSCN solution and the "jelly-like" layer below the GnSCN:CsCl interface was removed by aspiration (using a pasteur pipette), until approximately 1ml of CsCl remained. The bottom of the tube (containing the RNA pellet and 1ml of CsCl) was cut from the rest of the tube using a heated scalpel blade. The tube bottom was inverted and the pellet rinsed with 95% ethanol at room temperature and resuspended in 200µl of DEPC-treated water by gently pipetting up and down whilst keeping on ice. The resuspended pellet was transferred to an eppendorf tube and the suspension remains were rinsed into the eppendorf with a further 200µl of DEPC-treated water. The RNA was precipitated out of solution by the addition of 3M sodium acetate, pH 5.2, (to result in a final volume of 0.3M) and 2 volumes of ice-cold absolute ethanol, overnight at -20°C. The RNA was pelleted by spinning at 4°C, at maximum speed in a microfuge. The pellet was washed with 70% ethanol, the supernatant removed and the pellet was briefly air-dried*. The pellet was resuspended in 100µl of

DEPC-treated water and stored at -80°C.

* The pellet was not allowed to dry completely as this greatly decreases its solubility. The solubility of RNA can be improved by heating to 55-60°C with intermittent vortexing or by passing through a pipette tip.

Total RNA was also extracted as described in the technical bulletin for Tri Reagent (Sigma; T-9424). The DNA and protein fractions resulting from the total RNA isolation were discarded.

2.7.2 mRNA Extraction from Cultured Cell Lines

Adherent cells were grown in 175cm^2 tissue culture flasks until approximately 80% confluent. The medium was removed and the cells in two replica flasks were trypsined and centrifuged at 120g. The pellet was washed once with 25mls of ice cold PBS and centrifuged again at 120g. The supernatant was poured off and the cell pellet stored on ice until ready to continue. The mRNA extraction procedure was carried out as described in the technical manual for the PolyAtract System 1000 from Promega. (Cat. # Z5400). The extracted mRNA was stored at -80° C.

2.7.3 RNA Quantitation

RNA was quantified spectrophotometrically at 260nm and 280nm. An optical density of 1 at 260nm is equivalent to 40mg/ml RNA. An A_{260}/A_{280} ratio of 2 is indicative of pure RNA. Partially solubilised RNA has a ratio of <1.6 (Ausubel *et al.*, 1991). The yield of RNA from most lines of cultured cells is 100-200μg/90mm plate (Sambrook *et al.*, 1989). In these studies 200μgRNA/175cm^2 flask was retrieved.

2.7.3.1 RNA Quality

RNA quality was checked by running a quantity of RNA on a 1% formaldehyde/agarose gel. For a 100ml, 1% gel, 1g of agarose was combined with 73.4ml sterile distilled water and dissolved by heating in microwave. In a fume hood 10ml of 10X MOPS buffer (0.25M MOPS (Sigma; M8899), 0.05M Na-acetate,

0.01M EDTA, pH 7.0) and 16.6ml formaldehyde (BDH; 15513) were added to the molten agarose. 2.5µl of ethidium bromide (Sigma; E8751) (10mg/ml) was added and the gel was then poured. 1.0µl formaldehyde, 5.0µl formamide (BDH; 33272), 0.5µl loading buffer (50% glycerol (Sigma; G5576), 1mg/ml xylene cyanol FF (BDH; 44306), 1mg/ml bromophenol blue (Sigma; B5525), 1mM EDTA) was added to 1.75µl of RNA, and incubated at 65°C for 15 minutes, placed on ice and then loaded onto the gel. The gel was run in 1X MOPS buffer. Intact RNA is detected as two ribosomal (28S and 18S) RNA bands.

2.7.4 Micropipette Accuracy Tests

Accuracy and precision tests were carried out routinely on all micropipettes used in all steps of the RT-PCR reactions. The accuracy and precision of the pipettes was determined by standard methods involving repeatedly pipetting specific volumes of water and weighing them on an analytical balance. The specifications for these tests were supplied by Gilson

2.7.5 Reverse Transcription of RNA isolated from cell lines

The following components were used in the reverse transcriptase (RT) reaction for RNA isolated from cell lines. 1µl oligo $(dT)_{15}$ primers (1µg/µl) (Eurogentec), 1µl of total RNA (1µg/µl), and 3µl of DEPC-H_2O were mixed together and heated at 70°C for 10min and then chilled on ice to remove any RNA secondary structure formation and allow the oligo (dT) primers to bind to the poly $(A)^+$ tail on the mRNA. 4µl of a 5X buffer (consisting of 250mM Tris-HCl, pH 8.3, 375mM KCl and 15mM $MgCl_2$), 2µl of DTT (100mM), 1µl of RNasin (40U/µl) (Promega: N2511), 1µl of dNTPs (10mM each of dATP, dCTP, dGTP and dTTP), 6µl of water and 1µl of Moloney murine leukaemia virus-reverse transcriptase (MMLV-RT) (40,000U/µl) (Promega: *) was then added to the heat-denatured RNA complex and the mixture was incubated at 37°C for 1h to allow the MMLV-RT enzyme catalyse the formation of cDNA on the mRNA template. The enzyme was then inactivated

and the RNA and cDNA strands separated by heating to 95°C for 2min. The cDNA was used immediately in the PCR reaction or stored at -20°C until required for analysis.

2.7.6 Polymerase Chain Reaction (PCR) Analysis of cDNA

The cDNA formed in the above reaction was used for subsequent analysis by PCR for the expression of specific mRNAs.

2.7.6.1 PCR Analysis of cDNA formed from mRNA isolated from cell lines

Typical PCR reactions were set up as 50µl volumes using 2 to 5 µl of cDNA formed during the RT reaction (see Section 2.7.5). cDNA was amplified for varying cycle numbers but where possible amplification was carried out in the exponential phase of amplification. The sequences of all primers used for PCR in this thesis are shown in Figure 2.7.6.1.

Each PCR reaction tube contained 5µl 10Xbuffer (100mM Tris-HCl, pH 9.0, 50mM KCl, 1% Triton X-100), 3 or 9 µl 25mM $MgCl_2$**, 1µl of first strand target primer* (250ηg/µl), 1µl of second strand target primer* (250ηg/µl), 0.5µl of first strand endogenous control primer* (250ηg/µl), 0.5µl of second strand endogenous control primer* (250ηg/µl) and water to bring the volume to 35 to 38µl (depending on the volume of cDNA used). 2 to 5µl of cDNA (pre-heated to 95°C for 3min. to separate strands and remove any secondary structure if the sample had been stored at -20°C) was added to the above and a drop of liquid paraffin (BDH; 29436) was added to each reaction tube. The mixture was heated to 94°C for 5min (reduces non-specific binding of primers to template). 1 or 2 µl of 10mM dNTP**, 0.5µl of Taq DNA Polymerase enzyme (Promega; N1862) and water to a total volume of 10µl was then added to the above The cDNA was then amplified by PCR (Techne; PHC-3) using the following program:

- 94°C for 1.5min (denature double stranded DNA);
- 30-35 cycles 94°C for 1.5min (denature double stranded DNA);
  55 or 65°C for 1min (anneal primers to cDNA);
  72°C for 3min (extension);

- 72°C for 7min (extension).

* All oligonucleotide primers used throughout the course of this thesis were made to order on an "Applied BioSystems 394 DNA/RNA Synthesiser" by Eurogentec Ltd., 4-10 The Quadrant, Barton Lane, Oxon, England. Sequences of primers used are shown in Table 3.1.1 and Appendix C

**All PCR reactions used 3µl of $MgCl_2$ and 1µl of 10mM dNTP except for the amplification of LRP using the primers giving a 300 bp product. These reactions required 9µl of 25mM $MgCl_2$ and 2 µl of 10mM dNTP.

Figure 2.7.6.1 Sequences of primers used for PCR

Gene	Primer Length	T_m	Amplified length	Sequence
mdr1	20	58	157	GTT CAA ACT TCT GCT CCT GA
	20	60		CCC ATC ATT GCA ATA GCA GG
MRP	21	58	203	
	21	62		
β-actin (large)	29	84	383	GAA ATC GTG CGT GAC ATT AAG - GAG AAG CT
	22	64		TCA GGA GGA GCA ATG ATC TTG A
β-actin (small)	23	70	142	TGG ACA TCC GCA AAG ACC TGT AC
	22	64		TCA GGA GGA GCA ATG ATC TTG A
LRP	21	68	300	CAC AGG GTT GGC CAC TGT GCA
	21	64		CCT CGA GAT CCA TTG TGC TGG
Ribozyme	19	60	118	AGC ACA GAG CCT CGC CTT T
Expression	17	54		TCT GGA TCC CTC GAA GC
Antisense	19	60	108	AGC ACA GAG CCT CGC CTT T
expression	17	54		TCT GGA TCC CTC GAA GC
Vector	19	60	96	AGC ACA GAG CCT CGC CTT T
expression	17	54		TCT GGA TCC CTC GAA GC

All reaction tubes were then kept at 4°C until analysed by gel electrophoresis.

A 10µl aliquot of tracking buffer, consisting of 0.25% bromophenol blue (Sigma; B5525) and 30% glycerol in water, was added to each tube of amplified cDNA products. 20µl of cDNA products from each tube were then separated by electrophoresis at 100mV through a 2% agarose (Promega; V3122) gel containing ethidium bromide (Sigma; E8751), using TBE (22.5mM Tris-HCl, 22.5mM boric acid (Sigma; B7901), 0.5mM EDTA) as running buffer. Molecular weight markers "ϕ-X174" Hae III digest (Promega; G1761) were run, simultaneously, as size reference.

The resulting product bands were visualised as pink bands (due to the intercalation of the cDNA with the ethidium bromide) when the gels were placed on a transilluminator (UVP Transilluminator). The gels were photographed.

2.8 Detection of RNA expression by Northern Blotting

The RNA samples to be analysed were first separated by Formaldehyde-Agarose Electrophoresis

2.8.1 Formaldehyde-Agarose gel Electrophoresis

A 100ml 1% agarose gel was prepeared by dissolving 1g of agarose in 73.4 ml of sterile distilled water (SDW). The gel was then cooled to around 60°C and 10ml of 10X MOPS buffer (0.25M MOPS, 0.05M Na acetate, 0.01 EDTA, pH 7.0) was added along with 16.6 ml formaldehyde and mixed well before pouring. The running buffer for the gel was 1X MOPS containing 12.9 ml formaldehyde/300ml. 1 or 2µg of mRNA was freeze dried overnight in a 200µl eppendorf and dissolved in 5µl of SDW, to allow equal sample sizes. The RNA samples were mixed with RNA loading buffer (2.9µl 10X MOPS, 5µl formaldehyde, 14.3 µl formamide, 1.43µl tracking buffer (Section 2.7.4.1)) and heated to 65°C for 15min, placed on ice and loaded onto the gel. The RNA samples were run on the gels at 75mV for 2 hours alongside RNA size markers (Promega). The gels were washed in 3 changes of SDW over 30 minutes.

2.8.2 Northern Blotting

A sheet of Hybond-N (Amersham) was cut to the same size as the RNA gel. A tray or glass dish was half filled with the transfer buffer (20X SSC (8.823 % (w/v) tri-sodium citrate, 17.532 % (w/v) NaCl, pH 7-8)). A platform was made to stand in the tray above the level of the transfer buffer and a wick (3MM filter paper) was placed over the platform into the transfer buffer. The RNA gel was placed loading side down on the wick platform without trapping air bubbles. The Hybond-N was placed on top of the RNA gel and three sheets of 3MM filter paper placed upon the Hybond-N. A stack of absorbent tissue paper over 5cm high was placed on top of the filter paper and was covered with a glass plate. Finally a glass plate with a 750g weight were placed on top of the paper stack and the transfer was carries out overnight. After blotting, the transfer apparatus was dismantled and the gel loading tracks were marked on the Hybond-N to allow land identification. The nucleic acid was fixed to the membrane by baking at 80°C for 2 hours and stored until use between two sheets of dry filter paper.

After blotting, the gel was rehydrated in a 1 μg/ml EtBr solution. The gel was than viewed under a U.V. lamp. The efficiency of RNA transfer to the membrane could then be assessed by looking for remaining traces of 28 and 16S ribosomal bands. The lane on the gel conatining the RNA markers was removed from the gel before blotting and stained with EtBr alongside the blotted gel. The position of the RNA markers were photographed and used as a reference to size bands on the developed Northern Blots.

2.8.3 Slot-Blotting of RNA

Slot-Blots of mRNA samples was carried out using the Bio-Rad Bio-Dot SF cell slot blot. The protocol is as outlined in the accompanying manual. Briefly 1 μg of Poly A+ RNA was diluting to 500 μL in the denaturing blotting solution (0.5M NaOH). A piece of Hybond-N nylon membrane was cut to the same size as the blotter. Three pieces of thick filter paper were also cut to the same size as the blotter. The filter paper and the membrane were pre-wetted in 20X SSC and put into place on the blotter and the vacuum attached. The wells not required were covered with masking tape to allow even suction on the wells. The wells were washed with the denaturing blotting solution using the vacuum. The diluted samples were added to the

wells and transferred onto the membrane under vacuum. The wells were washed through under vacuum with a further 500 µL of the denaturing blotting solution. The membrane was then baked at 80°C for 2 hours and stored dry until required.

2.8.4 Preparation of Hybridisation probe

The LRP probe was prepared from a LRP cDNA plasmid (LHN42) kindly donated by Rik Scheper. A 1 kb fragment of the LRP cDNA was restricted from the plasmid using two restriction enzymes. The fragment was electrophoresed on a 1% low-melting point agarose gel containaing ethidium bromide at 75mV for 1 to 2 hours along with the molecular weight size markers IX and III (Boehringer Mannheim: 558 552 and 1 449 460) to check for the correct fragment size. The gel was viewed under a U.V. illuminator and the fragement band cut out of the gel with a scalpel. The 1 kb fragment was then extracted from the agaraose using the Qiaex II Agarose Gel Extraction kit (Qiagen: 20021) according to the given protocol. Similarly a 1.3kb MRP and a 1.4kB mdr-1 fragment was restricted and purified from a MRP cDNA and a mdr-1 cDNA containing plasmid respectively.

2.8.5 Radioactive Labelling of Probes

All DNA probes were labelled with [α-^{32}P]dCTP (Amersham) using the Prime-a-gene labelling kit (Promega : U1100) according to the supplied protocol. Riboprobes (RNA) were labelled with [α-^{32}P]CTP (Amersham) using the Riboprobe *In Vitro* Transcription Systems kit (Promega: P1440) according to the supplied protocol. The T7 promoter and RNA polymerase were used in this labelling reaction. 20 to 40 ng of cDNA fragment and 5µl of [α-^{32}P]dCTP or [α-^{32}P]CTP was used to make each probe.

To test the percentage incorporation of nucleotides into the DNA probes the following protocol was carried out. 1 µl out of the 50µl reaction mix was diluted 1 in100 with water. 1µl of the diluted probe was then blotted onto four 1cm^2 pieces of filter paper and air dried. Two of these pieces of filter paper were washed twice for 10 minutes in 10% Tri-chloro Acetic Acid (Riedel-del Haen: UN-No-1839), rinsed in 100% ethanol and air dried. Then the counts on the two washed and unwashed pieces of filter were measured using a scintillation counter. The filter paper was placed in scintillation counter tubes with 10ml of scintillation

fluid (Ecolite : *) and the Counts per minute (CPM) read. The CPM of the washed pieces of filter paper as a percentage of the unwashed pieces of filter paper gave the percentage incorporation of oligonculeotides into the probe. Probes with less than 50% incorporation were purified using NAP10 columns (Amersham ?) according to the manufacturuers protocol.

2.8.6 Hybridisation of labelled probes to RNA membranes

The baked Hybond-N membranes with the mRNA samples were prehybridised overnight at 65°C in 10ml of hybridisation buffer (In 100ml : 43ml 1 M Sodium phosphate pH 7.2, 33 ml 20% Sodium Dodecyl Sulphate (SDS), 20 ml 5% BSA, 4ml 0.5 M EDTA) per membrane. The hybridisation was carried out in glass hybridisation tubes in a hybridisation oven. The appropriate probe was heated to 94°C for 3 min before addition to 10ml of preheated (65°C) hybridisation buffer. Sufficient probe was used to give $1x10^6$ CPM/ml hybridisation buffer. The pre-hybridisation buffer was discarded from the hybridisation tubes and replaced with the fresh hybridisation buffer with the probe. Hybridisation was carried out at 65°C overnight. The membranes were then washed at 65°C for 5 min in 2X SSC, followed by 2 x 15 min washes in 0.5X SSC, 0.1% SDS and 2 x 15 min washes in 0.1X SSC, 0.1% SDS. The membranes were wrapped in cling film and exposed to X-ray film at -80°C for the desired length of time (typically 24h to 5 days).

2.9 Protein Analysis

Protein analysis was carried out by Western blotting using whole cell extracts and immunocytochemistry using cytospins of whole cells.

2.9.1 Whole Cell Extract Preparation

Cells were grown in 175cm^2 flasks until they reached 80-90% confluency. The cells were then trypsinised and centrifuged at 120g. for 5 min. The pellet was washed in PBS and re-pelleted twice. 1ml of lysis buffer (PBS, 1% NP-40 (Sigma; N-3516), 1X protease inhibitors and 0.2mg/ml PMSF(Sigma; P7626)) was added to the pellet and left on ice for

20 min. A 100X stock solution of protease inhibitors consisted of 400mM DTT (Sigma; D5545), 1mg/ml aprotonin (Sigma; A1153), 1mg/ml leupeptin (Sigma; L2884), 1mg/ml soybean trypsin inhibitor (Sigma; T9003), 1mg/ml pepstatin A (Sigma; P6425) and 1mg/ml benzamidine (Sigma; B6506). If cell lysis had not occurred after 20 min the cells were subjected to sonication. Whole cell extracts were aliquoted and stored at -80°C.

2.9.2 Quantification of Protein

Protein levels were determined using the Bio-Rad protein assay kit (Bio-Rad; 500-0006) with a series of bovine serum albumin (BSA) (Sigma; A9543) as standards. The dye reagent was provided as 5-fold concentrate. The appropriate standards (0.02ml) and test samples (0.02ml) were placed in clean, dry test tubes. The diluted dye reagent (1ml) was added and the mixture vortexed. After a period of 5 min to 1h, the OD_{595} was measured, against a reagent blank. From the plot of the OD_{595} of BSA standards versus their concentrations, the concentration of protein in the test samples was determined.

2.9.3 Gel electrophoresis

The protein present in the cell preparations were separated on a size basis using SDS polyacrylamide gel electrophoresis (SDS-PAGE) :-

Table 2.13.4.1 SDS-PAGE Recipes for 2 x 0.75mm Thick Gels

	Resolving Gel			Stacking Gel
	7.5%	12%	15%	5%
Acrylamide Stock*	3.8ml	5.25ml	7.5ml	0.8ml
Distilled H_2O	8.0ml	6.45ml	4.3ml	3.6ml
1.875M Tris, pH 8.8	3.0ml	3.0ml	3.0ml	.....
1.25M Tris, pH 6.8	.....	.....	.....	0.5ml
10% SDS	150µl	150µl	150µl	50µl
10% NH_4-persulphate	60µl	50µl	50µl	17µl
TEMED	9.0µl	10µl	7.5µl	8µl

* Acrylamide stock solution consists of 29.1g acrylamide (Sigma; A8887) and 0.9g NN'-methylene bis-acrylamide (Sigma; 7256) dissolved in 60ml UHP water and made up to 100ml final volume. The solution was stored in the dark at 4°C for up to 1 month. All components were purchased from Sigma; SDS (L4509), NH_4-persulphate (A1433) and TEMED, N,N,N',N'-tetramethylethylenediamine (T8133).

The resolving gel was immediately poured into two clean 10cm x 8cm gel cassettes comprising of a glass and aluminium plate separated by two 0.75cm spacers on either outer edge. The gel was overlayed with a 10% SDS solution and allowed to set. Once set, the SDS solution was poured off and the stacking gel layered on top of the resolving gel. A comb of appropriate thickness and well size was immediately inserted and the gel allowed to set. When the wells had formed, the gel combs were removed and the gels transferred to a mini-electrophoresis apparatus. The gels were flooded with running buffer (1.9M glycine (Sigma; G6761), 0.25M Tris, 0.1% SDS, pH 8.3 without adjustment).
Protein samples were loaded into the wells, based on equal protein loading. The samples to be loaded were diluted 1:1 with loading buffer (50mM Tris-HCl, pH 6.8; 0.1% SDS; 5% 2-mercaptoethanol (Sigma; M6350); 5% glycerol and 0.1% bromophenol blue) and boiled for 2 min. They were then loaded onto the gel, as were the appropriate molecular weight markers (New England Biolabs; 77085). The gels were run for approximately 1.5 hours with voltage set at 250V and current set at 45mA.

2.9.4 Western Blotting Procedure

Following electrophoresis, the acrylamide gels were equilibrated in transfer buffer (25mM Tris; 192mM glycine; pH 8.3 - 8.5 without adjustment). Nitrocellulose filter (Amersham; Hybond-ECL RPN2020D), which was cut to the same size as the gel, was soaked in transfer buffer for 5 min. If PVDF membrane (Boehringer Mannheim; 1 722 026) was used, the membrane, cut to the same size as the gel, was immersed in methanol for a few seconds, rinsed with water and then soaked in transfer buffer for 5 min. Six stacked sheets of gel-size Whatman 3mm filter paper were soaked in transfer buffer and placed on the cathode plate of a semi-dry blotting apparatus (BioRad). Excess air was removed from between the filters by sliding a pipette over and back on the filter paper. The nitrocellulose was placed over the filter paper, again ensuring no air bubbles became trapped. The acrylamide gel was placed on the nitrocellulose and the nitrocellulose was marked at the sites of the gel lanes and size markers. Six more sheets of pre-soaked filter paper was placed on top of the gel. The protein was transferred from the gel to the nitrocellulose at a current of 0.34mA/0.15V for 30 min. The nitrocellulose was then blocked in blocking buffer and exposed to specific antibodies. Negative blots were also performed whereby the primary antibody was replaced with antibody diluent.

2.9.4.1 P-glycoprotein

Total protein was separated on a 7.5% SDS polyacrylamide gel. Following transfer of the protein to the nitrocellulose, the nitrocellulose was blocked for 2 hr at room temperature in TBS (500mM NaCl; 20mM Tris, pH 7.5) containing 0.5% non-fat dried milk (Cadbury; Marvel skimmed milk). The nitrocellulose was rinsed twice with TBS and was exposed to the primary antibody (1 in 200 dilution of mouse mdr-1 ascites MAb(BRI)) at 4°C overnight. The nitrocellulose was washed three times in TBS containing 0.5% Tween-20 (Sigma; P1379). The nitrocellulose was exposed to the secondary antibody (horse radish peroxidase-conjugated (HRP)) (1:2000 dilution of goat anti-mouse-HRP (Dako; P0447) in TBS, 0.1% Tween-20) for 1.5 hours at room temperature. The nitrocellulose was again washed three times in TBS and was developed as outlined in Section 2.9.5.1.

2.9.4.2 MRP

The procedure was identical to that for P-Glycoprotein excpet for the primary and secondary antibodies used. The pimary antibody was 1/150 dilution of anti-human MRP Rat Mab (TCS; ZUMC-201). The secondary antibody was a 1/10,000 dilution of rabbit anti-rat-HRP immunoglobulin (Dako; P450). The membranes were washed 5 times in TBS (0.5% Tween). The membranes were developed using Pierce Super-Signal Ultra Chemiluminescence substarte as outlined in Section 2.8.5.2

2.9.5 Development of Western Blots

2.9.5.1 Development of Western Blots with ECL

Western blots were developed by a chemiluminescence method. An equal volume of ECL solutions 1 and 2 (Amersham; RPN2209) was mixed together (3ml/blot). The nitrocellulose was placed, protein side up, on a piece of flat cling-film and covered with the detection reagent for 1 min at room temperature. Excess detection reagent was poured off and the membranes were wrapped in cling-film, ensuring no air-pockets were created. A sheet of autoradiography film was placed on top of the membranes and exposure time

varied. Film was developed and fixed using standard methods.

2.9.5.2 Development of Western Blots with Super-Signal Ultra

An equal volume of Super-Signal Ultra Chemiluminescence substrate solutions 1 and 2 (Pierce; 34075) were mixed together (3ml/blot). The nitrocellulose was placed, protein side up, on a piece of flat cling-film and covered with the detection reagent for 5 min at room temperature. Excess detection reagent was poured off and the membranes were wrapped in cling-film, ensuring no air-pockets were created. A sheet of autoradiography film was placed on top of the membranes and exposure time varied. Film was developed and fixed using standard methods.

2.9.6 Cellular Labelling and Immunoprecipitation of LRP

Adherent cells were grown to 80% confluency in 175 cm^2 flasks. The medium was removed, the cells were trypsinised and centrifuged at 120g for 5 min. The supernatant was removed and the cell pellet was washed three times with PBS. The cell pellets were then stored at -80° C until required. The extraction and immunoprecipitation of the LRP protein from the cell pellets was carried out as detailed in the methods manual for the "Cellular Labelling and Immunoprecipitataion kit" (Boehringer Mannheim; 1 647 652). The antibody used for the immunoprecipitation was the LRP-56 monoclonal Antibody (TCS; ZIM 1001 and a gift from Rik Scheper). The samples containing the immunoprecipitated LRP protein were separated on a 7.5% SDS polyacrylamide gel by electrophoresis. After Western Blotting, the nitrocellulose or PVDF membrane was blocked for 2 h in 5% non-fat dried milk (Cadbury; Marvel skimmed milk). The membrane was rinsed twice with TBS (0.5% Tween). The membrane was then incubated with anti-biotin Antibody (HRP labelled; 1/3000 dilution) for 1 hr at room temperature. The membrane was rinsed again in TBS (0.5% Tween) for 10 min. The membranes were developed with ECL reagent as described in section 2.9.5.1.

2.9.7 Immunocytochemical analysis of protein expression

P-170 and LRP were detected on cytospins of the cells being tested using the ABC method

2.9.7.1 Preparation of cytospins.

Cells to be tested were trypsinised (Section 2.2.2) to form a single cell suspension, and washed three-times with Phosphate Buffered Saline (PBS) and resuspended at a concentration not exceeding $1x10^6$ cells/ml. Cells were spun onto Poly-L-lysine coated slides and left to dry overnight at room temperature. Slides were then wrapped in tinfoil and stored at -20^0 C until required. Before use, slides were brought to room temperature for at least 15-20 min. For the detection of LRP and P-170 cells were fixed for 10 and 1 minute respectively in ice-cold acetone. All slides were then air-dried for at least 15 min prior to immunostaining. Endogenous peroxidase activity was blocked by placing slides in 0.6% (v/v) H_2O_2 in methanol for 5 min at room temperature. Slides were then washed for 5 min with a washing buffer (1xTris Buffered Saline with 0.1% Tween). All cells were blocked with 20% normal rabbit serum (Dako; E0354) at room temperature. Primary antibodies were appropriately diluted (LRP-56 : 1/20, mdr MAb : 1/40) in washing buffer and applied overnight at 4^0 C. Biotinylated rabbit anti-mouse IgG secondary antibody (Dako), diluted in washing buffer (1/300), was applied for 30 min at room temperature. Finally, the StrepABC-complex/HRP was applied for 25min at room temperature. Slides were washed between each incubation in three changes of washing buffer within 15 min. The horseradish peroxidase substrate, DAB (3,3-diaminobenzidine tetrahydrochloride) containing 0.02% H_2O_2 was applied for 10 min at room temperature. Cells were then lightly counterstained with Cole's haematoxylin for 50 seconds, differentiated in 1% acid alcohol and blued in Scott's tap water. Negative controls on each slide were prepared using with either washing buffer alone or diluted control mouse ascites for LRP and P-170 respectively.

The stained cytospins were viewed under a microscope and the level of intensity gauged on a scale of 0 to 3. Photographs were also taken.

2.10 Transfection of Mammalian Cells with Exogenous DNA

Throughout the course of this thesis it was found necessary to introduce foreign DNA into host cells either to increase the level of expression of a particular gene (by transfecting with an expression plasmid) or decrease the level of expression of a particular gene (by transfecting with a plasmid containing a ribozyme or a antisense sequence or a free antisense oligonucleotide).

Sufficient plasmid was produced by transforming JM109 with the plasmid required, growing up a large stock of these cells and isolating the plasmid from them; this isolated plasmid was then transfected into the chosen cell line.

All the Phosphorothioate Antisense oligonucleotides used were made to order on an "Applied BioSystems 394 DNA/RNA Synthesiser" by Eurogentec Ltd., 4-10 The Quadrant, Barton Lane, Oxon, England. Sequences of antisense molecules used are shown in Table 3.4.1.

2.10.1 Plasmids and oligonucleotides used

The LRP ribozyme and the LRP antisense constructs were cloned into the pHβ expression plasmid and were a generous gift from Dr. Kevin Scanlon. (The constructs are shown in Section 3.1). All antisense molecules used were phosphorothioate oligonucleotides and were manufactured by Eurogentec as for RT-PCR primers.

2.10.2 $MgCl_2$ / $CaCl_2$ Transformation of JM109 Cells

10ml of LB broth (Appendix K) was inoculated with a single colony of JM109 bacteria from an agar plate and incubated overnight at 37°C at 200r.p.m. The following day 500µl of this suspension was inoculated into 50ml of LB broth and grown to an OD_{600nm} of 0.3. The cells were then pelleted at 3000r.p.m. for 10min, the supernatant removed and the pellet was resuspended in 10ml of 100mM $MgCl_2$, on ice for 15min. The cells were again precipitated at 3000r.p.m. for 10min and the pellet was resuspended in 10ml of 100mM $CaCl_2$ on ice for a further 15min. The precipitation step was then repeated and the pellet was resuspended in 1-2ml of 100mM $CaCl_2$ and left on ice for at least 15min. The cells

were now competent and ready for transformation with the foreign DNA required.
100µl of the competent cell suspension was mixed with 20ng DNA and placed on ice for 40min after which the mixture was heat-shocked at 42°C for 90sec and then placed on ice for 3min. 1ml of LB broth was added to the competent cell suspension and incubated at 37°C for 40min. 400µl of this suspension was spread on a selecting agar plate (Ampicillin/AMP (Boehringer Mannheim; 835 269)) and incubated overnight at 37°C. Single colonies which grew on these selecting plates were further colonised on another selecting plate and allowed to grow overnight.

2.10.2.1 Isolation of Plasmid from JM109 cells

A single colony (from 2.14.2) was inoculated into 10ml of LB AMP 50µg/ml and grown overnight; 2ml of this suspension was added to 200ml of TB AMP 50µg/ml and left to grow overnight at 37°C for large scale isolation of plasmid from JM109 cells. The following day the cells were pelleted and pZ523 spin columns (5 Prime → 3 Prime Inc.; 5-523523) were used to isolate the plasmid according to the manufacturer's instructions. This procedure involved lysing the pellet in 20ml of an ice-cold solution containing 50mM glucose, 25mM Tris-Cl, 10mM EDTA, pH8.0 and 5mg/ml lysozyme (Sigma; L6876) at room temperature for 10-15min. 40ml of a 0.2N NaOH and 1.0% SDS solution was gently mixed with the lysate until the suspension became clear and incubated on ice for 10min. 30ml of 3M K-Acetate, pH5.2 was added to the above and mixed gently until a flocculent precipitate appeared at which stage the mixture was stored on ice for at least 10min. The sample was centrifuged at 35,000g. for 1h at 4°C after which the supernatant was recovered and added to 0.6 volume of 100% Isopropanol, mixed gently and left at room temperature for 20-30min. The suspension was then centrifuged at 35,000g. for 30min at 20°C after which the supernatant was discarded and the pellet washed in ice-cold 70% ethanol and resuspended in 5ml of TE, pH8.0. To remove any contaminating RNA the plasmid solution was treated with RNase Plus (5 Prime → 3 Prime Inc.; 5-461036) (to a final dilution of 1:250) for 30min at 37°C followed by phenol:chloroform:isoamyl alcohol extractions. 10M ammonium acetate was added to the aqueous phase to a final concentration of 2.0M and 0.6 volume of 100% Isopropanol was added to the sample, mixed and stored at room temperature for 20-30min. The sample was centrifuged at maximum speed in an epifuge and the DNA pellet was washed in 70% ethanol and

resuspended in 3.6ml of 10mM Tris-Cl, 1mM EDTA, and 1.0M NaCl, pH8.0. 1.8ml of this sample was loaded into one of two pZ523 columns (following the manufacturer's instructions) and the column effluent was precipitated with 0.6 volume 100% Isopropanol, as described previously. The DNA was pelleted at maximum speed in an epifuge, washed in 70% ethanol and resuspended in TE. The DNA concentration was determined by measuring the OD_{260nm}.

2.10.3 $CaPO_4$ Transfection of Mammalian Cells

On the day prior to transfection the cells to be transfected with plasmid DNA were plated from a single cell suspension (Section 2.2.2) and seeded into a $75cm^2$ flask at $5x10^5$ cells per flask. The plasmid DNA was diluted to 1μg/μl in TE and 10μg DNA was stored overnight in 410μl H_2O at 4°C.

On the day of the transfection the diluted DNA was incubated at 37°C for 1h 60μl 2M $CaCl_2$ was added dropwise to the DNA with continual mixing. Immediately the DNA-$CaCl_2$ mixture was added dropwise into the 2XHBS (Appendix K) solution with continual mixing and left at room temperature for 30min to form a DNA-$CaPO_4$ mixture. The DNA-$CaPO_4$ mixture was added to the flask of cells (containing media) dropwise, swirling constantly to ensure even mixing. The cells were then incubated for 4h at 37°C after which time the cells were "shocked" with glycerol to aid the entry of the DNA into the cells. Glycerol-shocking was done by removing the media from the cells and adding 5ml of 10% glycerol in 1XHBS to the cells for 3min. The glycerol was then removed, the cells rinsed twice in 5ml serum-free media and then re-fed with 10ml fresh growth media and incubated for 2-3 days at 37°C.

2.10.3.1 Transfection of cells with Lipofection reagents

On the day prior to transfections, the cells to be transfected were plated from a single cell suspension (Section 2.2.2) and seeded into $25cm^2$ flasks at $1x10^5$ cells per flask. On the day of the transfection, the plasmid or oligonucleotides to be transfected were prepared along with the lipid transfection reagents according to the manufacturers protocols (DOTAP - Boehringer Mannheim; 1 202 375, Lipofectin - GibcoBRL ; 18292-011, Fugene6 - Boehringer Mannheim ; 1 814 443). The cells were either transfected for four hours in

the absence of serum after which the media was replaced with serum containing media, or for 24h to 48 h in the presence of 10% FCS. For all transfections the cells were incubated at 37°C.

2.10.3.2 Selection and Isolation of Colonies

In order to study the true effect of transfection studies, single colonies of stably transfected cells were selected and isolated. The selection process was carried out by feeding the "transfected" cells with media containing geneticin (Sigma; G9516) - the plasmids used had a geneticin-resistant gene, therefore, only those cells containing the plasmid will survive treatment with geneticin. 2 days after transfection the flask of cells was fed with 200μg/ml geneticin in complete media, when the cells grew readily in this concentration of selecting agent, the concentration was increased step-wise to a final concentration of 600μg/ml. At this stage the cells were plated at clonal density (see Section 2.6) and clonal populations were propagated, as described previously. Transfected cells were periodically challenged with geneticin to establish stability of transfectants.

3. RESULTS

3.1 Analysis of OAW42SR and OAW42s clones transfected with LRP-Ribozyme, LRP-Antisense or control plasmid pHβ.

OAW42SR and OAW42S cells were transfected with either the pHβ plasmid containing the anti-LRP ribozyme or the anti-LRP antisense constructs or the vector alone. The pHβ plasmid construct is shown in Figure 3.1.1. The anti-LRP ribozyme and antisense constructs are shown in Figure 3.1.2. The cells were transfected and selected as described previously (section 2.10.3.1). After selection with a concentration of 400 μg/ml of geneticin, cell stocks were frozen in liquid nitrogen. All clones were subsequently screened for decreased expression of LRP mRNA by RT-PCR and LRP protein by immunocytochemistry (Sections 2.7.5 and 2.9.7 respectively). Cytotoxicity assays with Adriamycin and Vinblastine or Vincristine were also carried out (Section 2.3). The clones, which were initially isolated, are shown in Table 3.1.

3.1.1 LRP RT-PCR Analysis of Transfected clones

To determine if any of the selected clones displayed a reduction in LRP mRNA levels as compared to the parental or control clones, RT-PCR was carried out on total RNA extracted from each clone. The primers used amplified a 300 bp fragment of LRP corresponding to the 5' end of the gene, and were designed as described previously (Section 2.7.6.1). Specific primers were used to produce a 142 bp band from β-actin, which was used as an internal control. The RT-PCR reactions were carried out at least twice. The molecular weight marker (MWM) used for all PCRs in this thesis is "ϕ–X174" Hae III digest (Promega: G1761). The results shown in Figure 3.1.1.1 for the OAW42SR clones, and in Figures 3.1.1.2 and 3.1.1.3 for the OAW42S clones, are representative of all the repeats. The results in Figures 3.1.1.2 and 3.1.1.3 were from the same PCR and so band intensities can be compared

From Figure 3.1.1.1 it can be seen that there were no observed decreases in LRP expression in any of the transfected OAW42SR clones. Figure 3.1.1.2 shows that there were LRP bands visible for all the OAW42S LRP-Ribozyme clones, except clone number 7. However, the β-actin control band was significantly reduced for this

Figure 3.1.1 The pHβApr-1-neo (pHβ) plasmid vector

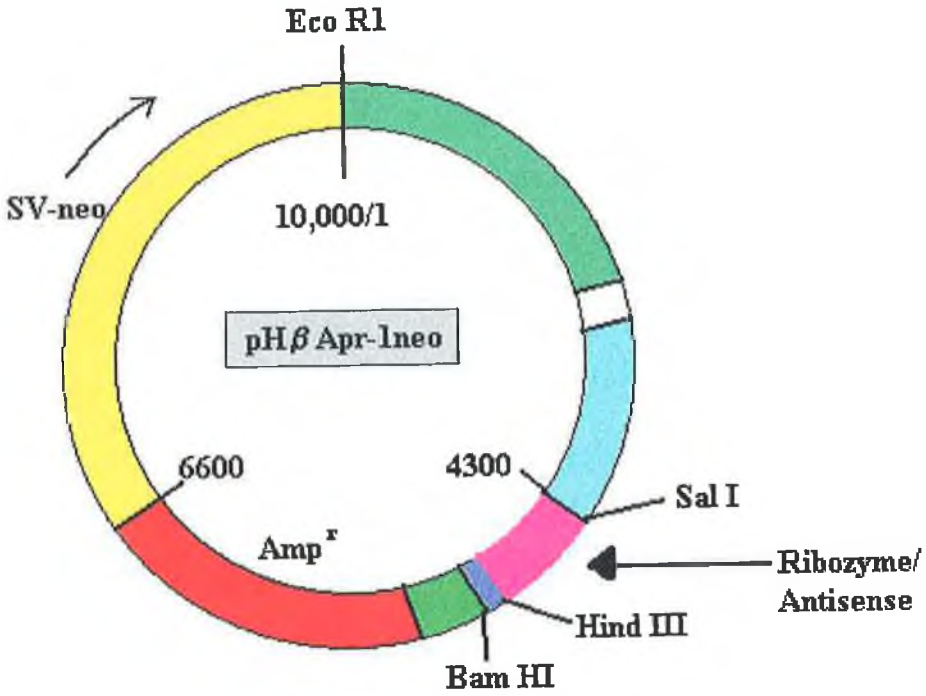


Figure 3.1.2 Diagram of anti-LRP Ribozyme and Antisense RNA

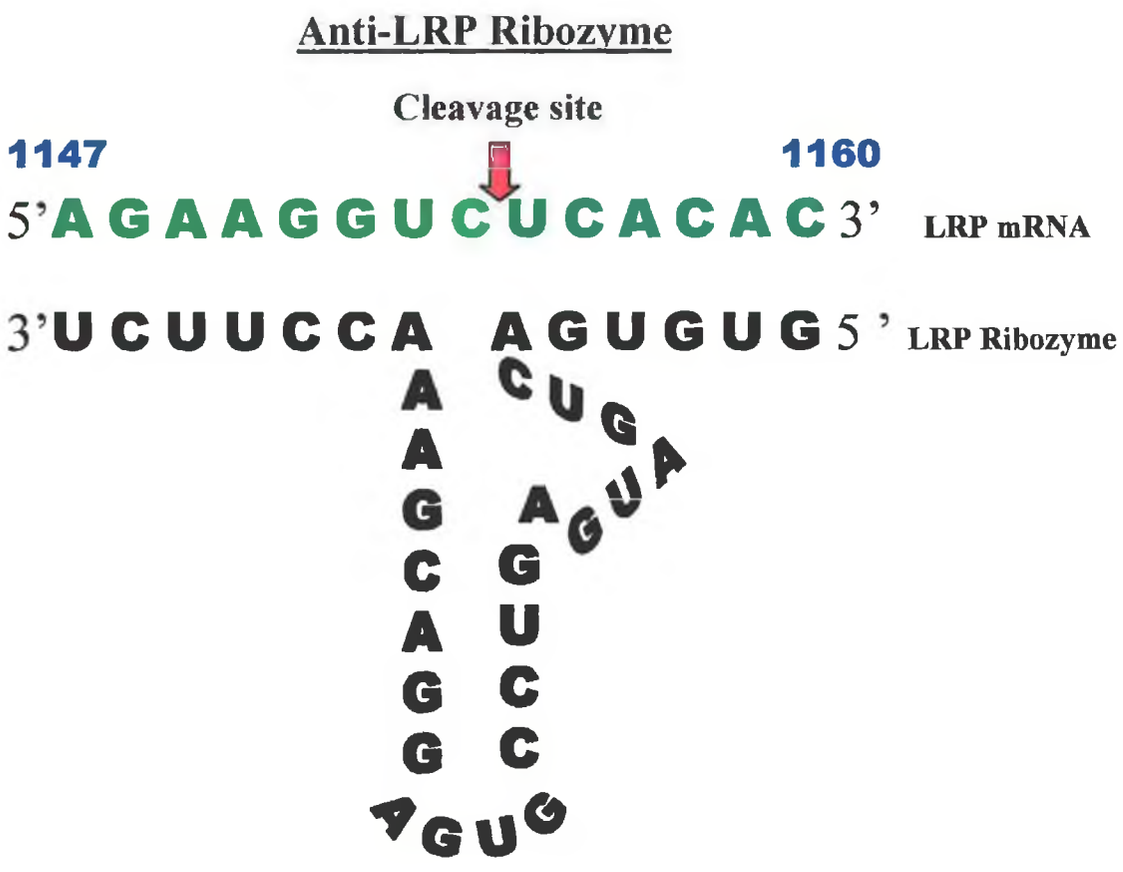


Table 3.1 Clones selected from transfection with LRP-Ribozyme, Antisense containing or control plasmid.

Plasmid Transfected	OAW42SR parent	OAW42S parent
pHβ-LRP-Ribozyme	SR-LRP-Rz 1	42S-LRP-Rz 2
	SR-LRP-Rz 2	42S-LRP-Rz 3
	SR-LRP-Rz 4	42S-LRP-Rz 4
		42S-LRP-Rz 7
		42S-LRP-Rz 8
		42S-LRP-Rz 10
		42S-LRP-Rz 15
pHβ-LRP-Antisense	SR-LRP-AS 1	42S-LRP-AS1
	SR-LRP-AS 4	42S-LRP-AS 4
	SR-LRP-AS 6	42S-LRP-AS 5
	SR-LRP-AS 7	42S-LRP-AS 9
	SR-LRP-AS 8	42S-LRP-AS 10
		42S-LRP-AS 13
		42S-LRP-AS 14
pHβ control	SR- pHβ 1	42S-pHβ 1
	SR- pHβ 3	42S-pHβ 2
	SR- pHβ 6	42S-pHβ 3
	SR- pHβ 7	42S-pHβ 4
		42S-pHβ 5

β-actin
LRP
MWM
OAW42S
42S-LRP-Rz 2
42S-LRP-Rz 3
42S-LRP-Rz 4
42S-LRP-Rz 7
42S-LRP-Rz 8
42S-LRP-Rz 10
42S-LRP-Rz 15
2R120
MWM

Figure 3.1.1.1 RT-PCR Analysis of LRP expression in OAW42SR clones

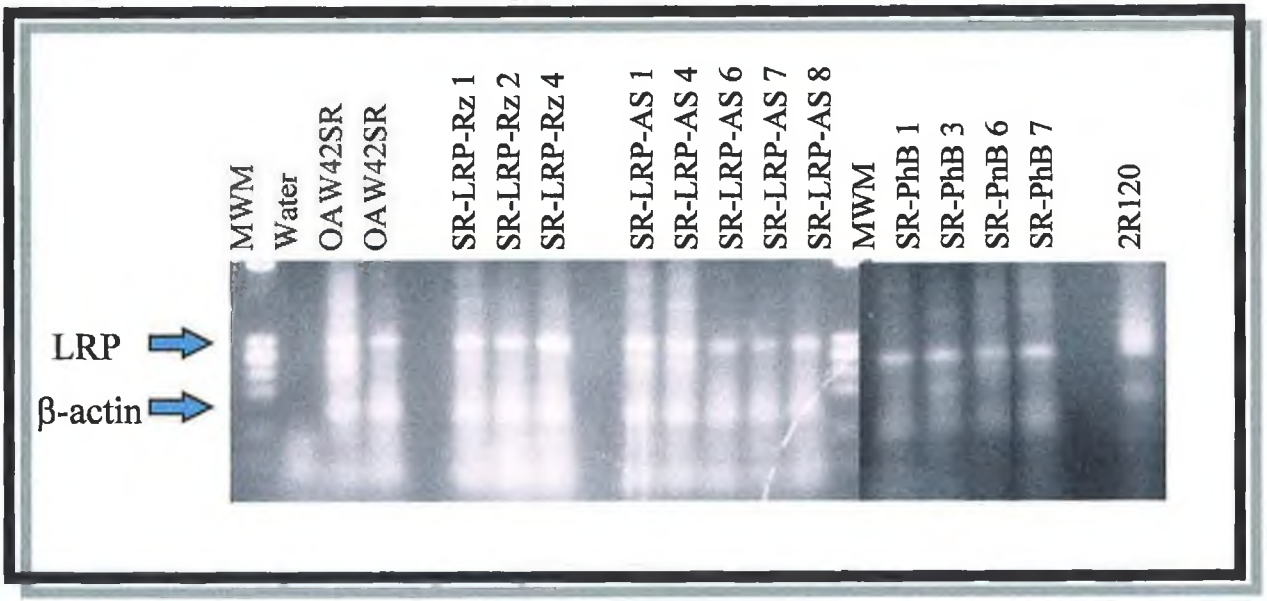
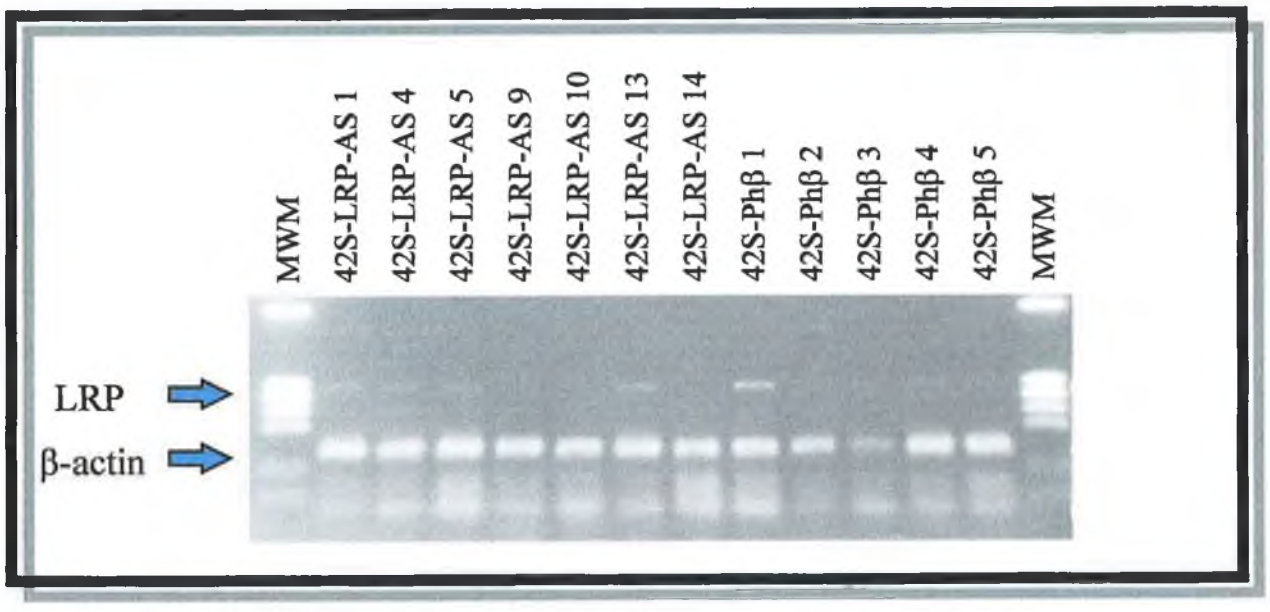


Figure 3.1.1.2 Rt-PCR Analysis of LRP expression in OAW42S LRP Ribozyme clones

Figure 3.1.1.3 RT-PCR Analysis of LRP expression in OAW42S LRP Antisense and Phβ clones



clone as compared to the rest. There appeared to be reduced expression in the LRP antisense transfected clones 42S-LRP-AS 10 and 14 (Figure 3.1.1.3). However, in other RT-PCRs, the decrease was not as dramatic (results not shown). Control plasmid transfectant 42S-pHβ 1 also appeared to have a decrease in LRP expression as compared to the parental cell line OAW42S.

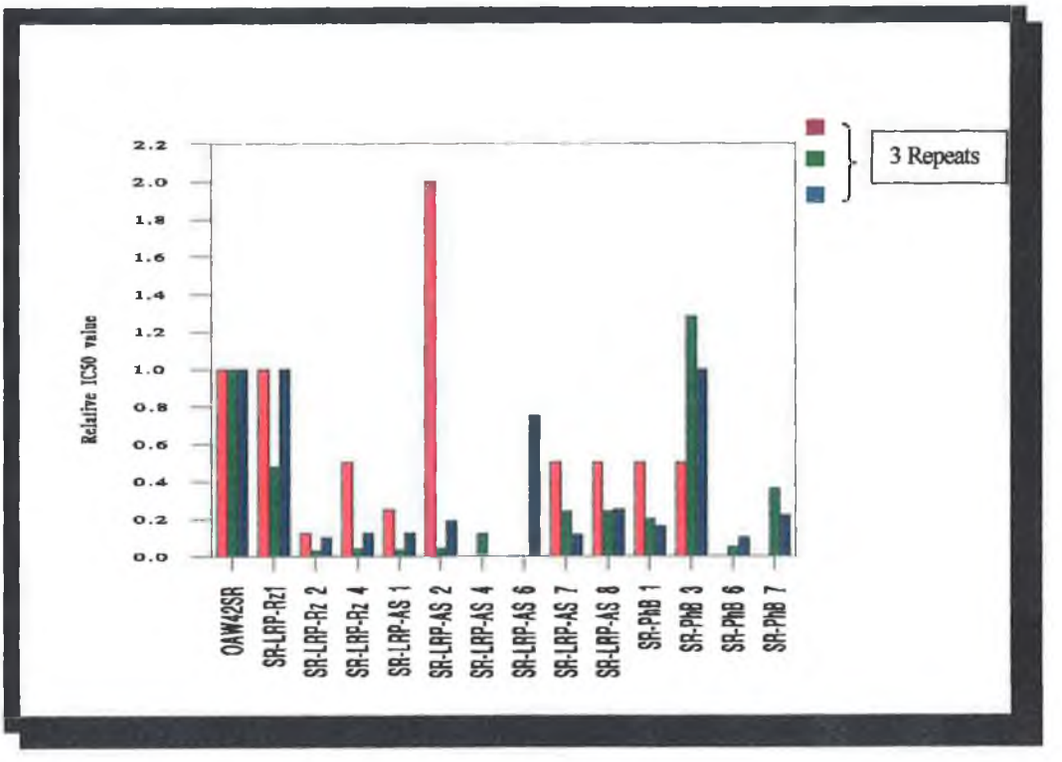
3.1.2 Analysis of Drug Toxicity Profiles of Transfected OAW42SR and OAW42S clones

Each of the selected clones was treated with a range of concentrations of Adriamycin and either Vinblastine (OAW42SR's) or Vincristine (OAW42S's) as described earlier (Section 2.3). Each toxicity assays was performed three times. The results for the OAW42SR clones for Adriamycin and Vinblastine are shown in Graphs 3.1.2.1 and 3.1.2.2 respectively. For OAW42SR clones, the IC50 values are only relative values, due to experimental variations, with the IC50 of the parental OAW42SRs given a value of 1. Graphs 3.1.2.3 and 3.1.2.4 show the IC50 values of Adriamycin and Vincristine respectively for the OAW42S clones. Averages of the IC50 values over three repeats were made and are shown in Graphs 3.1.2.5 and 3.1.2.6 for OAW42SR clones, and Graphs 3.1.2.7 and 3.1.2.8. for OAW42S clones.

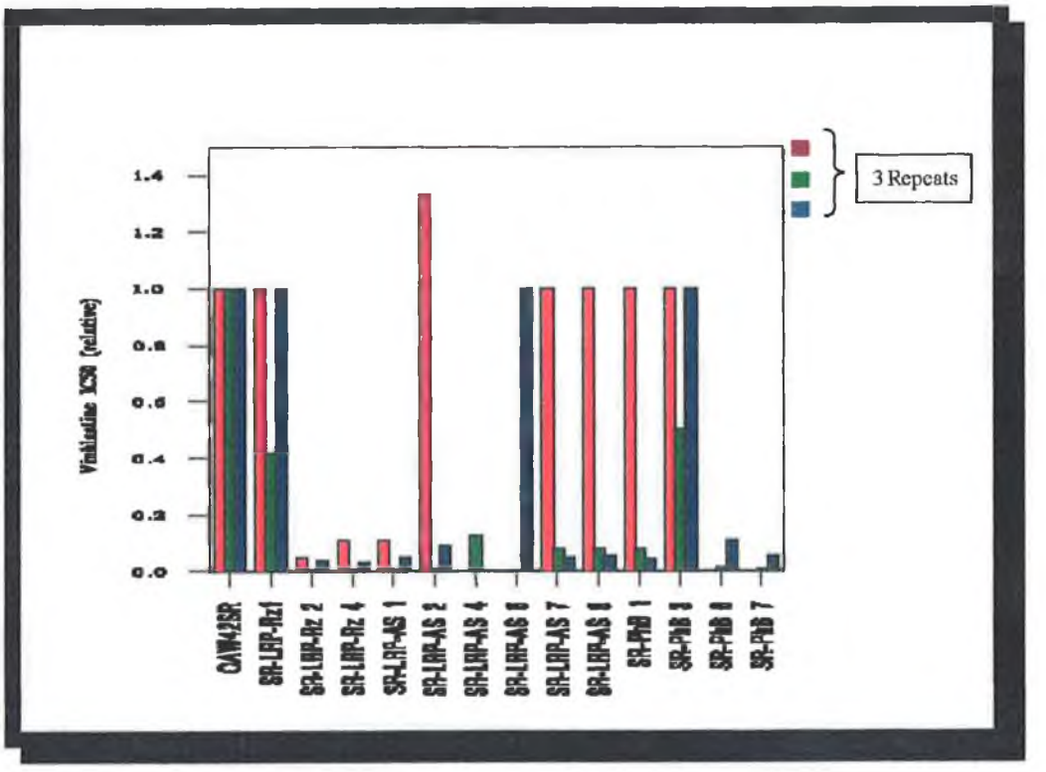
Graphs 3.1.2.1 and 3.1.2.5 illustrate clearly that there were four OAW42SR clones (SR-LRP-Rz 2 and 4, SR-LRP-AS 1 and 4) that exhibited a marked reduction in resistance to Adriamycin as compared to the parental OAW42SR cell line. The clone SR-LRP-Rz 2 exhibited the largest change, with an average 10-fold reduction in resistance to Adriamycin. Some of the other antisense clones (SR-LRP-AS 7 and 8) also displayed decreases in resistance, but the changes were not as significant as those mentioned above. The clones SR-LRP-Rz 1 and SR-LRP-AS 2 and SR-LRP-AS 6 displayed only very minor decreases in resistance to Adriamycin, possibly indicating that the transfected plasmids were not functioning in these clones.

Three of the control pHβ plasmid transfectants (SR-pHβ 1, 6 and 7) showed increased sensitivity to Adriamycin, with only SR-pHβ 3 showing no alteration. These

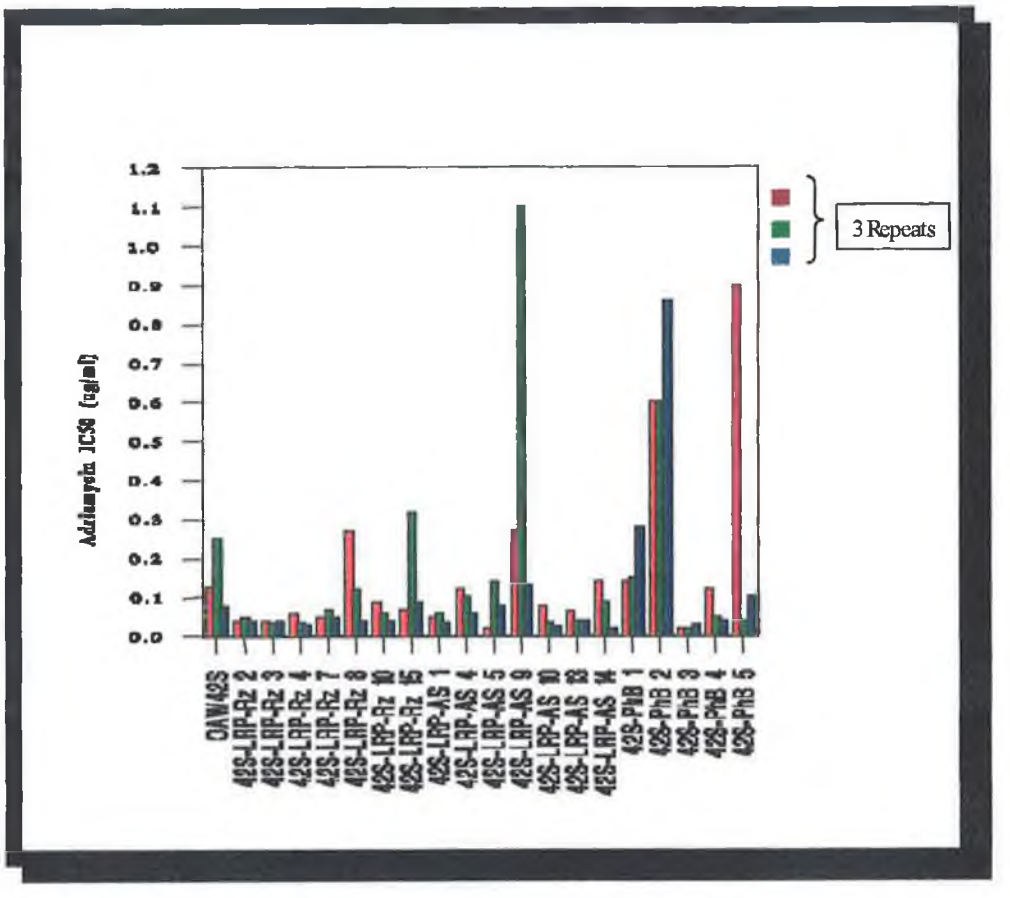
Graph 3.1.2.1 Adriamycin toxicity assay on OAW42SR clones : Relative IC50 values



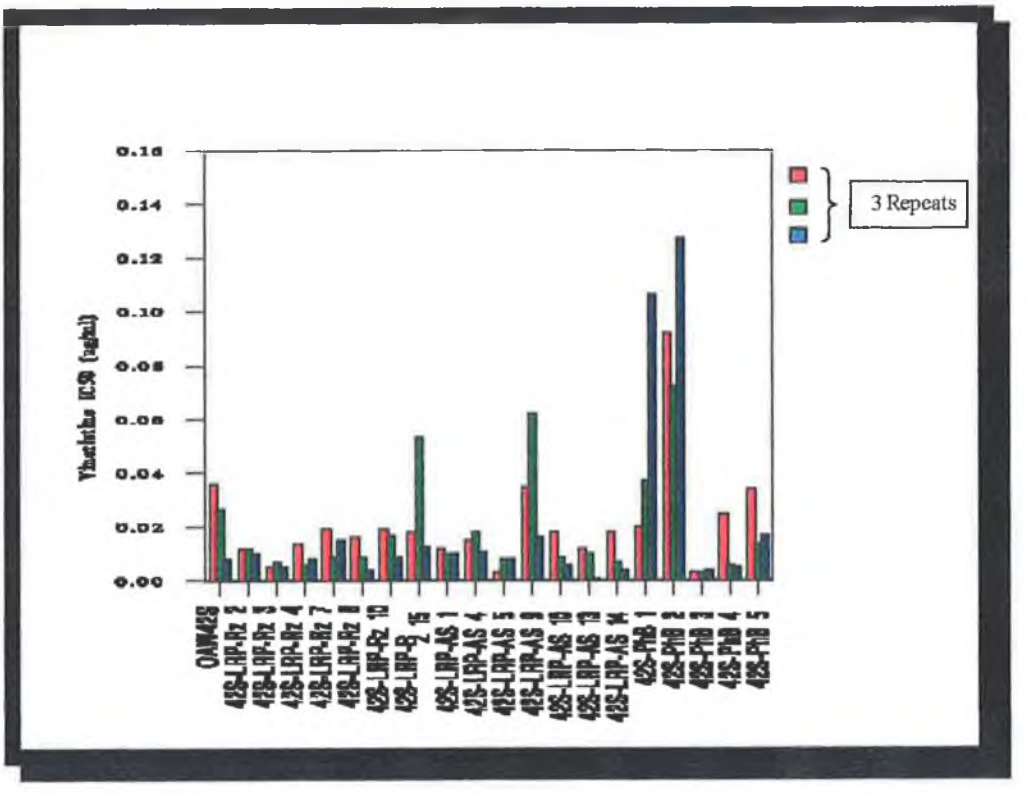
Graph 3.1.2.2 Vinblastine toxicity assay on OAW42SR clones : Relative IC50 values



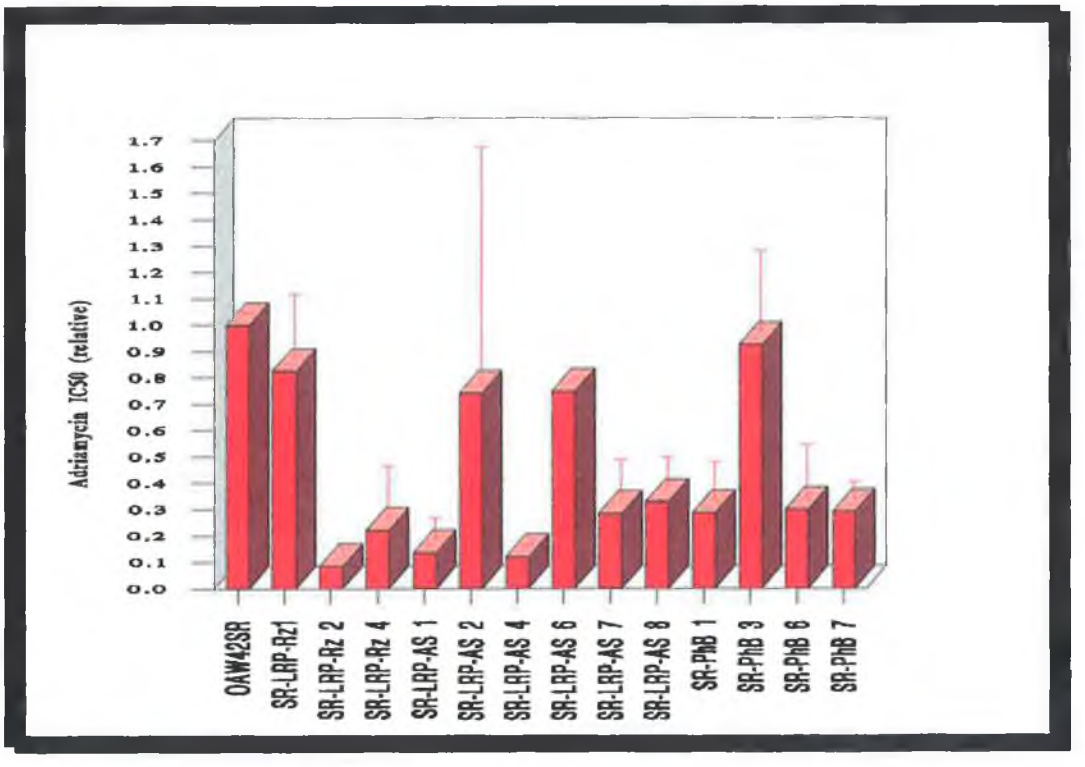
Graph 3.1.2.3 Adriamycin toxicity assay for OAW42S clones: IC_{50} values (μg/ml)



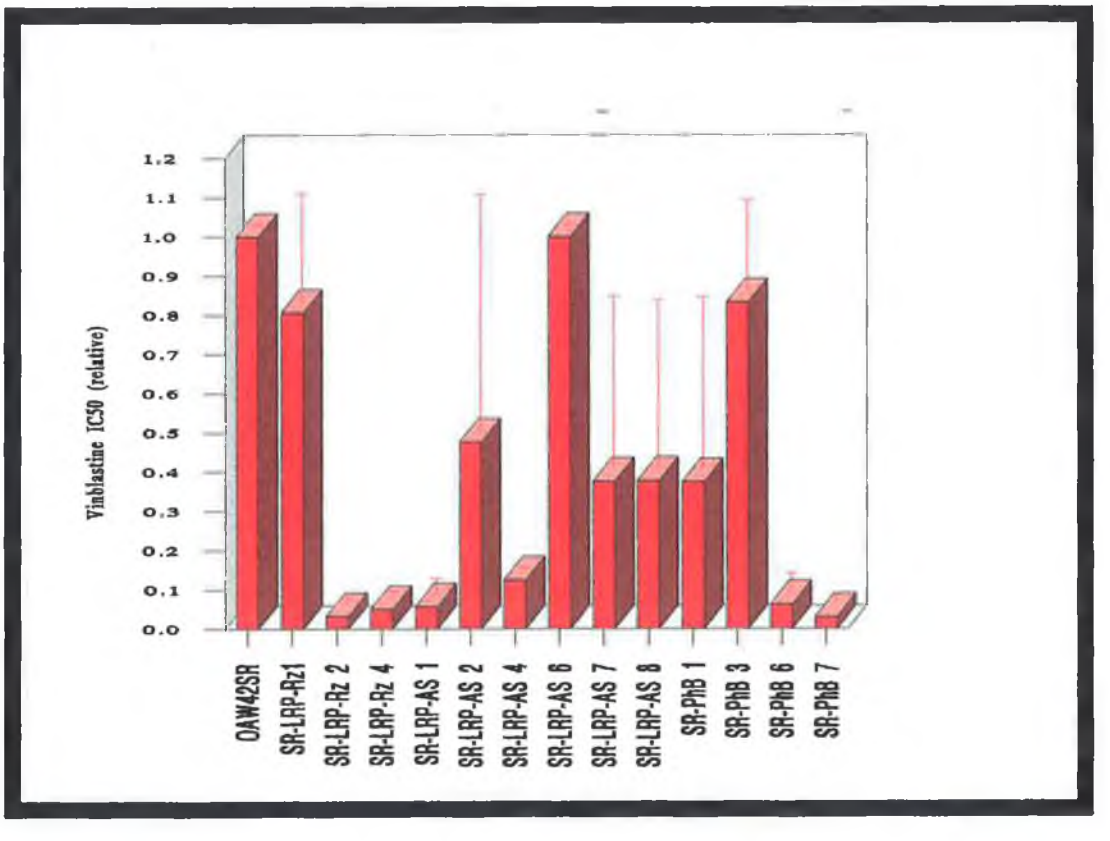
Graph 3.1.2.4 Vincristine toxicity assay on OAW42S clone : IC_{50} values (μg/ml)



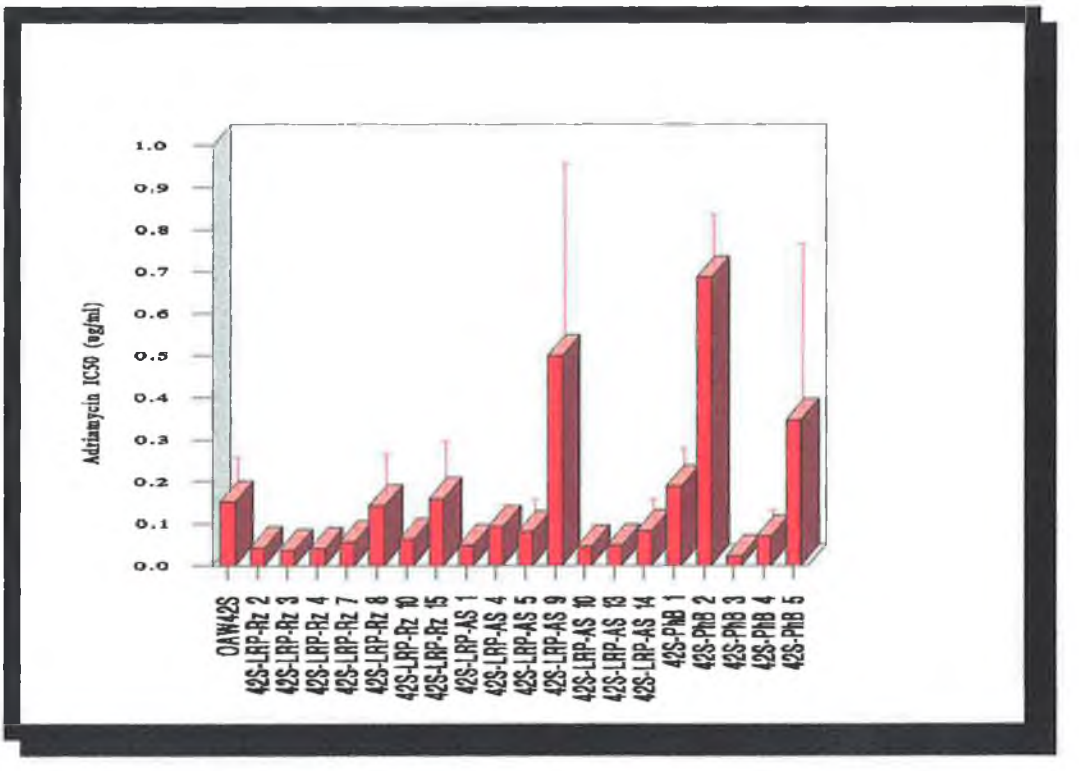
Graph 3.1.2.5 Adriamycin toxicity assay on OAW42SR clones : Average relative IC50 values



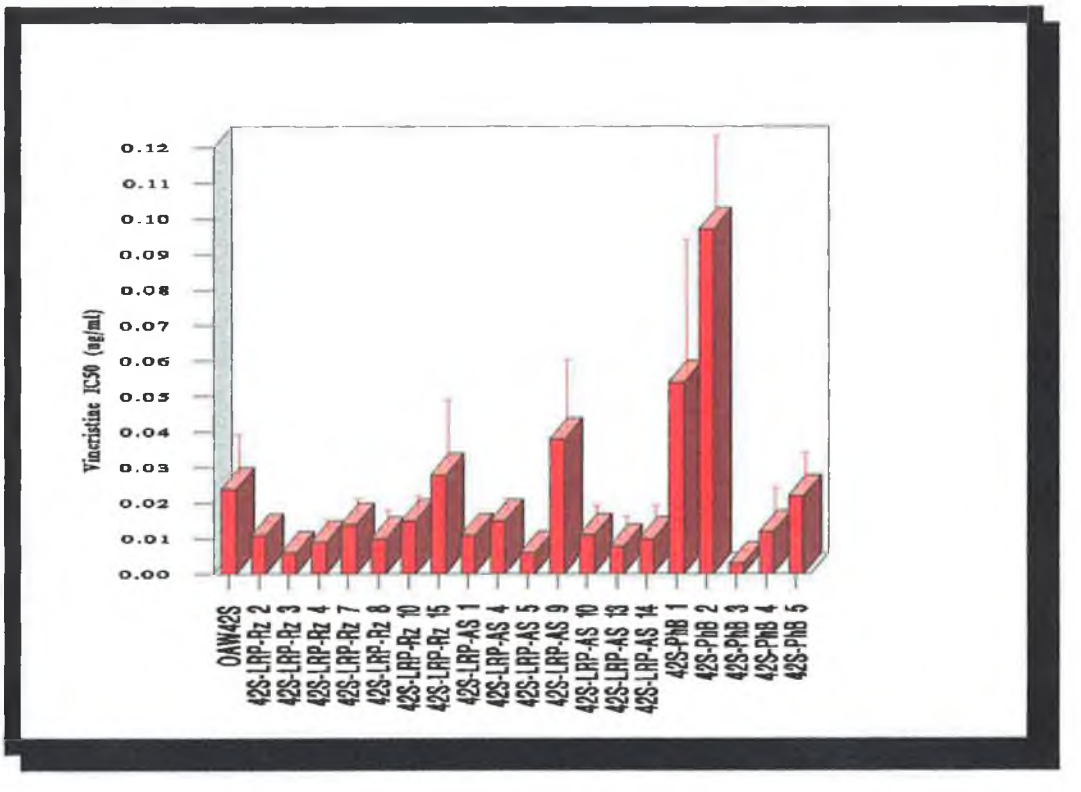
Graph 3.1.2.6 Vinblastine toxicity assay on OAW42SR clones : Average relative IC50 values



Graph 3.1.2.7 Adriamycin toxicity assay on OAW42S clones : Average IC_{50} values (μg/ml)



Graph 3.1.2.8 Vincristine toxicity assay on OAW42S clones : Average IC_{50} values (μg/ml)



decreases in resistance to Adriamycin corresponded to the decreases in Vinblastine resistance (Graphs 3.1.2.2 and 3.1.2.6) with clones SR-LRP-Rz 2 and 4, SR-LRP-AS 1 and 4, showing the largest increases in sensitivity.

Similarly to the OAW42SR clones, there were a number of OAW42S transfectant which displayed varying levels of increased sensitivity to Adriamycin (Graphs 3.1.2.3 and 3.1.2.7). However the magnitude of changes in resistance of these OAW42S clones (42S-LRP-Rz 2, 3, 4, 7 and 42S-LRP-AS 1, 4, 5, 10, 13 14) was not as great as for the OAW42SR clones with a maximum of around 5-fold decrease in IC50 values as compared to the parental cells. As with the OAW42SR clones, there were a number of control plasmid (pHβ) transfectants (42S-pHβ 3, 4) which also displayed reduced IC50 values. However, there were also a number of clones (42S-LRP-AS 9, 42S- pHβ 2 and 5) which exhibited a greatly increased resistance to the cytotoxic effects of Adriamycin.

Once again, the results from the Vincristine toxicity assays on OAW42S clones (Graphs 3.1.2.4 and 3.1.2.8) strongly reflected those for Adriamycin.

3.1.3 Immunocytochemical staining of OAW42SR and OAW42S clones with LRP-56 MAb

In order to test if the observed reductions in cytotoxic drug resistance (Section 3.1.2.) and decreases in LRP mRNA level (Section 3.1.1) for the various clones corresponded with the level of expression of LRP at the protein level, cytospins were prepared from the clones and were stained with the LRP-56 monoclonal antibody (MAb) (Sections 2.9.7). Each of the OAW42SR clones was examined while only a selection of OAW42S clones were analysed, due to the high number of these clones and time constraints. Which OAW42S clones to analyse by immunocytochemistry, was decided from the results of the LRP RT-PCRs and the toxicity assays (Sections 3.1.1 and 3.1.2). The clones chosen in Table 3.1.3.2 were thought to be interesting in terms of LRP protein down-regulation, as judged by decreased LRP mRNA levels and decreased resistance to adriamycin and vincristine.

Cytospins from each clone were prepared on two separate occasions, and immunocytochemistry with the LRP-56 MAb repeated on each set of cytospins at least twice. The intensity of LRP staining for each OAW42SR clone is given in Table 3.1.3.1, while the corresponding photographs of the stained cytospins shown in Figure 3.1.3.1. The OAW42S clones were also tested for LRP expression on two occasions, with the levels of staining as shown in Table 3.1.3.2. and Figure 3.1.3.2. The values in Tables 3.1.3.1 and 3.1.3.2 are averaged from a number of cytospins and not just the ones shown in Figures 3.1.3.1 and 3.1.3.2.

From Table 3.1.3.1 and Figure 3.1.3.1, it is evident that a number of OAW42SR clones had reduced levels of LRP expression. Over the two repeats, SR-LRP-Rz 2 and SR-LRP-AS 1 displayed an almost total elimination of LRP expression. The clones SR-LRP-Rz 4, SR-LRP-AS 6, 7, SR- pHβ 1 and 7 all showed significant reductions in LRP staining intensity as compared to the parental cells. The clones SR-LRP-Rz 1, SR-LRP-AS 2 and 4 and SR-pHβ 3 and 6 exhibited very slight, if any, variations in the levels of LRP compared to parental OAW42SR cells.

A number of the OAW42S clones also showed reductions in the staining intensity compared to the parental cells (Table 3.1.3.2 and Figure 3.1.3.2). LRP expression appeared almost totally eliminated in 42S-LRP-Rz 2, 42S-LRP-AS 1 and 42S- pHβ 1.

Table 3.1.3.1 Immuncytochemical cytospin staining intensity with the LRP-56 MAb on OAW42SR clones.

Clone	1st Repeat	2nd Repeat
OAW42SR	+++	+++
SR-LRP-Rz 1	++	+++
SR-LRP-Rz 2	0/+	0
SR-LRP-Rz 4	++	+/++
SR-LRP-AS 1	0	0/+
SR-LRP-AS 2	++	++
SR-LRP-AS 4	++	++
SR-LRP-AS 6	+	0/+
SR-LRP-AS 7	0/+	+/++
SR-LRP-AS 8	++	+/++
SR- pHβ 1	+	++
SR- pHβ 3	++	++
SR- pHβ 6	++/+++	++/+++
SR- pHβ 7	++	+/++

Intensity of staining was as follows: 0 - No cells staining; 0/+ - few cells lightly stained; + - significant number of cells lightly stained; ++ - most cells stained moderately; +++ - intense staining on almost all cells.

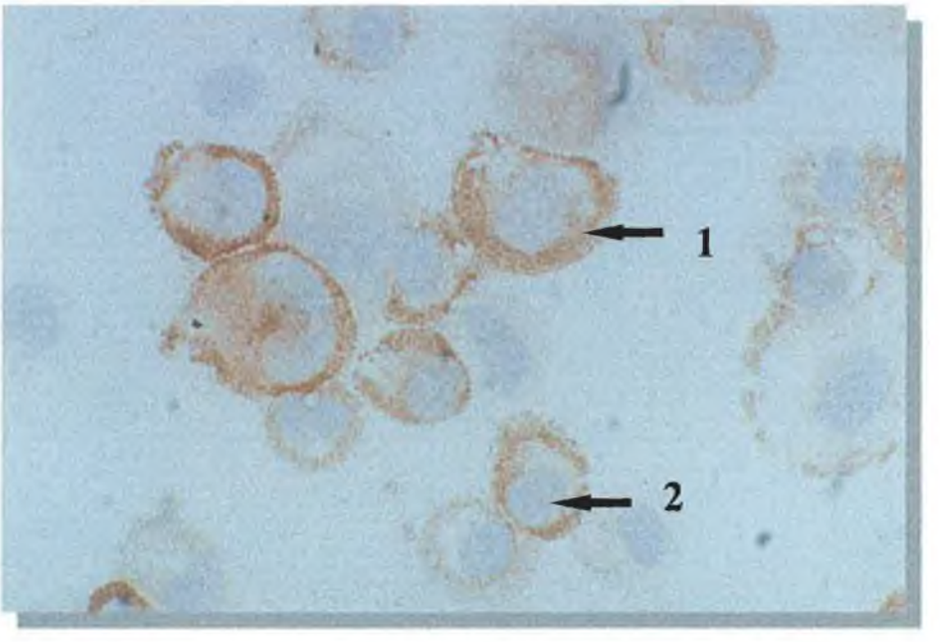
Table 3.1.3.2 Immuncytochemical cytospin staining of OAW42S clones

Clone	1st Repeat	2nd Repeat
OAW42S	+/++	+++
42S-LRP-Rz 2	0/+	+
42S-LRP-Rz 4	+++	+++
42S-LRP-Rz 7	+++	+++
42S-LRP-AS 1	0/+	0/+
42S-LRP-AS 10	+++	+++
42S-LRP-AS 13	+++	+++
42S-LRP-AS 14	+++	+++
42S-pHβ 1	0	0
42S-pHβ 4	+++	+++

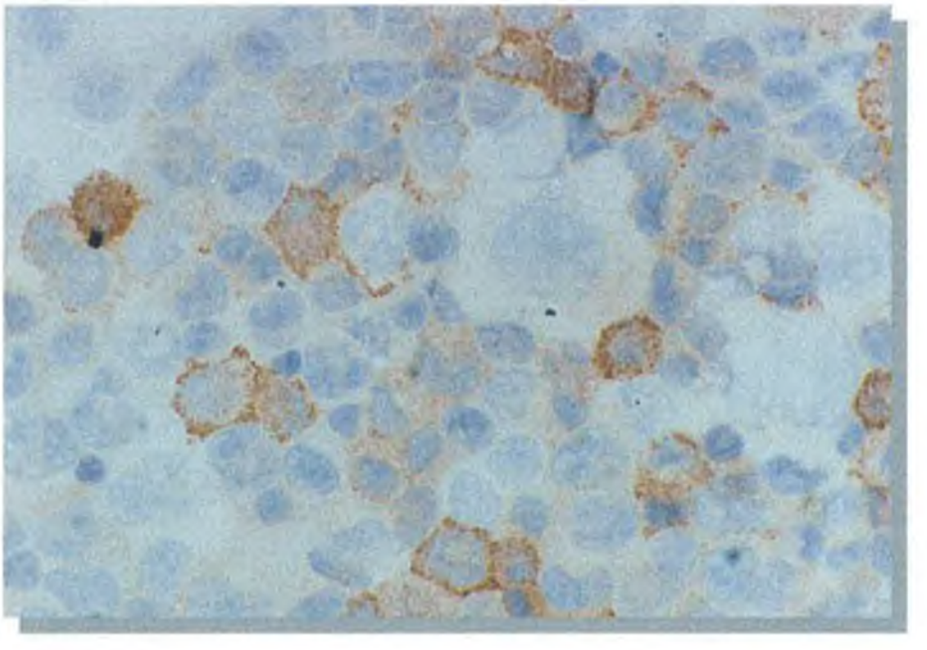
Staining intensity as in legend for Table 3.1.3.1

Figure 3.1.3.1 Immunocytochemical staining of OAW42SR clone cytospins with LRP-56 MAb

(a)



(b)



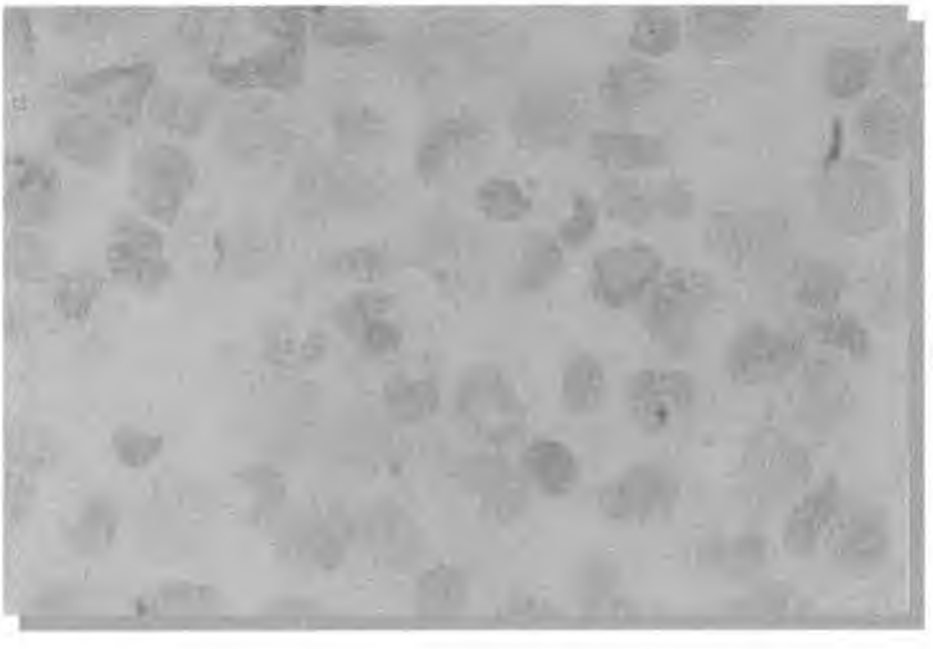
(a) OAW42SR; (b) SR-LRP-Rz 1

1- Punctate cytoplasmic LRP staining; **2-** Blue nucleus counter stain

Figure 3.1.3.1 (cont'd) Immunocytochemical staining of OAW42SR clone cytospins with LRP-56 MAb

(c)

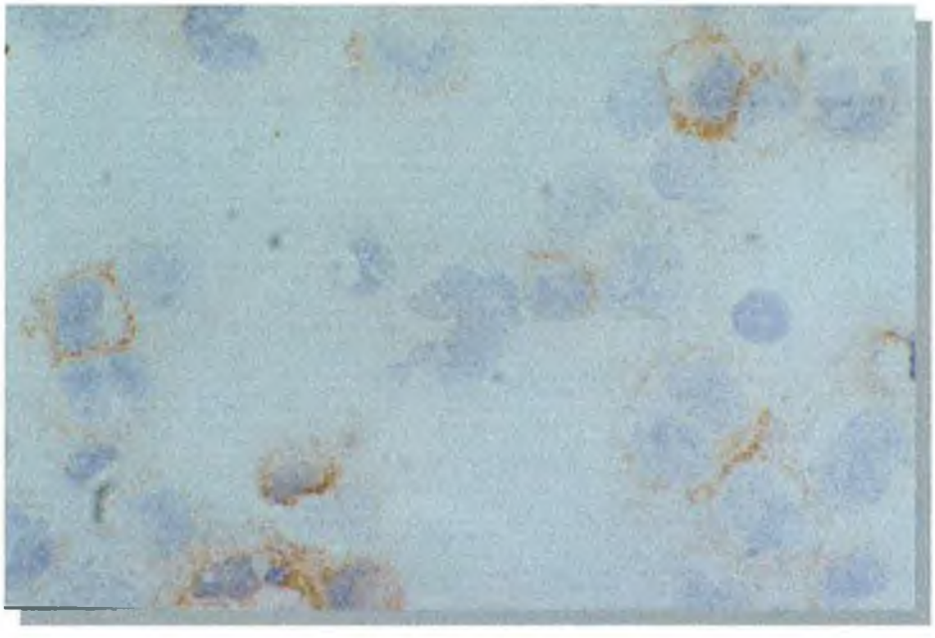
(d)



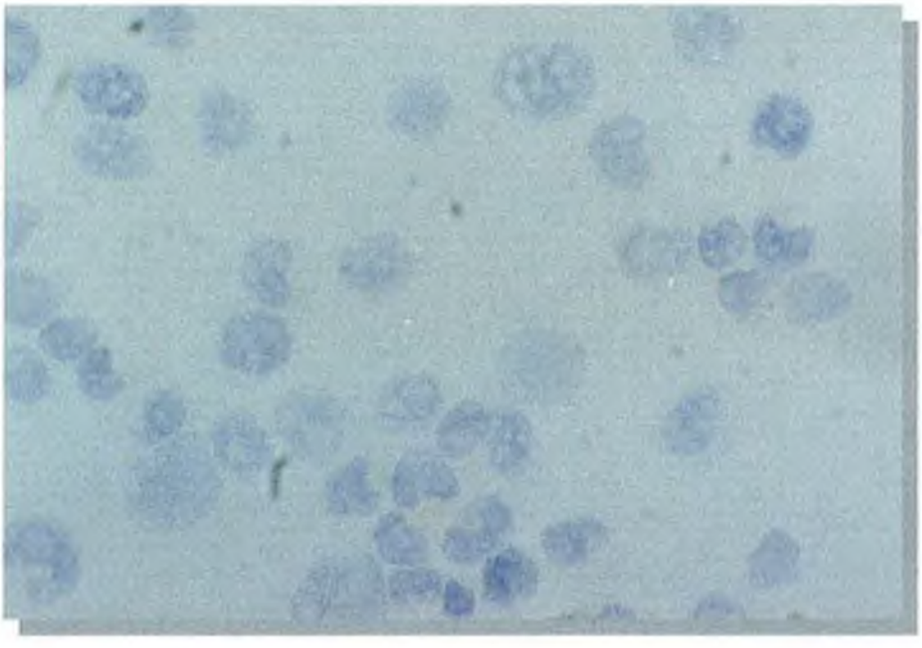
(c) SR-LRP-Rz 2; (d) SR-LRP-Rz 2 negative control

Figure 3.1.3.1 (cont'd) Immunocytochemical staining of OAW42SR clone cytospins with LRP-56 MAb

(e)



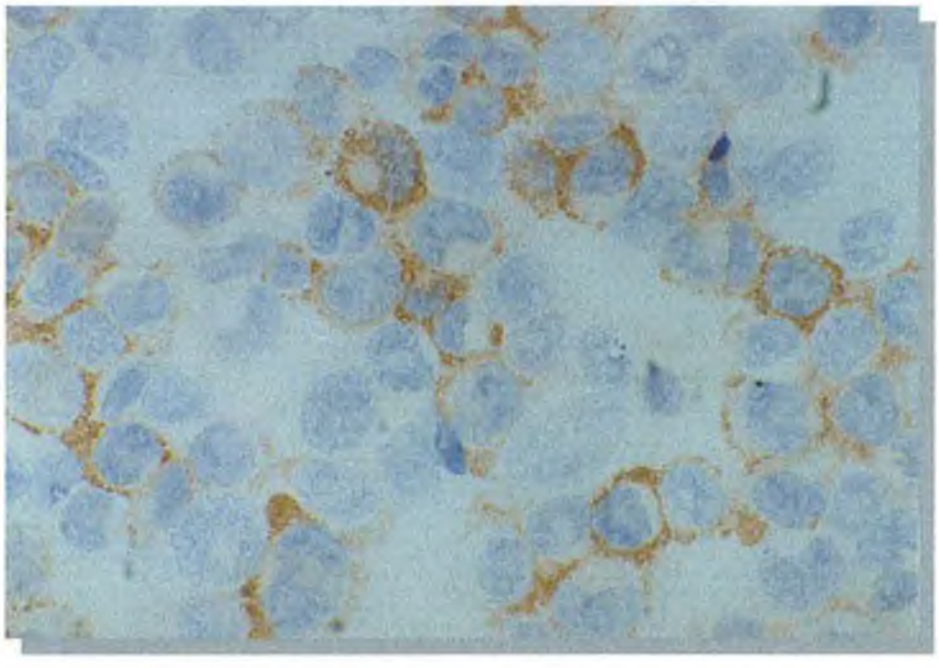
(f)



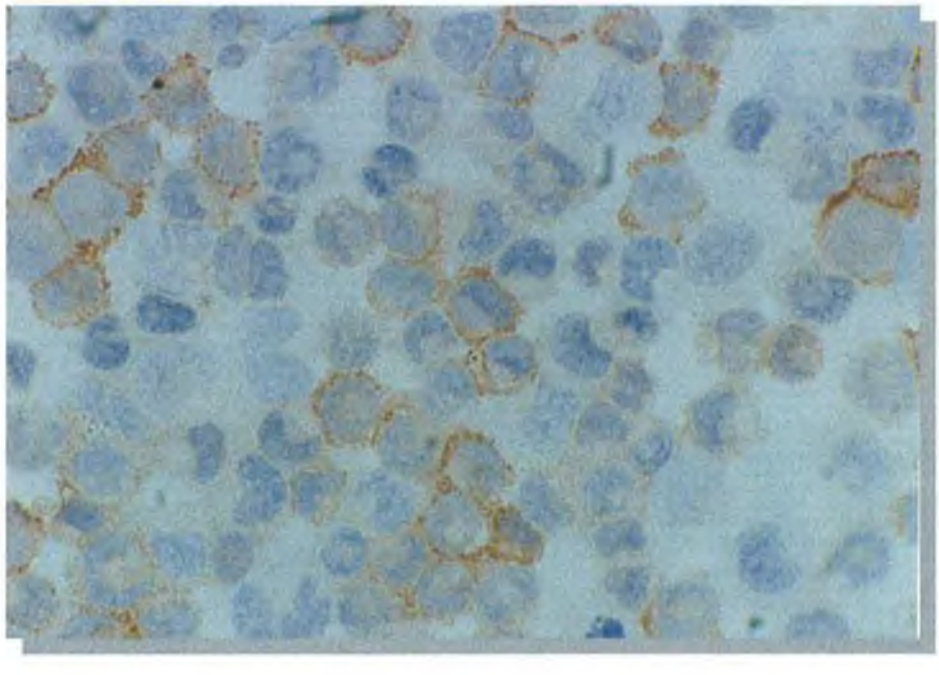
(e) SR-LRP-Rz 4; (f) SR-LRP-AS 1

Figure 3.1.3.1 (cont'd) Immunocytochemical staining of OAW42SR clone cytospins with LRP-56 MAb

(g)



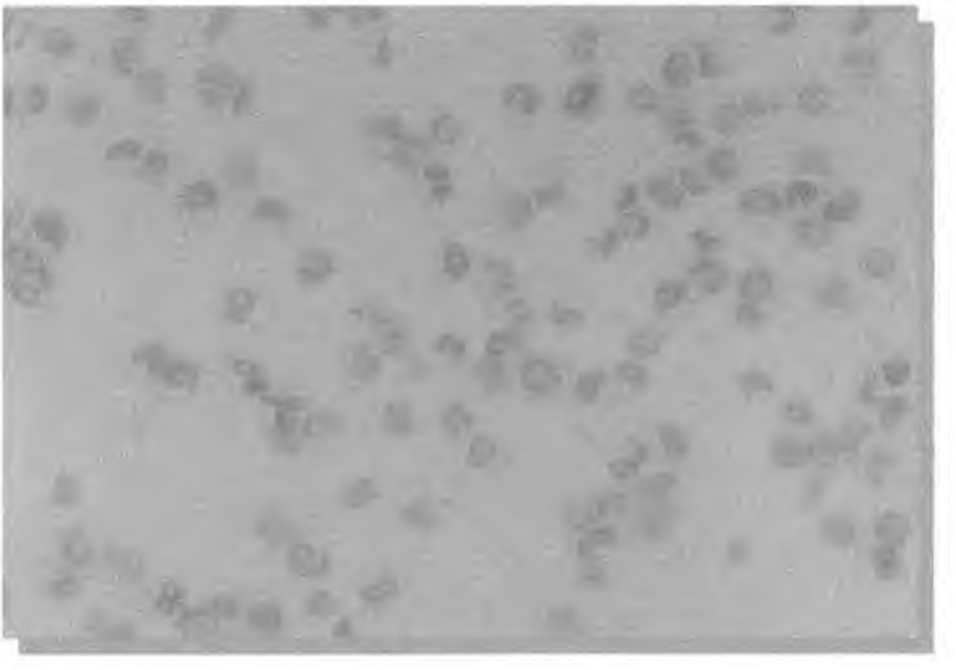
(h)



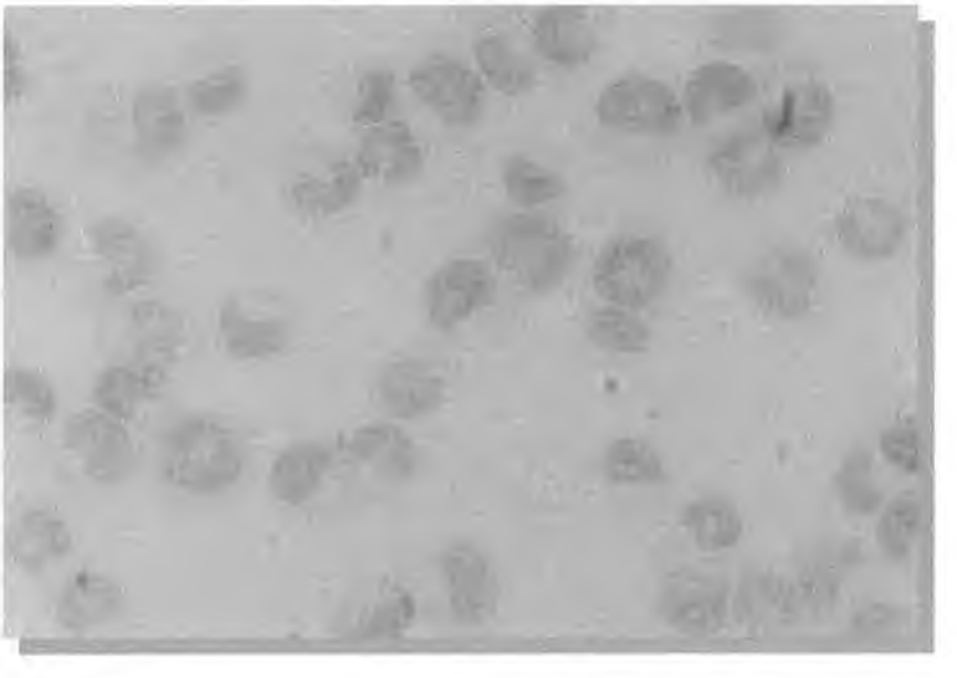
(g) SR-LRP -AS 2; (h) SR-LRP-AS 4

Figure 3.1.3.1 (cont'd) Immunocytochemical staining of OAW42SR clone cytospins with LRP-56 MAb

(i)



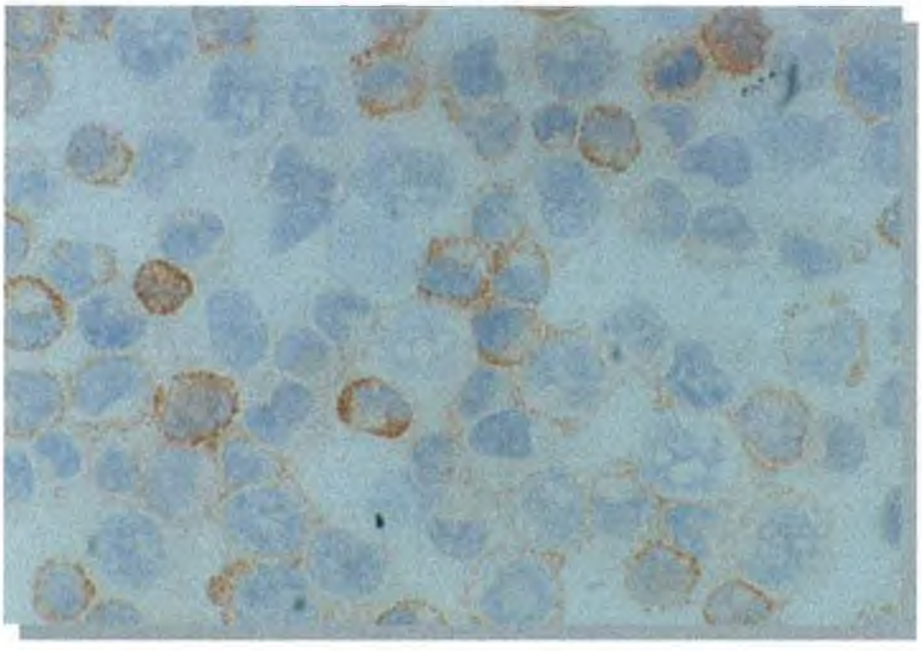
(j)



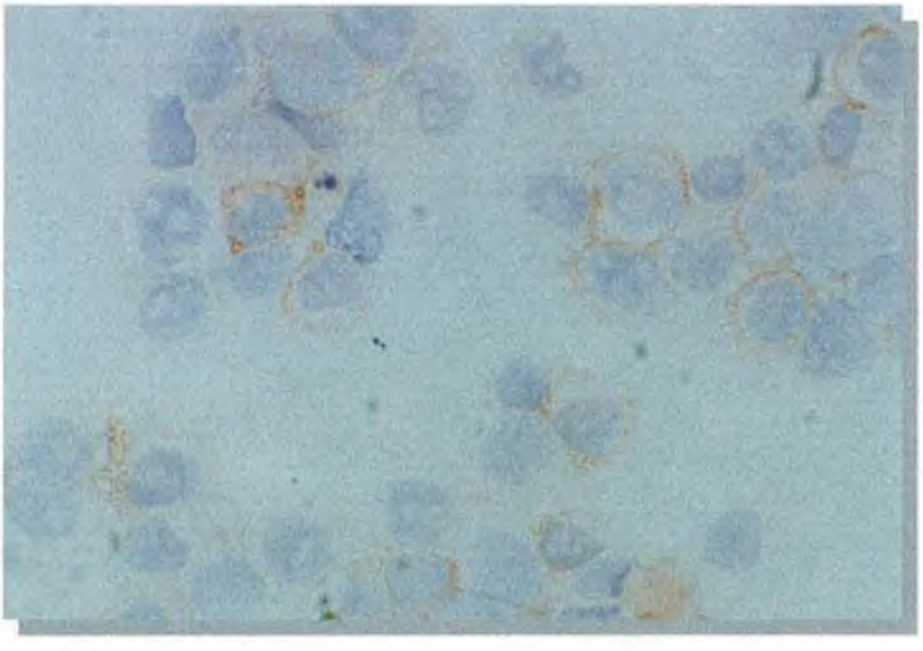
(i) SR-LRP-AS 6; (j) SR-LRP-AS 7

Figure 3.1.3.1 (cont'd) Immunocytochemical staining of OAW42SR clone cytospins with LRP-56 MAb

(k)



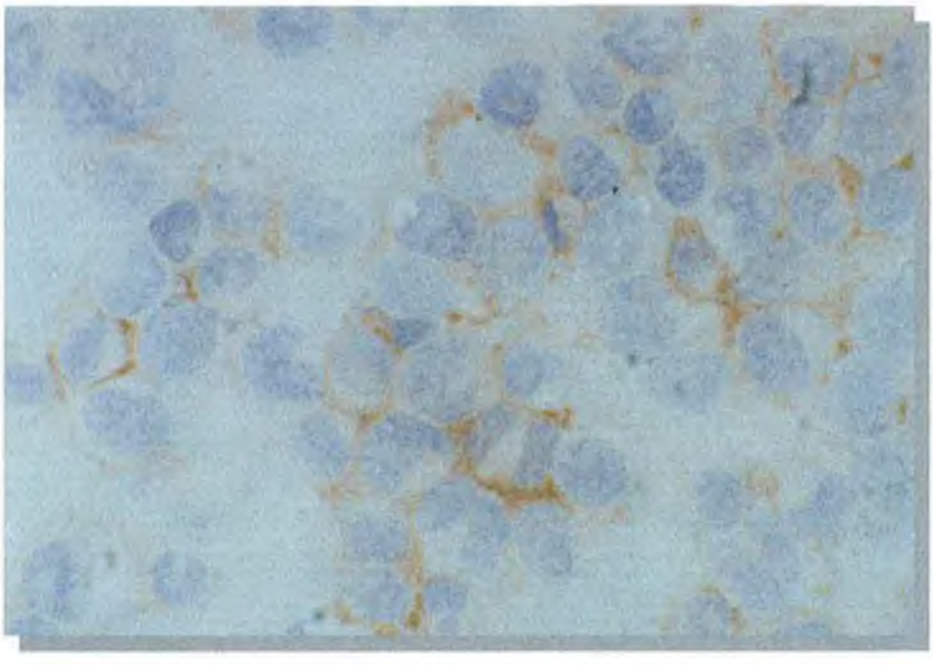
(l)



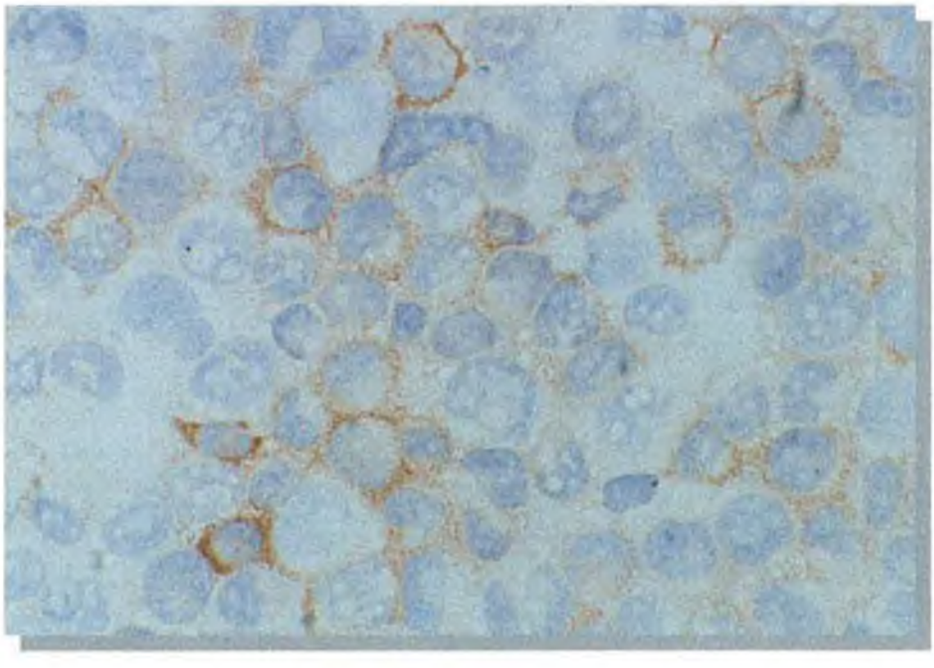
(k) SR-LRP-AS 8; (l) SR-pHβ 1

Figure 3.1.3.1 (cont'd) Immunocytochemical staining of OAW42SR clone cytospins with LRP-56 MAb

(m)



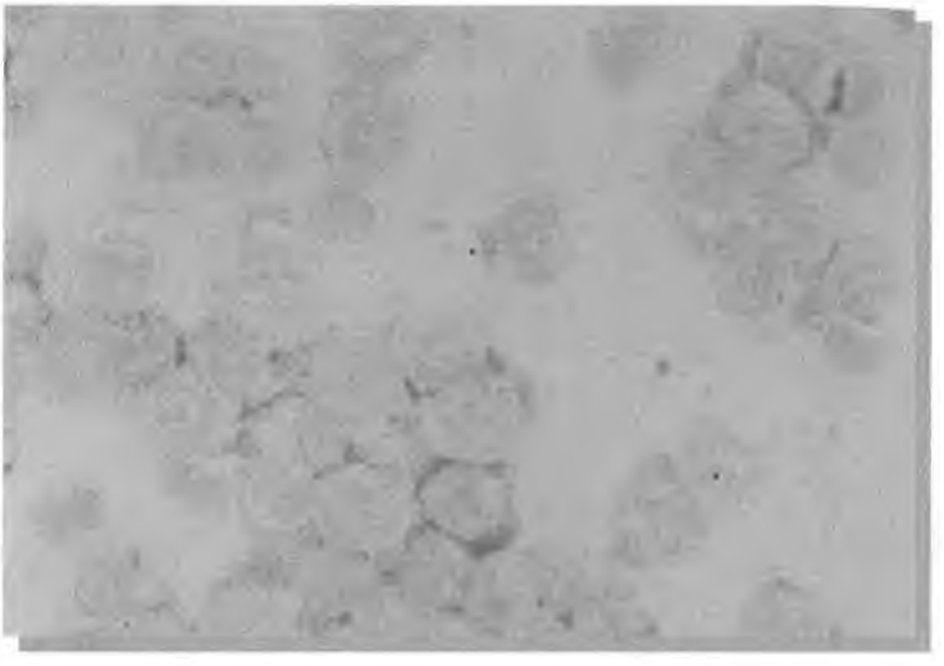
(n)



(m) SR-pHβ 3; (n) SR-pHβ 6

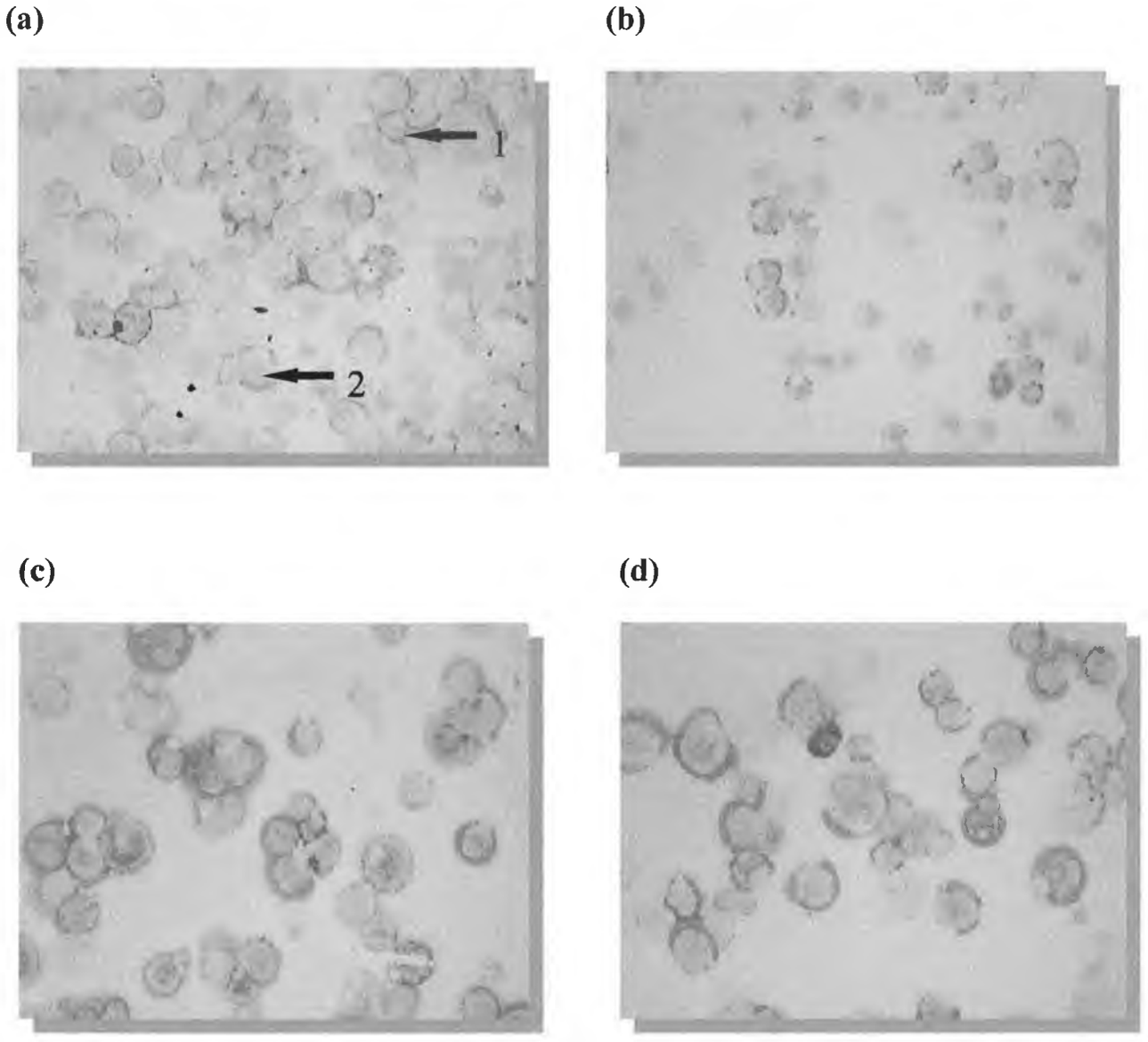
Figure 3.1.3.1 (cont'd) Immunocytochemical staining of OAW42SR clone cytospins with LRP-56 MAb

(o)



(o) SR-pHβ 7

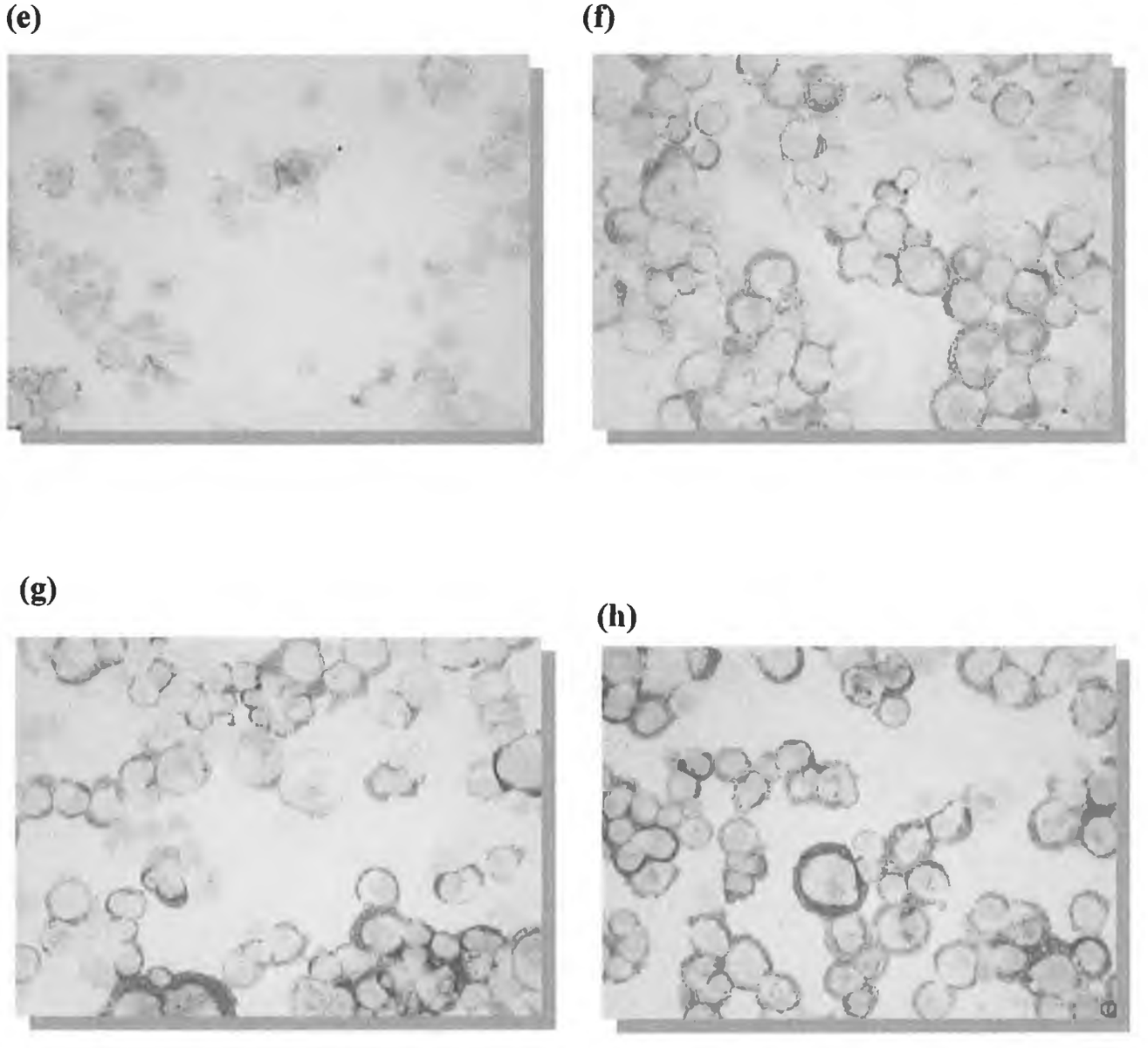
Figure 3.1.3.2 Immunocytochemical staining of OAW42S clone cytospins with LRP-56 MAb



(a) OAW42S; (b) 42S-LRP-Rz 2; (c) 42S-LRP-Rz 4; (d) 42S-LRP-RZ 7

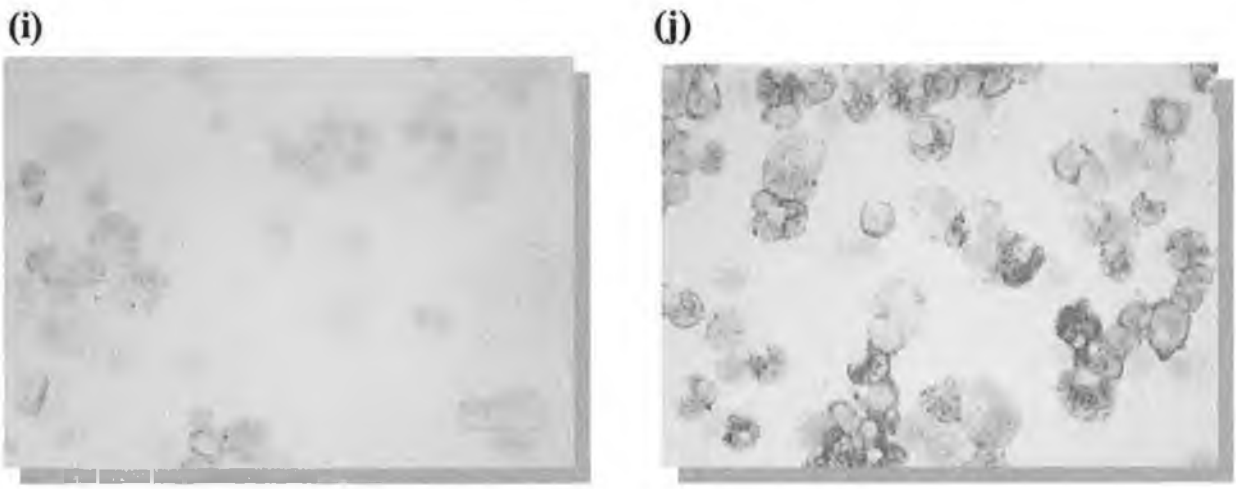
1- Punctate cytoplasmic LRP staining; **2-** Blue nucleus counter stain

Figure 3.1.3.2 (cont'd) Immunocytochemical staining of OAW42S clone cytospins with LRP-56 MAb



(e) 42S-LRP-AS 1; (f) 42S-LRP-AS 10; (g) 42S-LRP-AS 13; (h) 42S-LRP-AS 14

Figure 3.1.3.2 (cont'd) Immunocytochemical staining of OAW42S clone cytospins with LRP-56 MAb



(i) 42S-pHβ 1; (j) 42S-pHβ 4

3.1.4 Assessment of results

The results up to this point illustrated that in a number of ribozyme and transfectant clones there was a significant reduction in LRP protein levels. This indicated that, in at least some of the clones, the ribozyme and antisense constructs were functioning effectively. However, a number of control vector (pHβ) transfectants also displayed reductions in LRP expression. There, therefore, appears to be quite a high level of clonal variation arising out of the heterogeneous OAW42SR parental population. As a result, the reductions in LRP expression observed in the ribozyme and antisense transfectants may not be wholly attributed to the action of these constructs.

This clonal variation is also apparent at the level of drug resistance, where a number of control vector transfectants display a reduction in resistance to adriamycin and vinblastine/vincristine. The changes in IC_{50} values varied from a 10 to 20-fold reduction for SR-LRP-Rz 2 and SR-LRP-AS 1, to no change for the clones SR-LRP-Rz 1 and SR-pHβ 3. The magnitude of the changes was not as great in the OAW42S clones as for the OAW42SR clones. This may reflect the lower intrinsic resistance of the OAW42S cells.

For a number of the clones, there appears to be good correlation between the level of LRP expression and drug resistance. The clones SR-LRP-Rz 2 and SR-LRP-AS 1 exhibit large reductions in LRP levels as well as IC_{50} values. SR-LRP-Rz 1 shows no reduction in LRP expression and no reduction in resistance as compared to the parental cells. However, some of the other clones display no obvious correlation. For instance, SR-LRP-Rz 4 and SR-LRP-AS 4 show large decreases in drug resistance, with relatively small decreases in LRP expression. SR-LRP-AS 6 exhibits no reduction in IC50 values, but has greatly reduced LRP expression. It is clear that there is a large degree of variability in these results, and that much more detailed work was required.

3.1.5 Choice of OAW42SR and OAW42S clones for detailed analysis.

In order to feasibly carry out a detailed analysis of the OAW42SR and OAW42S transfection clones at the mRNA and protein level, it was necessary to limit the number of clones being investigated. Therefore a number of clones which exhibited varying levels of LRP expression were chosen in order to allow the correlation of LRP expression and cytotoxic drug resistance. The clones chosen are shown in Table 3.1.4.

A number of clones were chosen, as they appeared to display reduced LRP expression levels. From the OAW42SR clones, SR-LRP-Rz 2 and 4 and SR-LRP-AS 1, displayed decreased resistance to adriamycin and vinblastine and reduced levels of LRP expression as determined by immunocytochemical staining (Sections 3.1.2. and 3.1.3). The clones SR-LRP-Rz 1 and SR-pHβ 3 were chosen as they showed an apparent lack of LRP down-regulation.

Similarly, 42S-LRP-Rz 2 and 42S-LRP-AS 1 were chosen from the OAW42S clones for their apparent decreases in LRP levels and cytotoxic drug resistance, while 42S-pHβ 4 was selected for its lack of LRP variation (Section 3.1.2 and 3.1.3). In addition to the above, the clones SR-pHβ 1 and 42S-pHβ 1 were included in order to investigate their mechanism of LRP down-regulation and decreased drug resistance in the absence of an anti-LRP ribozyme or antisense molecules.

Table 3.1.4 OAW42SR and OAW42S clones chosen for detailed protein and mRNA analysis.

OAW42SR clones	OAW42S clones
OAW42SR	OAW42S
SR-LRP-Rz 1	42S-LRP-Rz 2
SR-LRP-Rz 2	42S-LRP-AS 1
SR-LRP-Rz 4	42S-pHβ 1
SR-LRP-AS 1	42S-pHβ 4
SR-pHβ 1	
SR-pHβ 3	

3.2 Analysis of OAW42SR and OAW42S clones at the mRNA and protein level.

In order to clarify the role of LRP in multidrug resistance, it is important to show a clear and unambiguous decrease in the levels of LRP at the protein level, which may also, but not necessarily, be reflected at the mRNA level.

In this respect, LRP expression levels were determined at the mRNA level by further RT-PCR analysis and northern blots (Sections 2.7.5-2.7.6 and 2.8). At the protein level, LRP expression was examined by immunocytochemistry and immunoprecipitation (Sections 2.9.7 and 2.9.7). As a proposed functional assay, cytotoxic drug toxicity assays were carried out with a variety of classic MDR and non-MDR drugs (Section 2.3).

When attempting to ascertain a role for a certain protein in the functioning of a cell through its down-regulation, it is imperative to ensure that other proteins, which exhibit a similar function, are not also down-regulated. A decrease in the levels of related proteins could easily lead to a mis-interpretation of results. To avoid this, the levels of expression of two classic MDR related genes, mdr-1 and MRP (Section 1.1), were examined in parallel to that of LRP. Elimination of a possible role for these proteins in the observed decreases in drug resistance would clarify the role of LRP in the observed MDR. Additionally, for the RT-PCR reactions and northern blotting, the levels of gene expression were compared to those of internal controls, the house-keeping genes β–actin and GAPDH, to ensure equal loading of samples.

3.2.1 RT-PCR Analysis of mRNA expression levels in OAW42SR and OAW42S clones.

Total RNA was extracted from each clone by the method described earlier (Section 2.7.1). The primers used for detection of LRP, mdr-1, MRP, Ribozyme/Antisense and β-actin (short or long) expression by RT-PCR are as detailed earlier (Section 2.7.6.1).

3.2.1.1 Analysis of LRP mRNA expression by RT-PCR

The primers used to detect LRP mRNA expression yield a band of 300 bp (Section 2.7.6.1). The expression of the housekeeping gene β-actin was used as a control, and the primers chosen (β-actin short 1 and 2) gave a band of 142 bp. Each RT-PCR reaction was repeated a minimum of three times on separate occasions with different RNA preparations. Total RNA extracted from SW1573-2R120 (2R120) cells was used as a positive control for LRP expression, as this cell line displays characteristically high levels of LRP mRNA (see Section 1.2). Water was used instead of cDNA as a negative control in the PCR reactions. The results shown in Figures 3.2.1.1.1 and 3.2.1.1.2, for OAW42SR and OAW42S clones respectively, are representative.

From Figure 3.2.1.1.1, it can be seen that the only SR-LRP-Rz 2 shows a significant n reduction in LRP mRNA expression. All the other clones displayed similar LRP levels to the parental OAW42SR cell line, while the positive and negative controls were as expected.

The OAW42S clones tested (Figure 3.2.1.1.2) failed to display decreases in LRP mRNA levels as determined by RT-PCR, while the positive (2R120) and negative (water) controls were as expected.

Figure 3.2.1.1.1 RT-PCR Analysis of LRP expression in OAW42SR clones

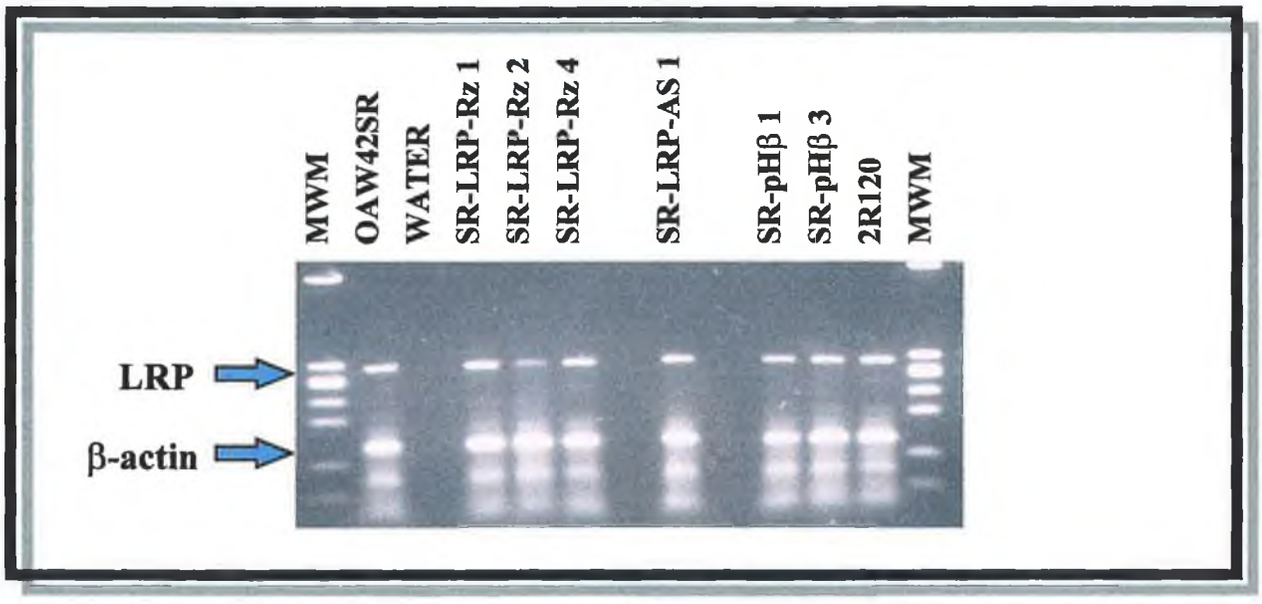
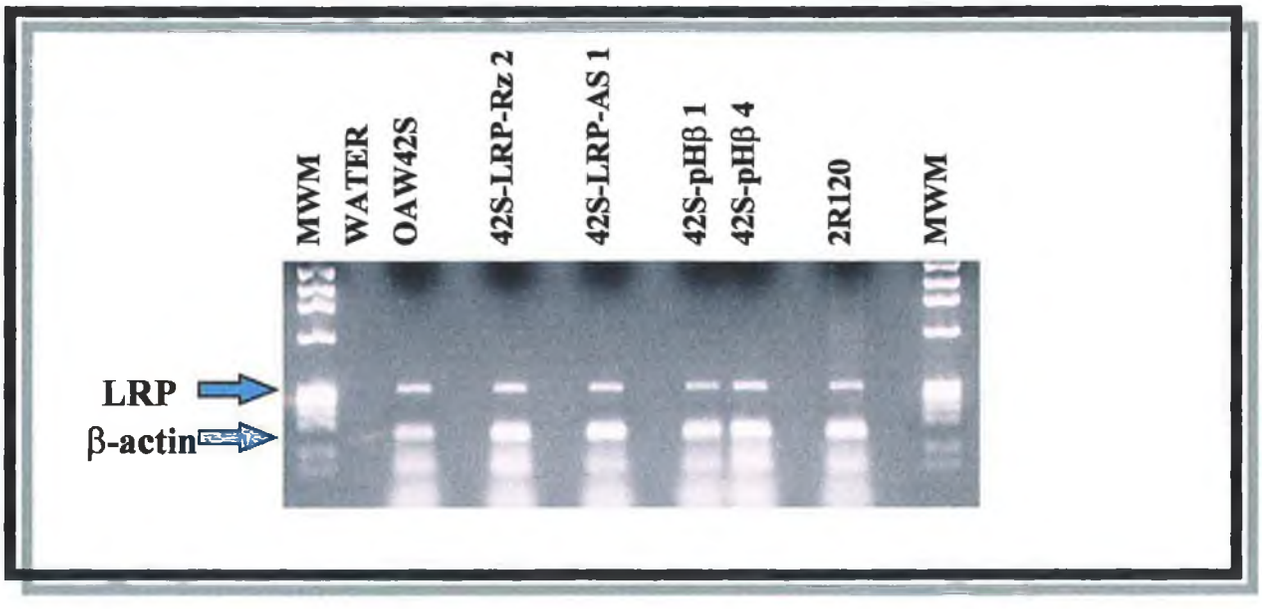


Figure 3.2.1.1.2 RT-PCR Analysis of LRP expression inOAW42S clones



3.2.1.2 Analysis of mdr-1 mRNA expression by RT-PCR

The primers used to amplify a 157 bp fragment of mdr-1 cDNA were as described earlier (Section 2.7.6.1). The control gene used was β-actin, with the primers (long) amplifying a 383 bp fragment (Section 2.7.6.1). Once again, each RT-PCR reaction was repeated at least three times on separate occasions with different RNA samples from the OAW42SR and OAW42S clones, and the results shown in Figure 3.2.1.2.1 and Figure 3.2.1.2.2 are representative.

The mdr-1 mRNA levels in the OAW42SR clones all appear to be equal to if not higher than those of the parent cells (Figure 3.2.1.2.1). The clone with the lowest observable levels of mdr-1 mRNA expression, SR-LRP-Rz 2, has equal levels to that of the parent, indicating no significant down-regulation of mdr-1 in this clone. The mdr-1 negative control (2R120) shows equal β-actin expression to the OAW42SR clones, but no mdr-1 expression as expected.

Figure 3.2.1.2.2 shows the results of mdr-1 RT-PCR on total RNA extracted from the OAW42S clones. No completely satisfactory results were obtained for mdr-1 expression in OAW42S cells. In the results shown, however, both 42S-LRP-Rz 2 and 42S-LRP-AS 1 exhibit higher mdr-1 levels than the control vector transfectant 42S-pHβ 1, indicating that mdr-1 expression was probably not reduced in these clones. Once again, the mdr-1 negative control (2R120) showed no observable mdr-1 expression.

3.2.1.3 Analysis of MRP mRNA expression by RT-PCR

The primers designed to amplify a fragment of the MRP cDNA give a band of 203 bp (see section 2.7.6.1). The internal control gene is β-actin, with an amplified product of 383 bp (long primers, see Section 2.7.6.1). Total RNA extracted from the MRP-positive cell line COR-L23R was used as a positive control for this RT-PCR. All reactions were repeated three times and the results shown (Figure 3.2.1.3.1 and 3.2.1.3.2) are representative.

Figure 3.2.1.2.1 RT-PCR Analysis of mdr-1 expression in OAW42SR clones

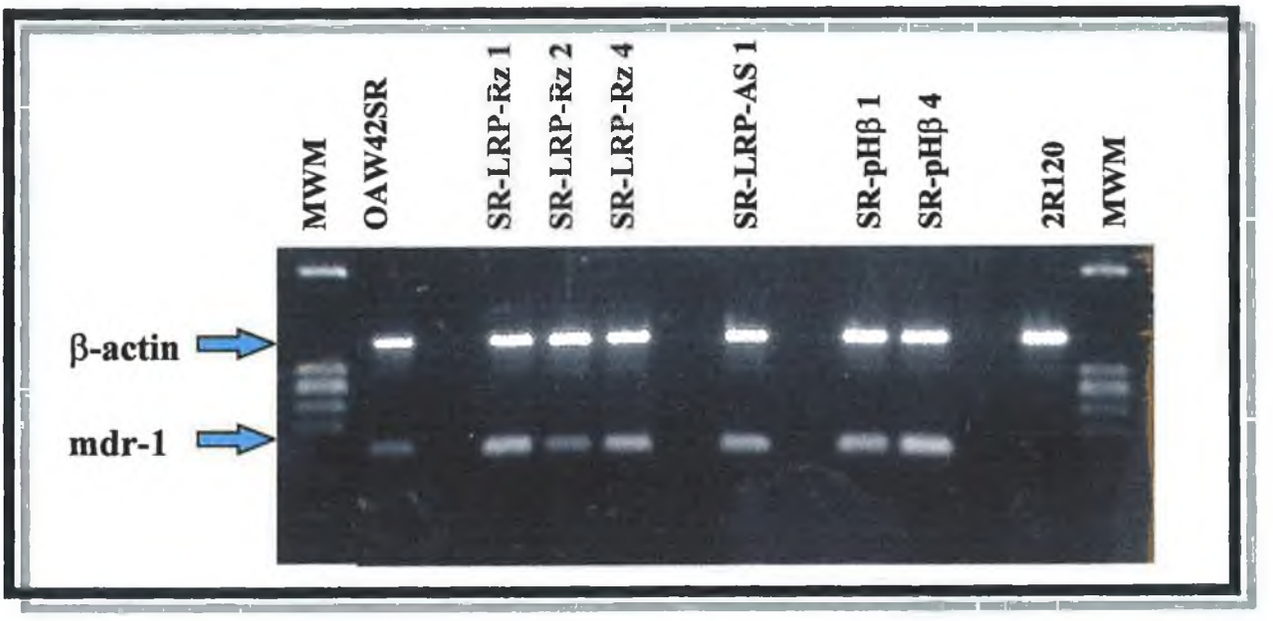


Figure 3.2.1.2.2 RT-PCR Analysis of mdr-1 expression in OAW42S clones

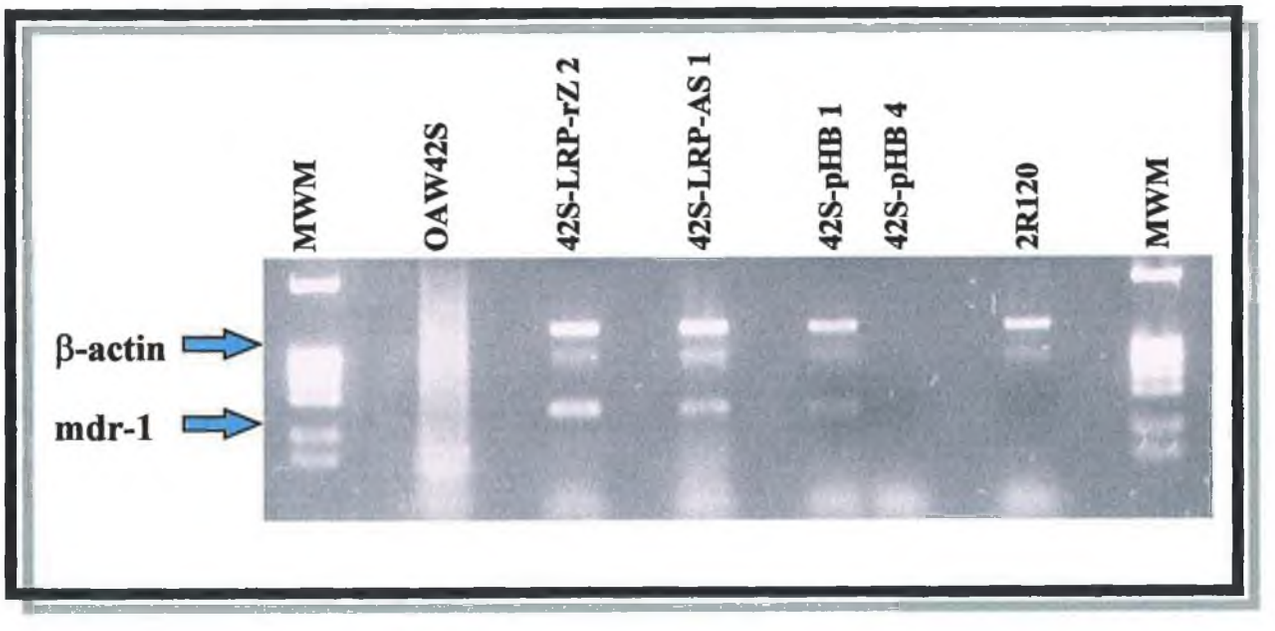


Figure 3.2.1.3.1 RT-PCR Analysis of MRP expression in OAW42SR clones

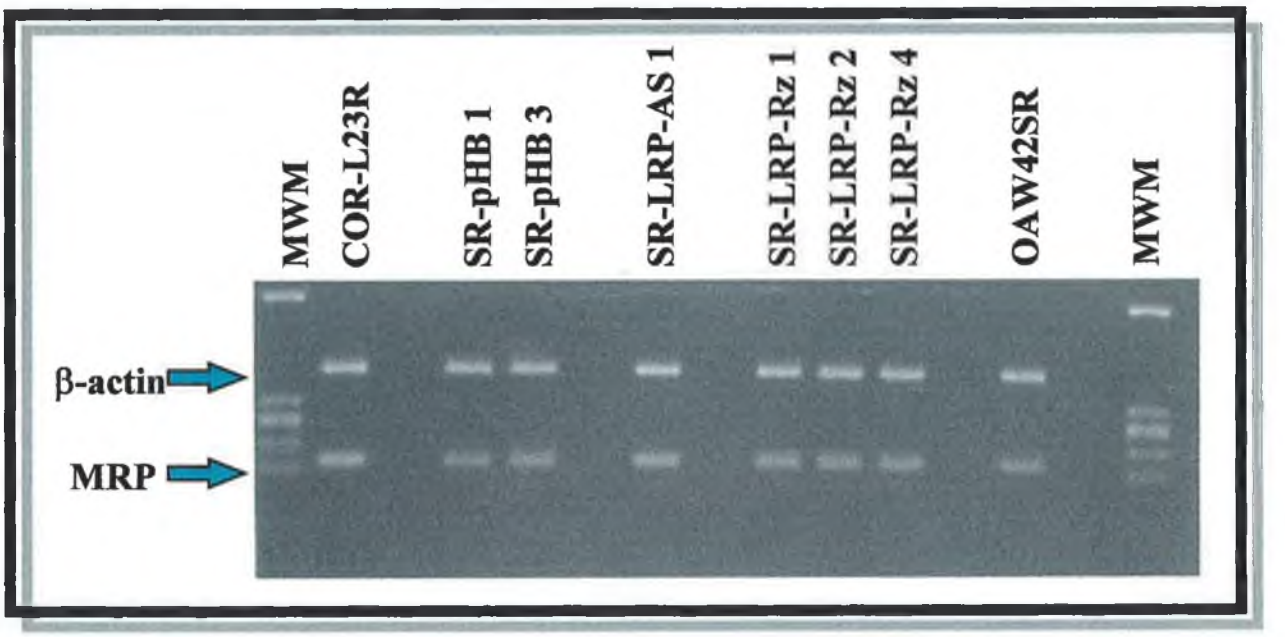


Figure 3.2.1.3.2 RT-PCR Analysis of MRP expression in OAW42S clones

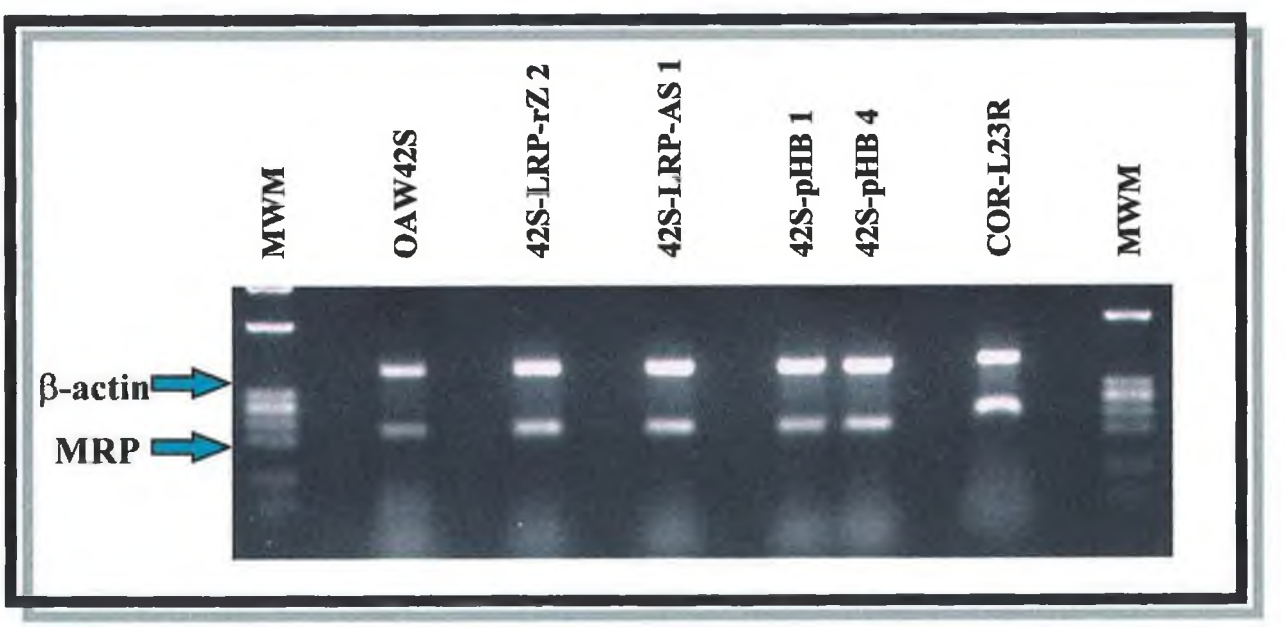


Figure 3.2.1.3.2 shows that there is no observable variation in MRP expression between the various OAW42SR clones and the parent cells. There is a strong band present for the COR-L23R sample, which is as expected. As with the OAW42SR clones, none of the OAW42S clones display reduced MRP levels as compared to the parental cells (Figure 3.2.1.3.2). The clones, in fact, all appear to have slightly higher levels of MRP expression than the parental cell line.

3.2.1.4 Analysis of Ribozyme/Antisense expression by RT-PCR

The primers used to amplify fragments of the ribozyme or antisense expression plasmids yield bands of lengths 118 and 108 bp respectively (see Section 2.7.6.1).

No control gene was used in these PCRs due to the high cycle number and stringent reaction mixture formulation required to amplify the ribozyme/antisense bands. Figure 3.2.1.4.1 shows the results of amplification of total RNA samples from the OAW42SR clones. Expression of the ribozyme is clearly evident in the clones SR-LRP-Rz 1 and 2, while the band for SR-LRP-Rz 4 is somewhat fainter.

Expression of the antisense construct is evident in SR-LRP-AS 1, with the band being of smaller length than the ribozyme band as expected. The bands for the two control pHβ plasmid transfectants confirm that the plasmid is present and functioning, while there is no amplification product for the untransfected OAW42SR parental cells, which acted as a negative control.

Figure 3.2.1.4.2 shows the ribozyme and antisense expression in the OAW42S clones. The ribozyme band in 42S-LRP-Rz 2 is clearly visible at 118bp. There is only a faint band visible for the antisense expression in 42S-LRP-AS 1. There are faint bands present for the control vector clones indicating plasmid presence. There appears to be an unexpected larger band present in the 42S-pHβ 4 sample. It is not the right size to be a ribozyme or antisense expression band, or indeed a plasmid presence band, and may just be an artefact of the PCR, due to some contaminant particle. There are no bands present for OAW42SR or 2R120 cells,both of which acted as negative controls.

Figure 3.2.1.4.1 RT-PCR Analysis of ribozyme/antisense expression in OAW42SR clones

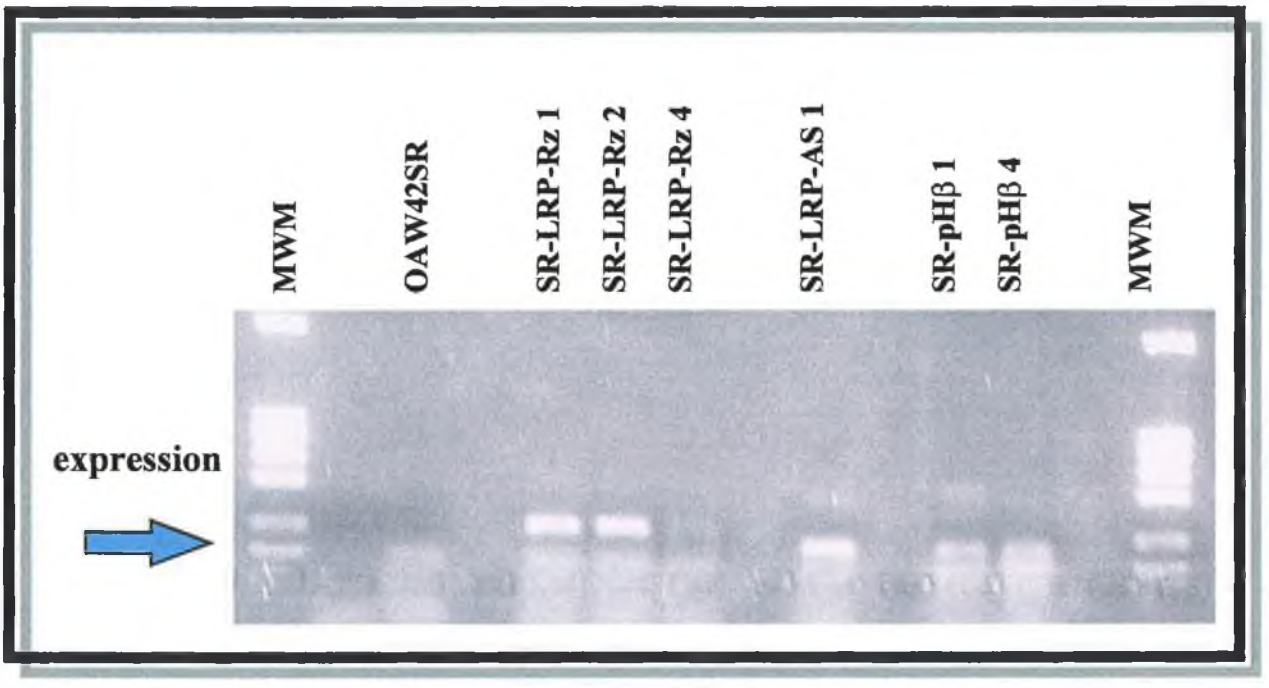
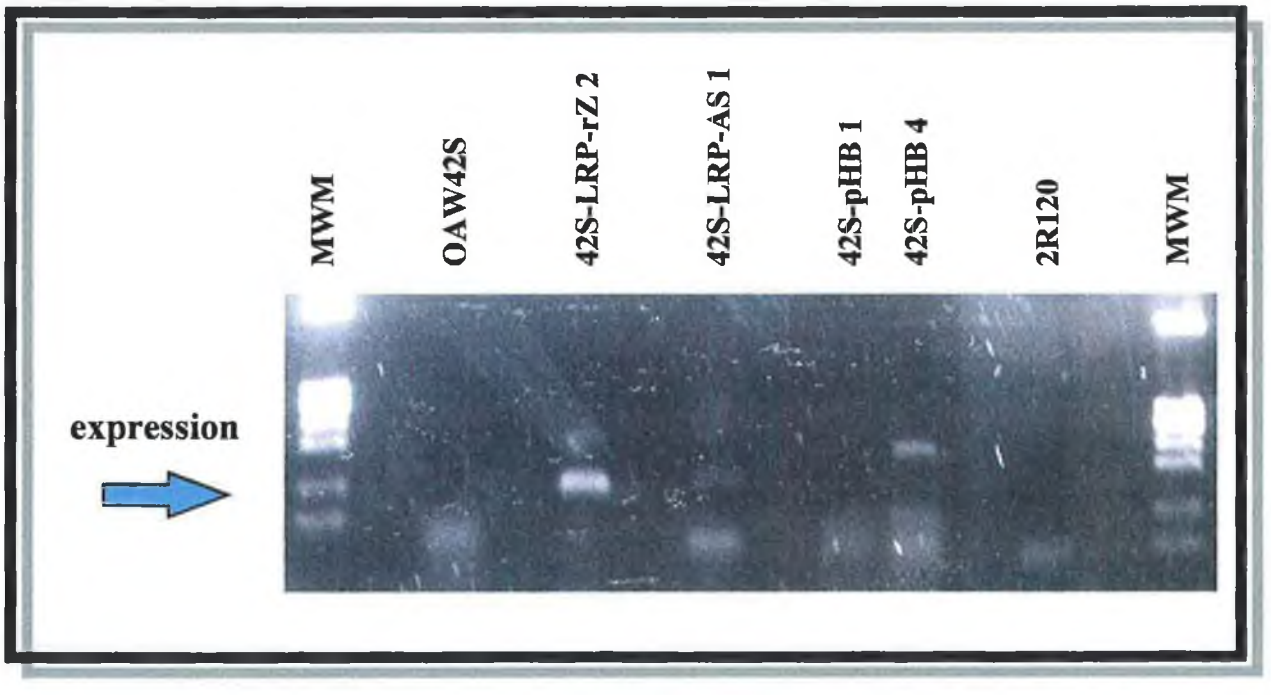


Figure 3.2.1.4.1 RT-PCR Analysis of ribozyme/antisense expression in OAW42SR clones



3.2.2 Analysis of MDR-related gene expression by northern/slot blot.

Analysis of the levels of mRNA expression of LRP, mdr-1 and MRP was carried out by preparing either northern blots or slot blots of Poly A+ RNA isolated from each clone by the methods described earlier (Section 2.8). The probes used for hybridisation were isolated from the cDNA expression plasmids of the various genes. The LRP probe was a 1360 bp fragment isolated from the LHN42 plasmid. The mdr-1 probe was a 1.38 kb fragment, while the MRP probe was 1 kb long. The probes were labelled with [α-^{32}P]dCTP and hybridised overnight as described previously (Section 2.8). An LRP Riboprobe, in which the probe is composed of [α-^{32}P]-labelled RNA, as opposed to DNA, was also used to detect LRP. As RNA-RNA interactions are stronger than the corresponding RNA-DNA interactions, a higher signal strength would be expected. Hybridised filters were exposed with X-ray films for various time lengths, depending on the strength of signal. After exposure, filters were stripped of the probes and rehybridised with a probe for GAPDH as a housekeeping gene internal control. Densitometry was carried out on the bands obtained on the X-ray films in order to quantitatively compare the levels of mRNA expression present. The levels of LRP expression, as measured by densitometry, were normalised to the levels of GAPDH to give a comparison of the levels of LRP mRNA expression between the parental cells line and the clones.

Figure 3.2.2.1 shows the results of northern blots of 1 μg of mRNA from each clone, probed with LRP, (a) and GAPDH, (b). The DNA probe used for these blots afforded only a weak signal for LRP, even in the positive control sample from 2R120 cells. It is nonetheless evident that there is a virtual absence of an LRP band for the SR-LRP-Rz 2 clone (Figure 3.2.2.1 (a)). SR-pHβ 1 also displayed a very weak signal. The levels of LRP expression appeared similar for the other clones and the parent, while the band for the 2R120s was, as expected, the strongest.

The GAPDH control hybridisation on the same filter (Figure 3.2.2.1 (b)), shows that the loading of the RNA gels was uneven, probably due to inaccuracies in the measuring of RNA concentration. The very weak GAPDH band for SR-pHβ 1, indicates that less RNA was loaded as compared to the other clones. This

Figure 3.2.2.1 Northern Blot and Densitometry analysis of LRP expression in OAW42SR clones

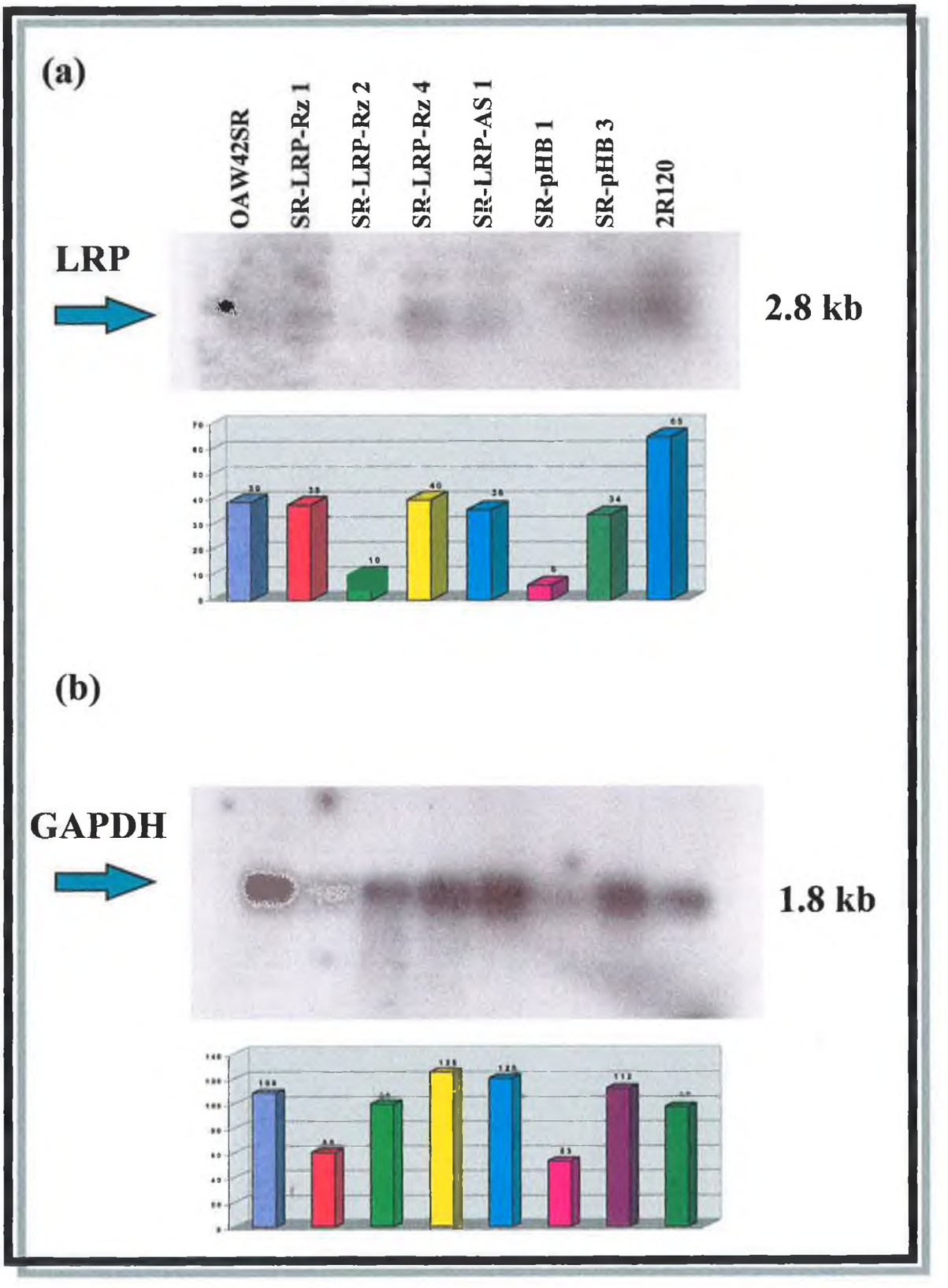
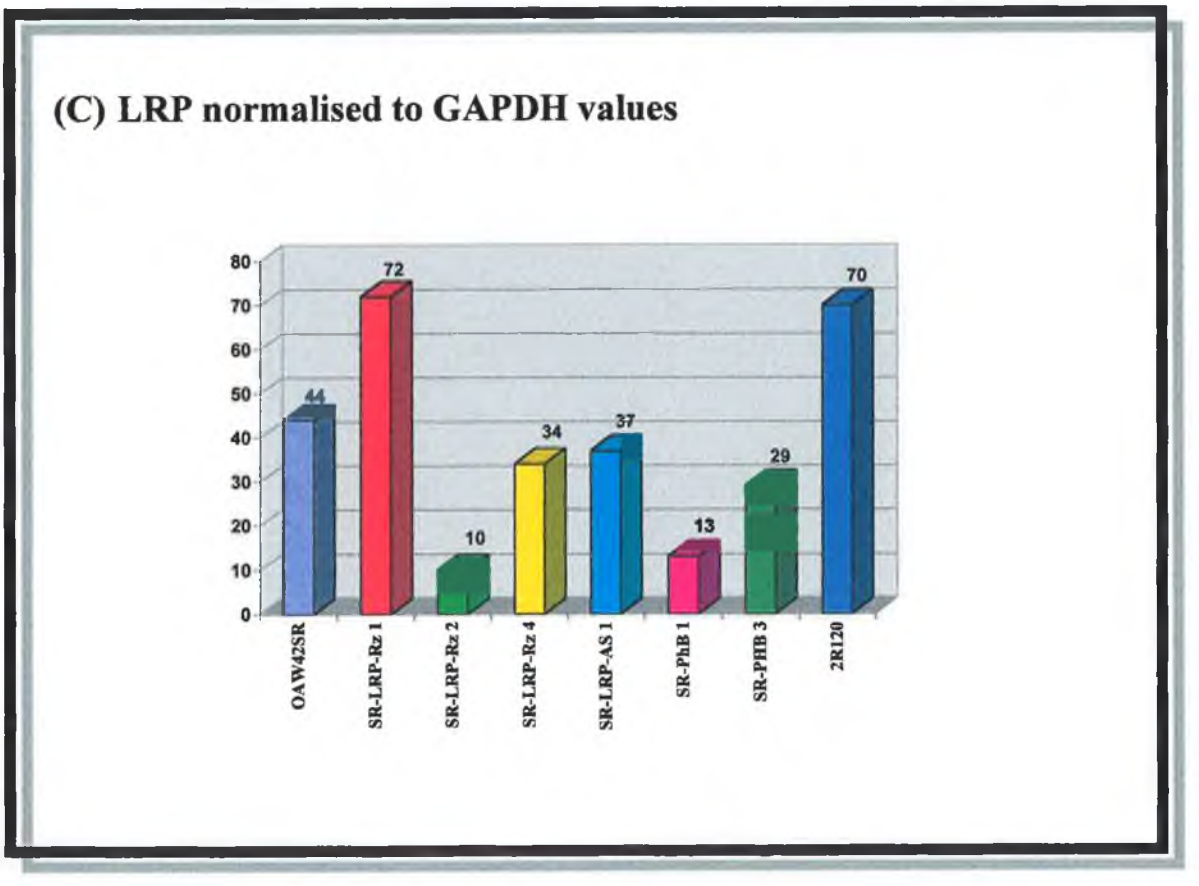


Figure 3.2.2.1 (cont'd)Northern Blot and Densitometry analysis of LRP expression in OAW42SR clones



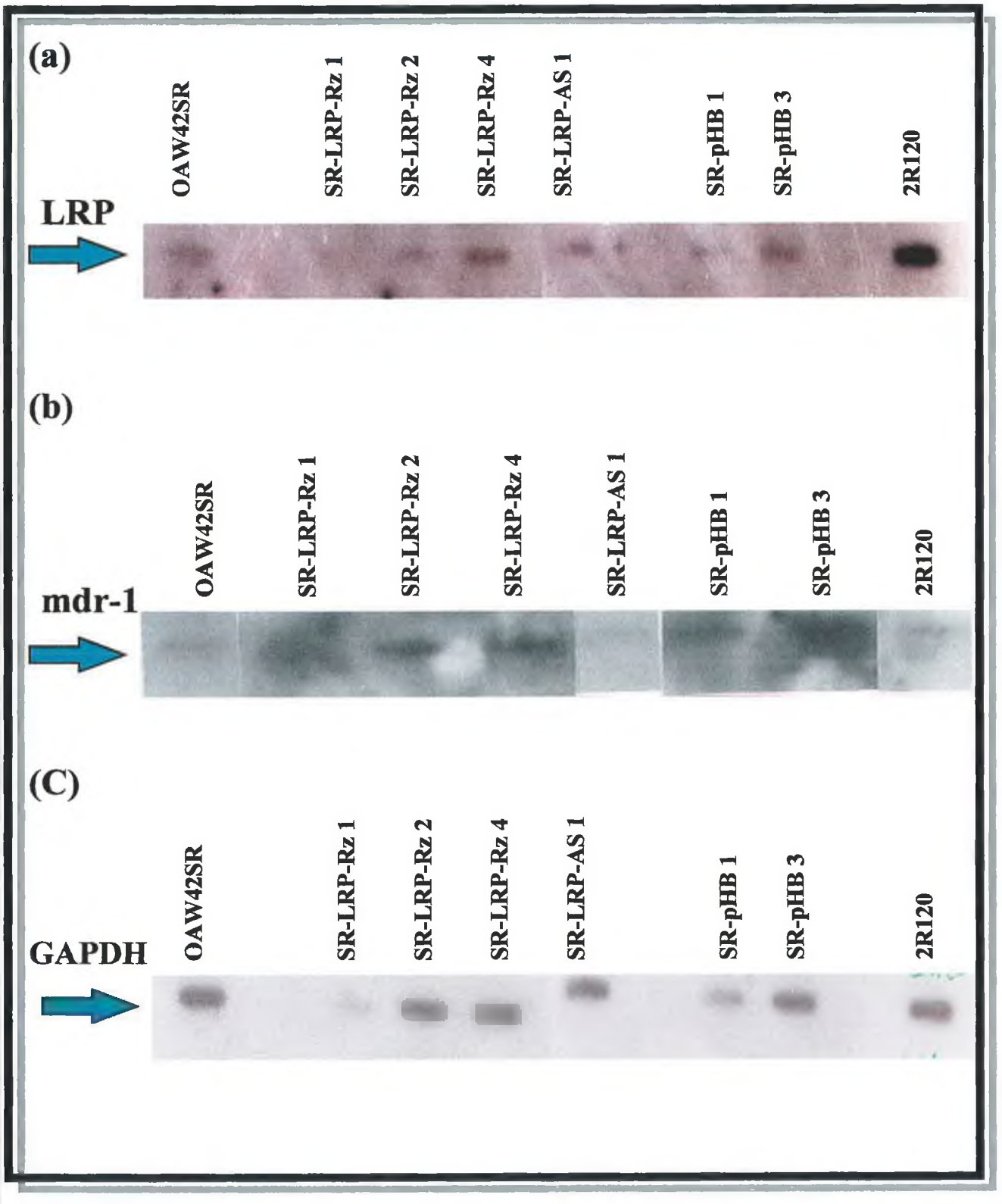
accounts for the lack of LRP signal for this clone. SR-LRP-Rz 1 also displays a weak band for GAPDH, once again indicating that a lower amount of RNA was loaded.

When the levels of LRP expression are normalised to those of GAPDH (Figure 3.2.2.1 (C)), it is evident that the clones SR-LRP-Rz 2 and SR- pHβ 1 have significantly reduced levels of LRP mRNA expression. SR-LRP-Rz 2 displays over a 4-fold decrease in the levels of LRP mRNA expression compared to the parental cells, and a 7-fold decrease as compared to the SR-LRP-Rz 1 clones. The clones SR-LRP-Rz 4 and SR-LRP-AS 1 also display reduced levels of LRP mRNA expression compared to the parental OAW42SR cells. However, the control plasmid transfectant SR-pHβ 3 also appears to have a decreased amount of LRP, while SR-LRP-Rz 1 shows an increase in LRP levels as compared to the parent. As expected, the positive control, 2R120, as expected gave a strong signal for LRP expression.

The hybridisations of the probes to slot blots of 1 μg of mRNA from the OAW42SR clones are shown in Figure 3.2.2.2. Once again, the signal from the DNA LRP probes is very weak, possibly indicating low expression levels or copy number of the mRNA (Figure 3.2.2.2. (a)). It is still possible to make out that the signals for most of the clones look fairly similar, being similar to the parent OAW42SR cells, with the exception of SR-LRP-Rz 2 and SR-pHβ 1, which have slightly weaker signals, and SR-LRP-Rz 1, for which there is no visible signal. These results are in keeping with the previous findings, that SR-LRP-Rz 2 and SR-pHβ 1 have the lowest levels of LRP mRNA (see above), except for that of SR-LRP-Rz 1. However, when the levels of LRP expression are compared with the control gene GAPDH (Figure 3.2.2.2 (C)) it is evident that there was far less SR-LRP-Rz 1 RNA loaded than the other clones. The signal for the LRP positive control SW1573-2R120 was clearly visible, being much stronger than the other signals even though the GAPDH signal for this sample was of similar strength to the OAW42SR clones.

Figure 3.2.2.2 (b) shows the results of the hybridisation of the slot blot with a mdr-1 probe. The background over a number of the bands was quite strong, clouding somewhat the ability to judge the signal strength. However, the essential point is that none of the clones appear to exhibit lower mdr-1 expression levels than the

Figure 3.2.2.2 Slot Blot analysis of mRNA expression in OAW42SR clones



OAW42SR parental cells. This is clearly evident for SR-LRP-Rz 2 , which displays mdr-1 levels equal to that of the other ribozyme clones and the control plasmid transfectant. This is especially important, as SR-LRP-Rz 2 displayed slightly decreased mdr-1 levels, as determined by mdr-1 RT-PCR (Section 3.2.1.2).

The same slot blot was used to examine MRP mRNA expression levels. However, the MRP signal strength was, as expected in OAW42SR cells, too weak to be visible by this method (result not shown).

Figure 3.2.2.3 (a) shows the results of probing 1μg mRNA from the OAW42SR clones with a [α-^{32}P] labelled RNA probe (Riboprobe) against LRP. The LRP band intensity varies between the clones, but is clearly much stronger as compared to the use of a DNA LRP probe (Figure 3.2.2.1 (a)). The weakest signals are present in the SR-LRP-Rz 2 and SR-pHβ 1 samples. The band for OAW42SR was also relatively weak, while the signals for SR-LRP-Rz 1 and SR-pHβ 3 are very strong, even compared to that of the positive control sample, 2R120. These results are reflected in the densitometry values shown. However, probing with the internal control GAPDH (Figure 3.2.2.3 (b)) showed that there was unequal loading of samples. When the densitometry readings for the LRP bands were normalised to those for GAPDH (Figure 3.2.2.3 (c)), it can be seen that SR-LRP-Rz 2 exhibits a greatly reduced level of LRP expression as compared to the parental cells and the other clones. This points towards the effectiveness of the anti-LRP ribozyme in reducing cellular LRP mRNA levels in this clone. However, while the signals for SR-LRP-Rz 4 and SR-pHβ-1 are also reduced compared to the OAW42SR cells, it is only a slight reduction. The signal for SR-LRP-AS 1 is actually slightly increased compared to the parental cells, as is SR-LRP-Rz 1.

Figure 3.2.2.4 (a) and (b) show the results of probing 1 μg of mRNA from the OAW42S clones with a LRP riboprobe and a GAPDH probe respectively. As can be seen, there are no visible bands for either OAW42S or 42S-LRP-Rz 2 for either LRP or GAPDH. Therefore, no comparisons can be made with the rest of the clones. However, it can be seen from Figure 3.2.2.4 (a), that the LRP signal for 42S-LRP-AS

Figure 3.2.2.3 Northern Blot and Densitometry analysis of LRP expression in OAW42SR clones

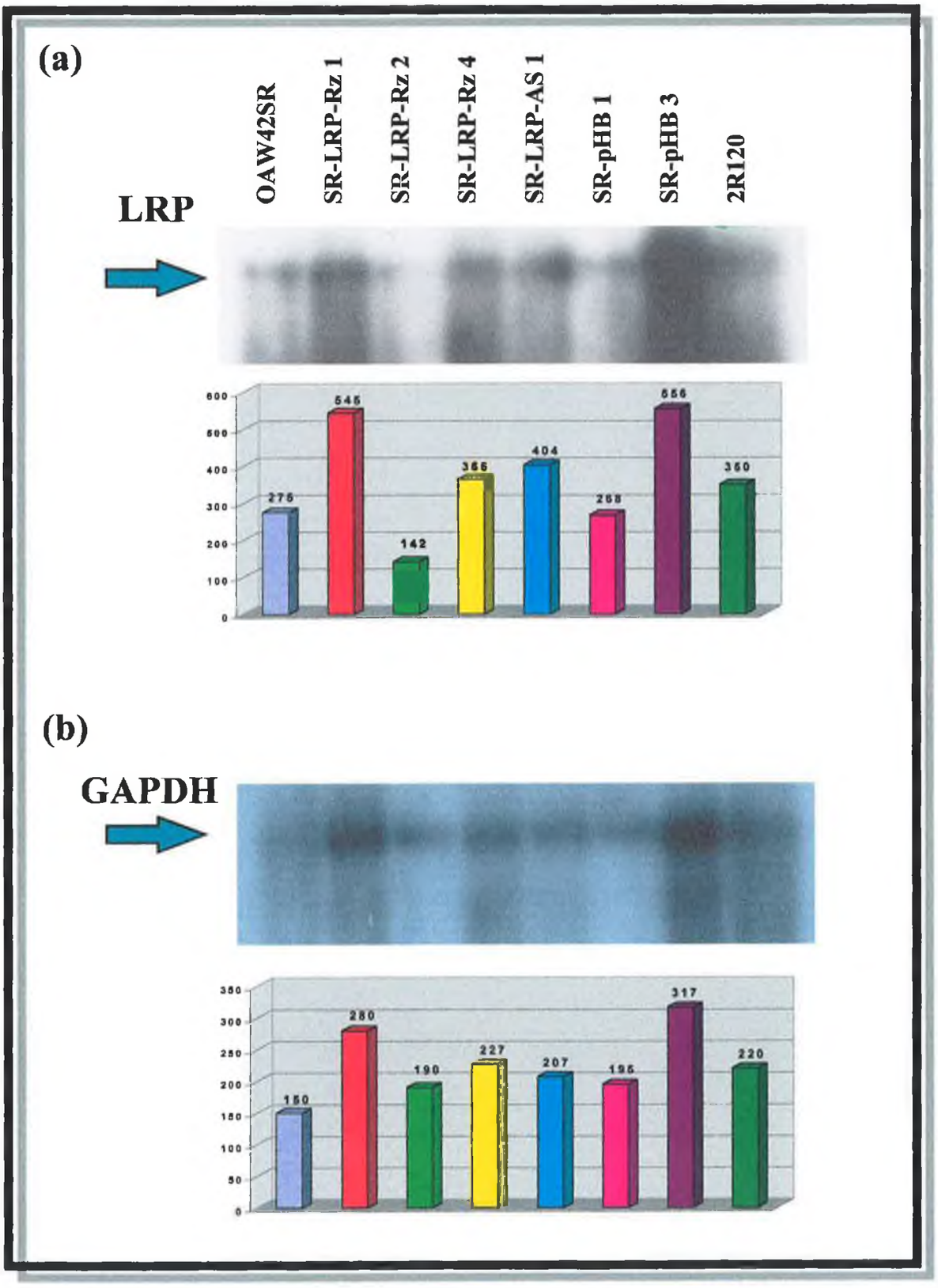


Figure 3.2.2.3 (cont'd) Northern Blot and Densitometry analysis of LRP expression in OAW42SR clones

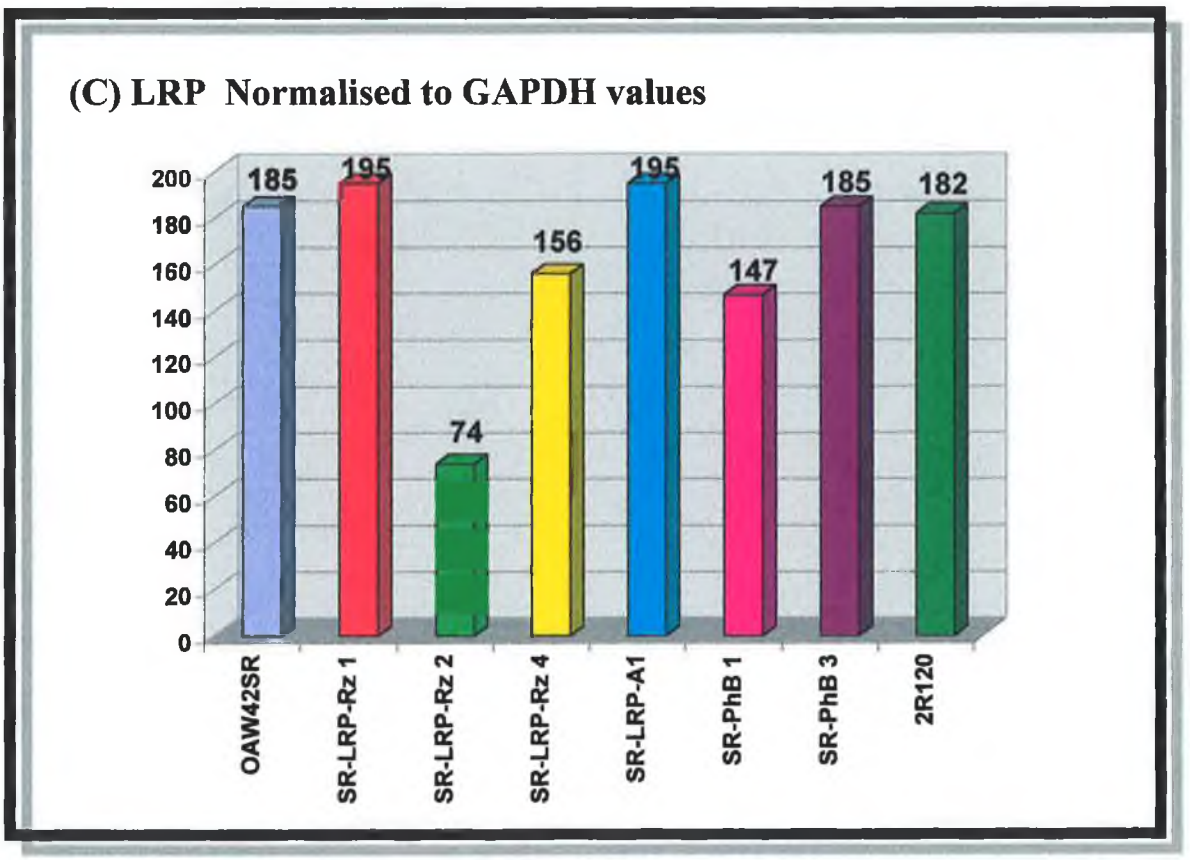
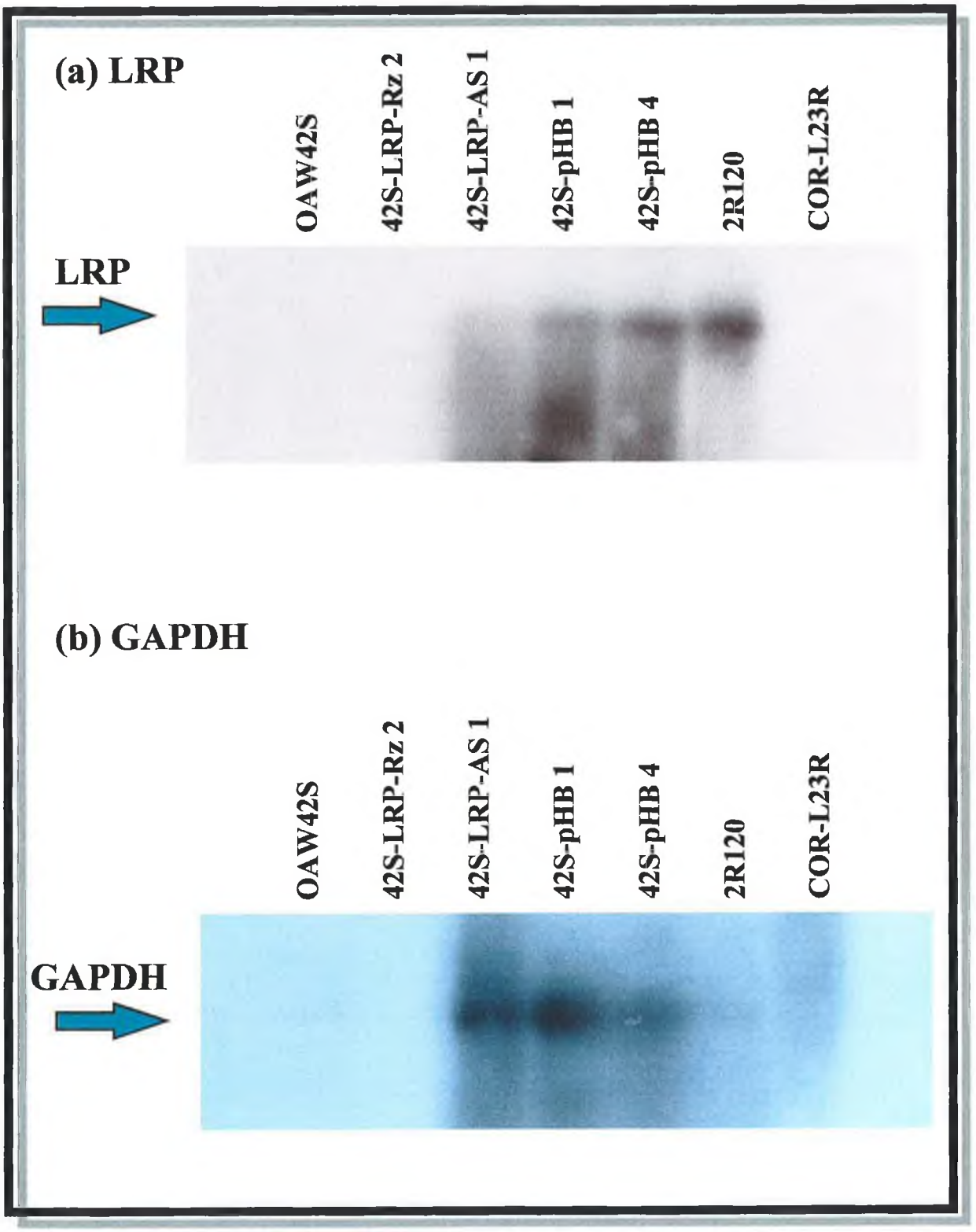


Figure 3.2.2.4 Northern Blot and Densitometry analysis of LRP expression in OAW42S clones



1 is much weaker than for 42S- pHβ-1 or 42S- pHβ-4. The signal for the positive control (2R120) is the strongest, as expected. This result is more significant when the levels of GAPDH are taken into account. Figure 3.2.2.4 (b) shows that 42S-LRP-AS 1 has comparable amounts of GAPDH to 42S- pHβ-1 and 4, indicating equal loading of mRNA samples.

3.2.2.1 Assessment of LRP mRNA expression

The above results show that a reduction in LRP mRNA is only evident in SR-LRP-Rz 2, SR-LRP-Rz 4 and SR-pHβ 1. The marked reduction in LRP expression in SR-LRP-Rz 2 demonstrates the ability of the ribozyme to inhibit LRP expression. The small reduction in LRP mRNA in SR-pHβ 1 indicates that the clonal variation already observed (Section 3.1.4) is also exhibited at the transcriptional level, and is not due solely to post-transcriptional modifications. The absence of a reduction in LRP mRNA for the antisense transfectant, SR-LRP-AS 1, is not totally unexpected. Antisense RNA cannot elicit the action of RNase H when bound to the target RNA and acts predominantly through steric inhibition of the translation process (see Section 1.4). Therefore, reduced protein levels are not necessarily reflected by reduced RNA levels.

3.2.3 Immunocytochemical Analysis of OAW42SR cytospins

Cytospins of the various clones were once again stained by immunocytochemistry with the LRP-56 MAb, to reconfirm the earlier findings for LRP protein expression levels (Section 3.1.3). Staining was repeated a minimum of three times on different cytospin preparations. The average level of LRP staining is indicated in Table 3.2.3. As can be seen, the pattern of staining is very similar to that found previously (Table 3.1.3.1). The clones exhibiting the lowest levels of LRP expression are once again SR-LRP-Rz 2, SR-LRP-AS 1 and SR-pHβ-1. The clone SR-LRP-Rz 4 shows a slight reduction in staining intensity, while the other clones are all largely unchanged from the parental OAW42SR cells.

Cytospins of the OAW42SR clones were also stained with an mdr-1 monoclonal antibody isolated from mouse ascites (see section 2.9.7.1). The staining patterns are shown in Table 3.2.3. Photographs taken of the staining patterns for OAW42SR, SR-LRP-Rz 2 and SR-pHβ-3 are shown in Figure 3.2.3.2 and are representative of the staining patterns observed. It can be seen from Table 3.2.3 and Figure 3.2.3.2 that the level of mdr-1 staining is similar in all of the clones and in the parental cells. Therefore it appears that the levels of mdr-1 protein expression are unaltered in any of the clones. This reflects the findings in Section 3.2.2 of unchanged levels of mdr-1 mRNA expression in the OAW42SR clones

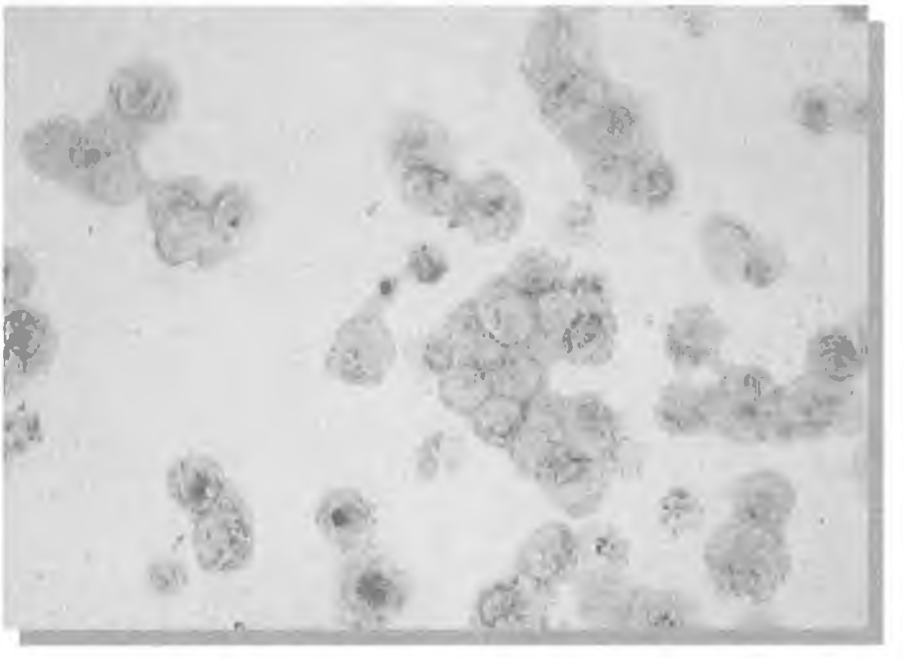
Immunocytochemical staining of cytospins with a MRP antibody was not carried out due to the high background and associated 'stickiness' of the MRP antibody when used for immunocytochemistry.

Table 3.2.3 Immunocytochemical Staining of OAW42SR clone cytospins

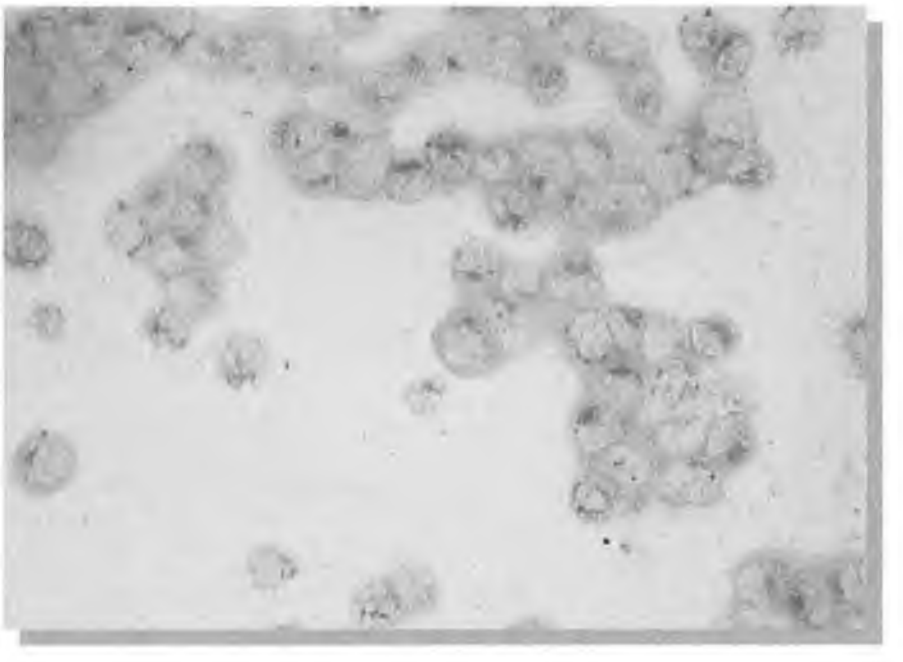
Clone	LRP-56 MAb	mdr-1 MAb
OAW42SR	++/+++	+++
SR-LRP-Rz 1	+++	+++
SR-LRP-Rz 2	+	+++
SR-LRP-Rz 4	++	+++
SR-LRP-AS 1	+	+++
SR-pHβ 1	+	+++
SR-pHβ 3	++/+++	+++
SW1573-2R120	+++	

Figure 3.2.3.2 Immunocytochemical staining of OAW42SR clone cytospins with mdr-1 MAb

(a)



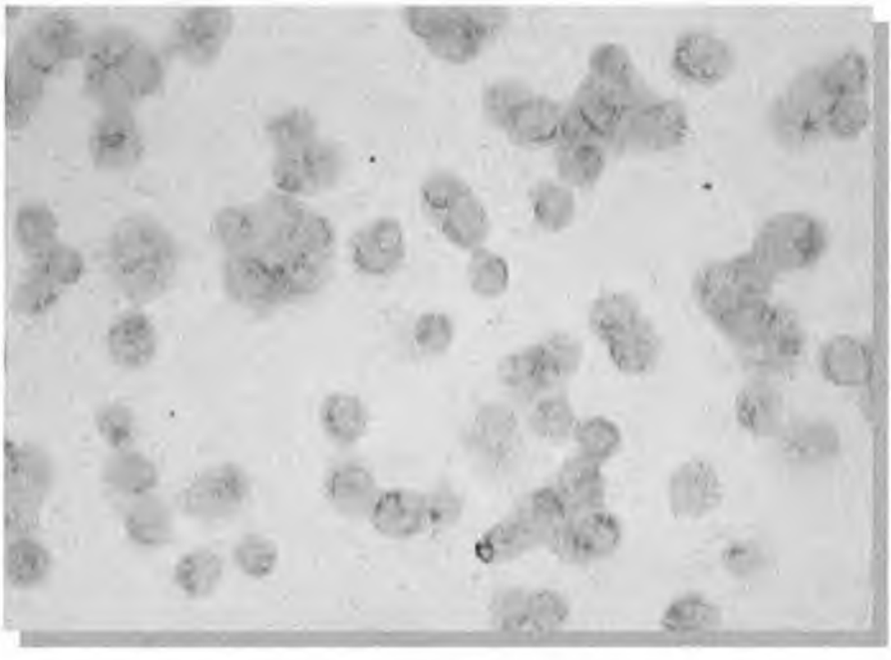
(b)



(a) OAW42SR; (b) SR-LRP-Rz 2

Figure 3.2.3.2 (cont'd) Immunocytochemical staining of OAW42SR clone cytospins with mdr-1 MAb

(c)



(C) SR-pHβ 3

3.2.4 Analysis of LRP expression level by Immunoprecipitation and Western Blotting.

The LRP protein was immunoprecipitated from extracts of 10^7 cells from each clone on at least three separate occasions as described earlier (Section 2.9.6). The precipitates were subsequently electrophoresed on a 7.5% polyacrylamide gel, which was blotted onto PVDF membrane. This membrane was blocked with a bovine serum albumin (BSA) solution, probed with an anti-biotin antibody and developed with either ECL reagent or SuperSignal Ultra prior to exposure to X-ray film (see Sections 2.9.3-2.9.5). 120kD protein size markers were run simultaneously during electrophoresis as a size reference for the 110kD LRP band.

The results from two repeats of the entire procedure for both OAW42SR and OAW42S clones are shown in Figures 3.2.4.1 to 3.2.4.4. Densitometry analysis was carried out on the LRP bands to determine their intensity and allow comparison between expression levels. All the densitometry results are given in arbitrary values, and the value for each band was normalised against the background staining.

Figure 3.2.4.1 shows the results of the first repeat of the immunoprecipitation from OAW42SR clones. In Figure 3.2.4.1 (a), a large reduction in LRP protein expression can be clearly seen for both SR-LRP-Rz 2 and SR-LRP-AS 1, and to a lesser extent SR-LRP-Rz 4. This reduction is reflected in the densitometry analysis (Figure 3.2.4.1 (b)), where SR-LRP-Rz 2 has a value of 36 units, while the parent OAW42SR has a value of 306 units, reflecting an almost 10-fold reduction in LRP protein expression levels. The clone SR-LRP-AS 1 exhibits an even larger reduction with a value of only 12 units, while SR-LRP-Rz 4 is given a value of 121 units. The level of LRP expression appears only slightly reduced in SR-LRP-Rz 1 and largely unchanged in SR-pHβ-1. However, SR-pHβ-3 does exhibit a marked reduction in LRP expression. The lane containing the sample from SR-pHβ-1 appears to be overloaded, judging by the strength of the background signal. No judgement should be passed, therefore, on this result alone.

Figure 3.2.4.1 Immunoprecipitation and Densitometry analysis of LRP expression in OAW42SR clones : Repeat 1

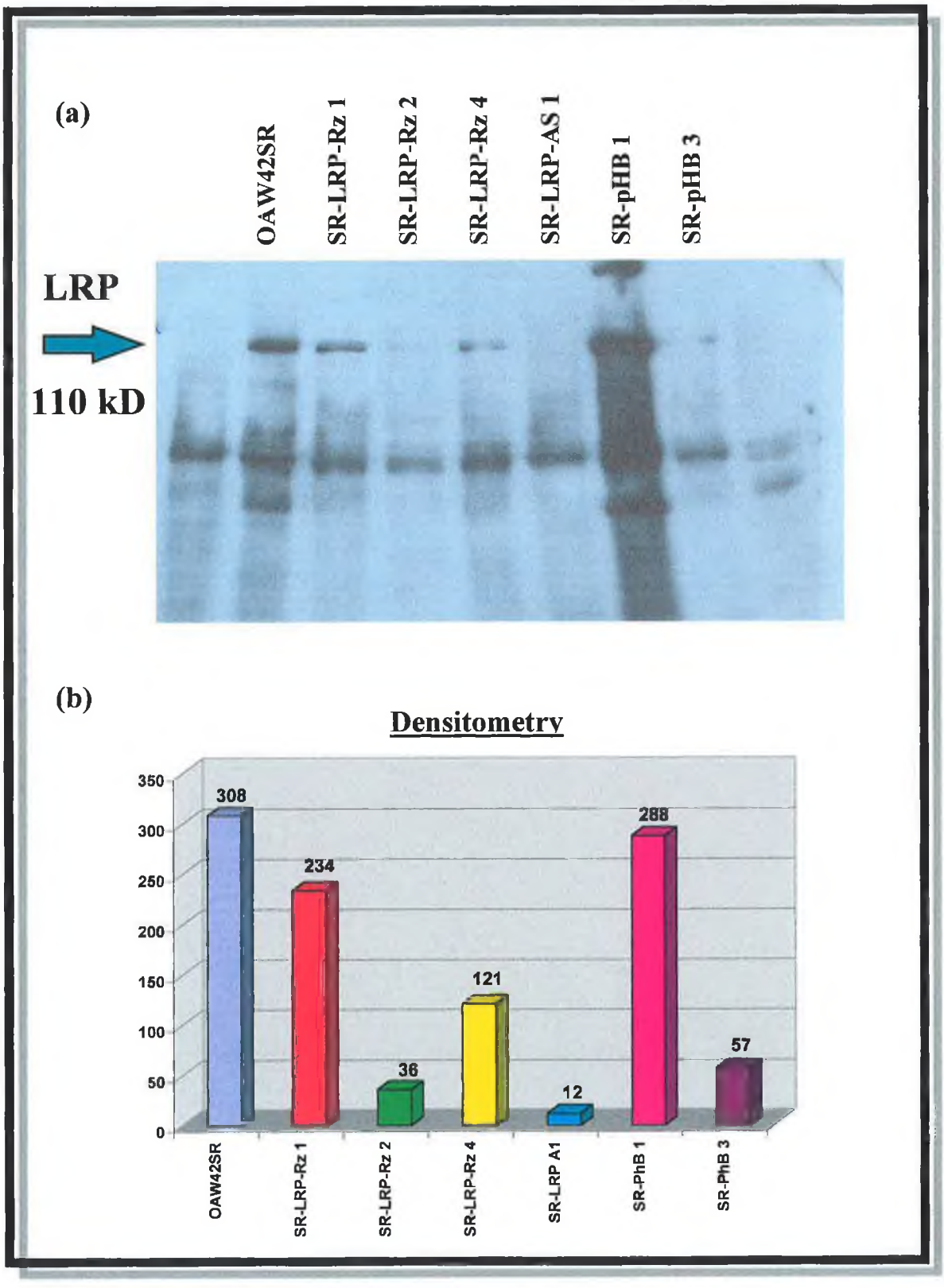


Figure 3.2.4.2 Immunoprecipitation and Densitometry analysis of LRP expression in OAW42SR clones : Repeat 2

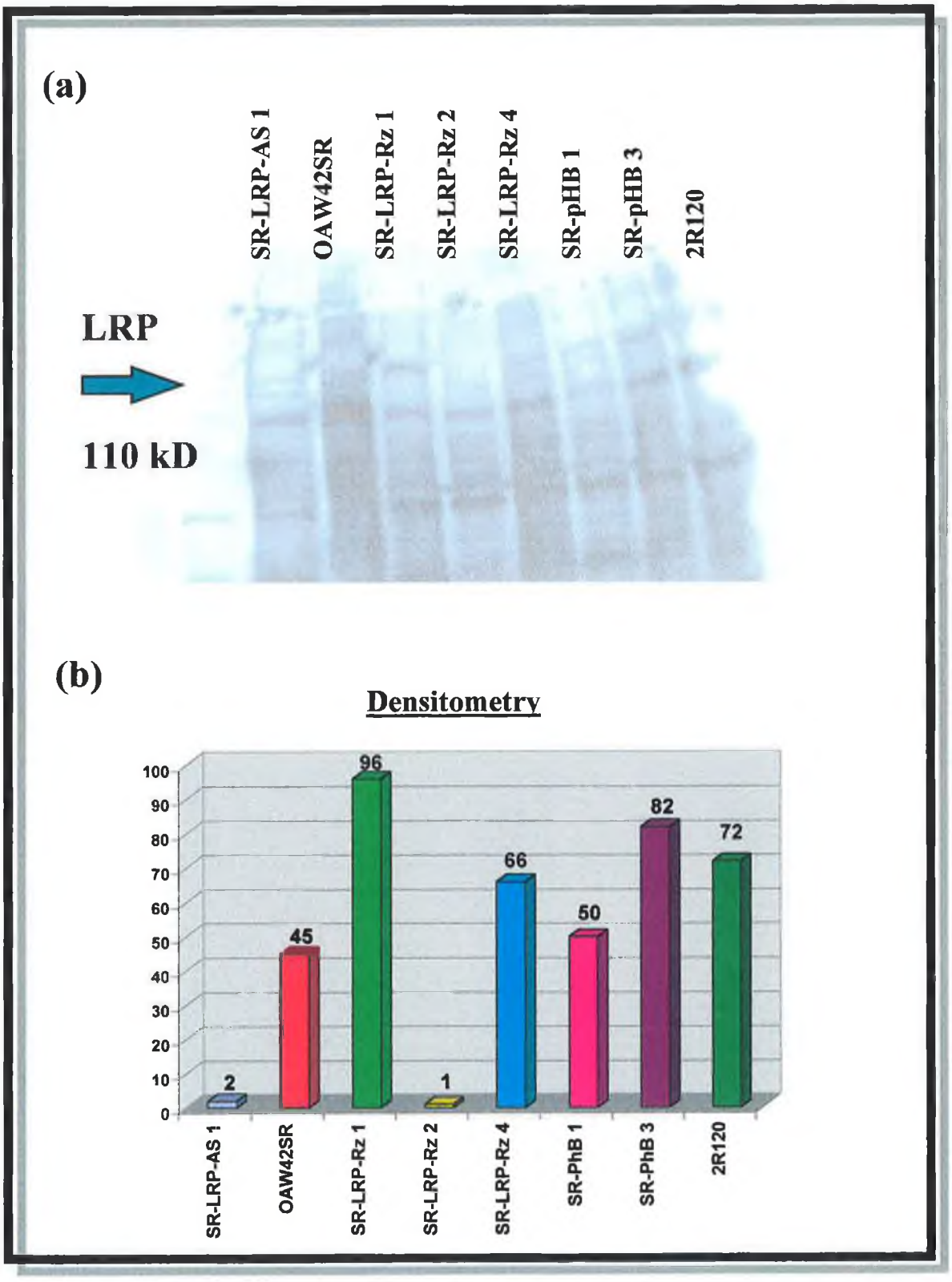


Figure 3.2.4.3 Immunoprecipitation and Densitometry analysis of LRP expression in OAW42S clones ; Repeat 1

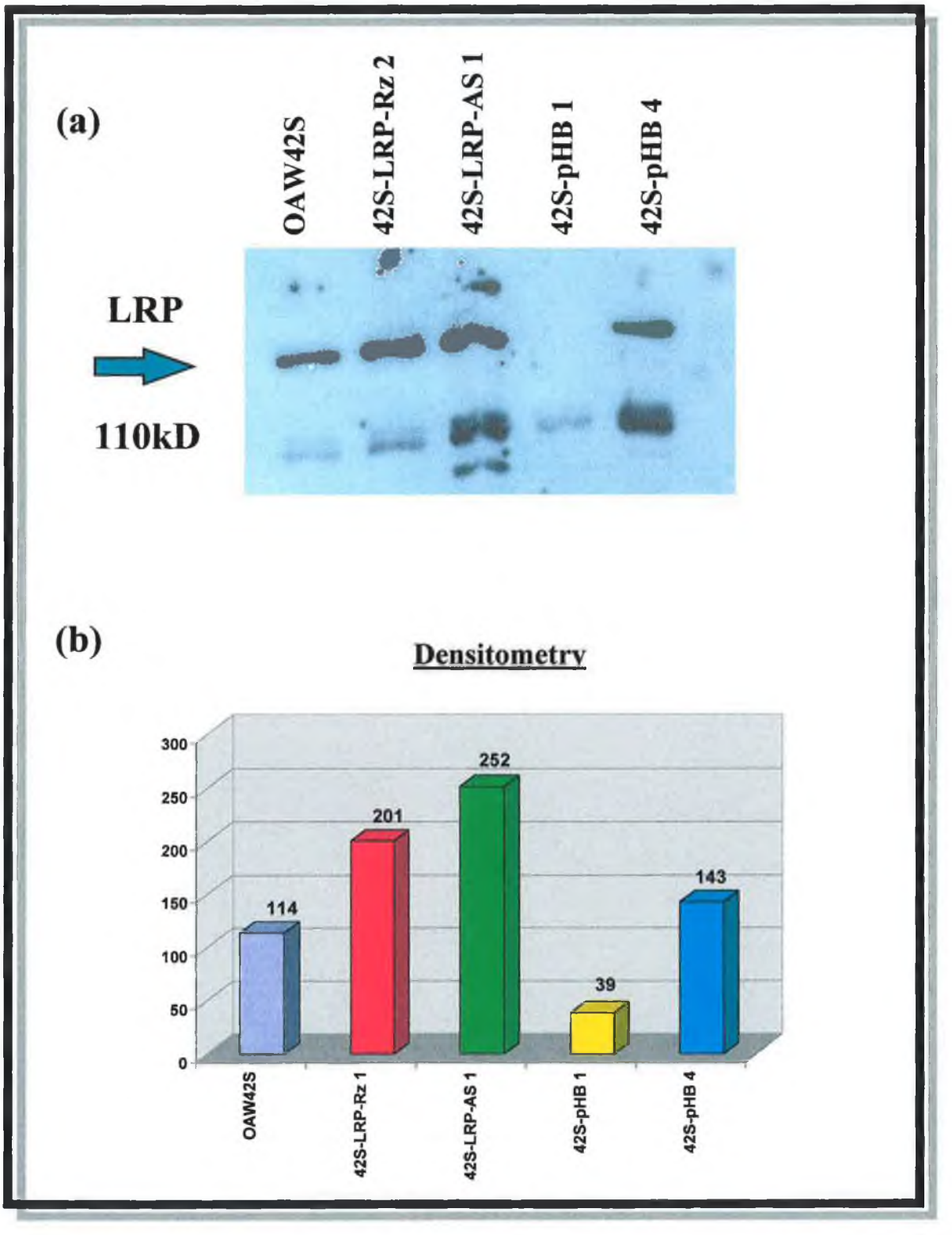
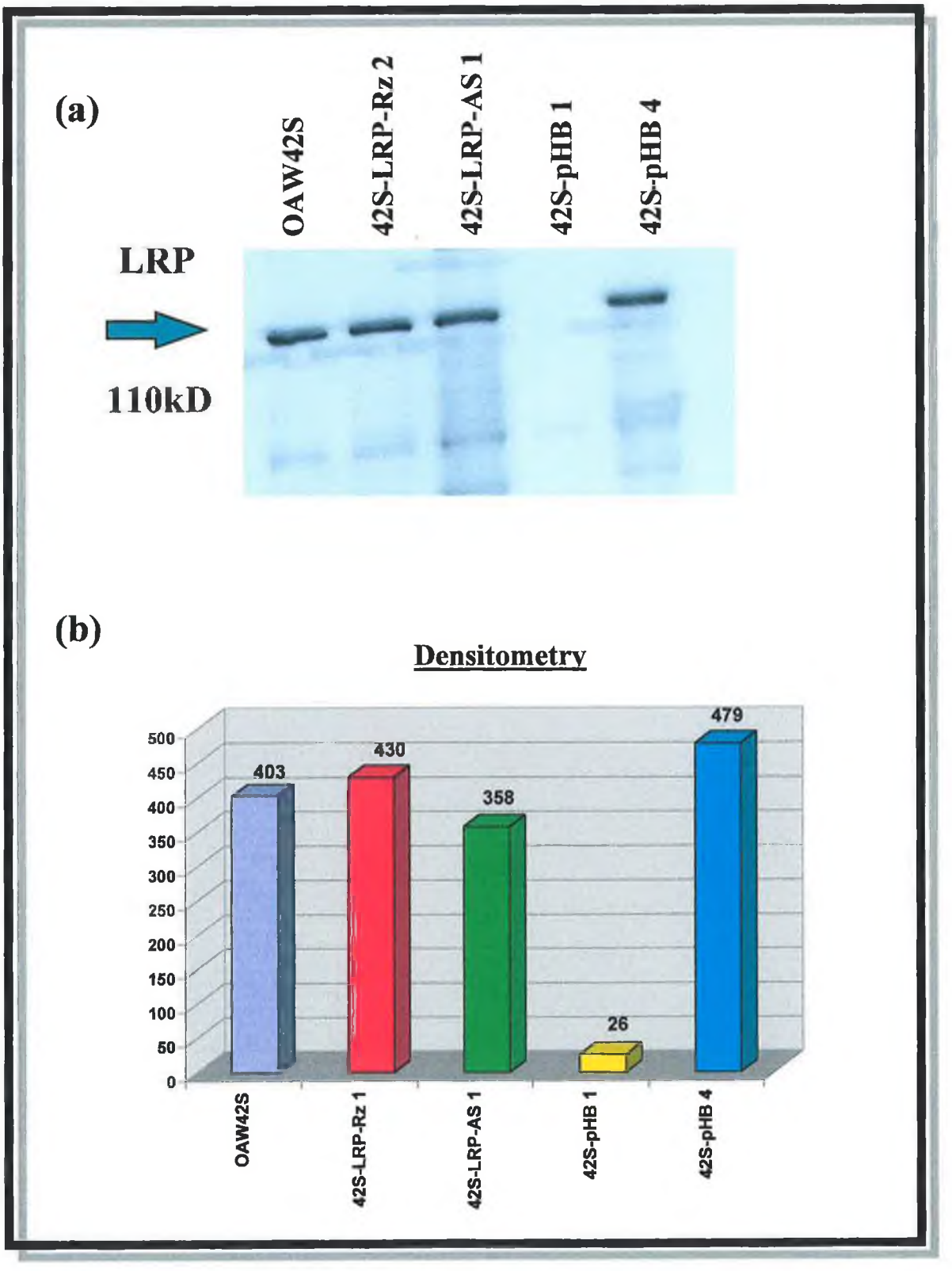


Figure 3.2.4.4 Immunoprecipitation and Densitometry analysis of LRP expression in OAW42S clones : Repeat 2



The results of another repeat of the procedure are shown in Figure 3.2.4.2. Once again there are significant reductions in LRP expression levels in SR-LRP-Rz 2 and SR-LRP-AS 1 as compared to the parental cells and the other clones. However, there are a number of variations from the previous result. SR-LRP-Rz 1, SR-LRP-Rz 4 and SR-pHβ-3 all exhibit increased LRP expression over the parental cells, while SR- pHβ-1 shows a similar level to the OAW42SRs.

Figures 3.2.4.3 and 3.2.4.4 show the results from two repeats of the immunoprecipitation/western blot procedure on the OAW42S clones, along with the densitometry analysis. The expected decrease in LRP expression for 42S-LRP-Rz 2 and 42S-LRP-AS 1, is not evident. In fact, there appears to be an increase in LRP expression in both of these clones, despite the lack of LRP expression as detected by immunocytochemistry (section 3.1.3). There is no band evident for 42S- pHβ-1. This is reflected in the second repeat of this procedure (Figure 3.2.4.4), where there is once again no band for 42S- pHβ-1. However, the levels of LRP expression in the other clones and the parent cells all appear similar, possibly indicating unequal protein loading in Figure 3.2.4.3.

3.2.5 Analysis of MDR1 and MRP protein levels by Western Blotting

Cell extracts were made from each of the OAW42SR and OAW42S clones and electrophoresed on a 7.5% polyacrylamide gel, which was blotted onto PVDF membrane. This membrane was blocked with a 5% marvel (milk powder) solution and probed with an anti-mdr-1 or anti-MRP primary antibody. Subsequently the membrane was exposed to a secondary antibody and developed with either ECL reagent or SuperSignal Ultra prior to exposure to X-ray film (see Sections 2.9.3-2.9.5). Protein size markers were run simultaneously during electrophoresis as a size reference for the mdr-1 and MRP bands.

Figures 3.2.5.1 and 3.2.5.2 show the results of representative western blots of OAW42SR and OAW42S cell extracts respectively, with the mdr1 MAb. As can be seen from Figure 3.2.5.1, there are some variations in the levels of mdr-1

Figure 3.2.5.1 Western Blot and Densitometry analysis of mdr-1 expression in OAW42SR clones

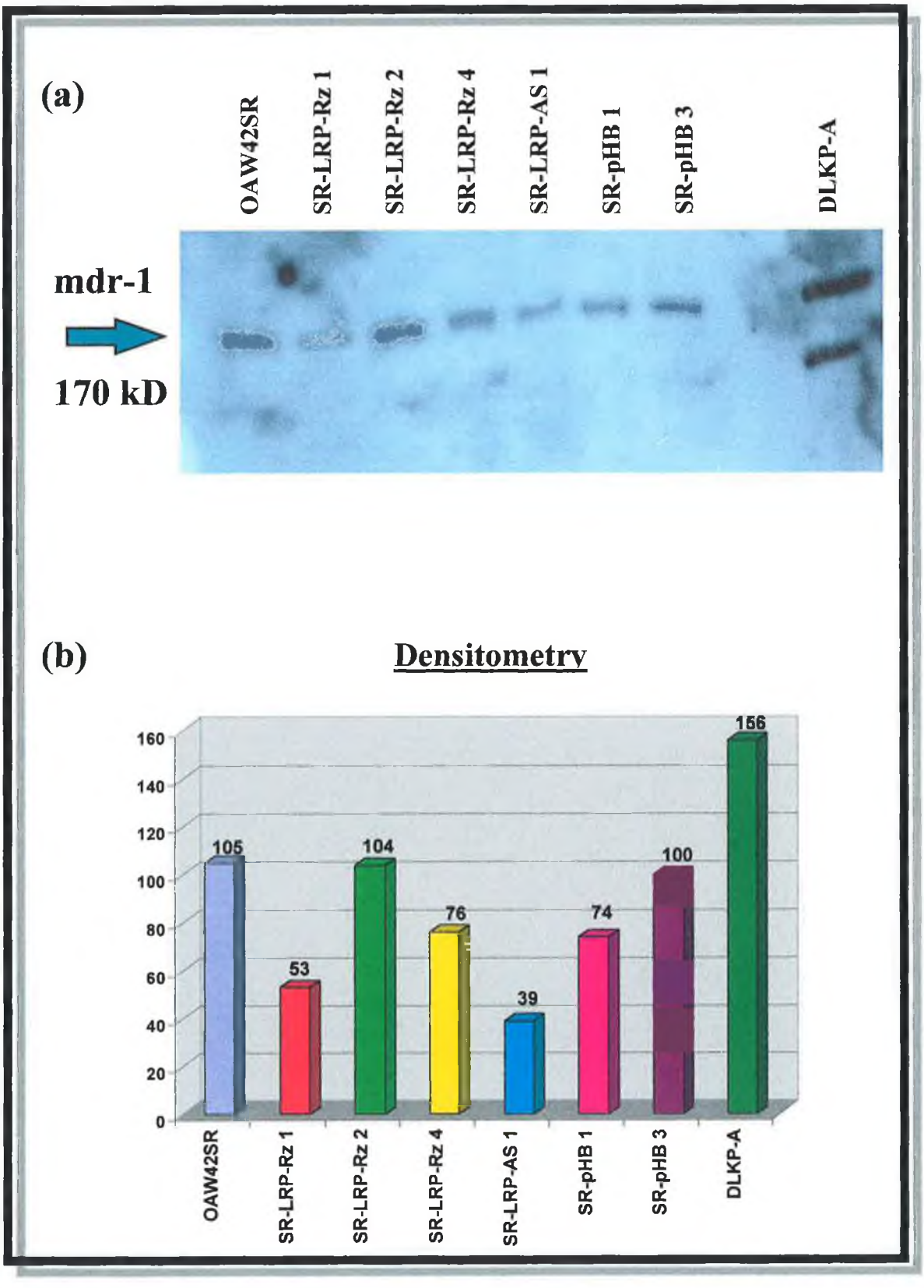


Figure 3.2.5.2 Western Blot and Densitometry analysis of mdr-1 expression in OAW42S clones

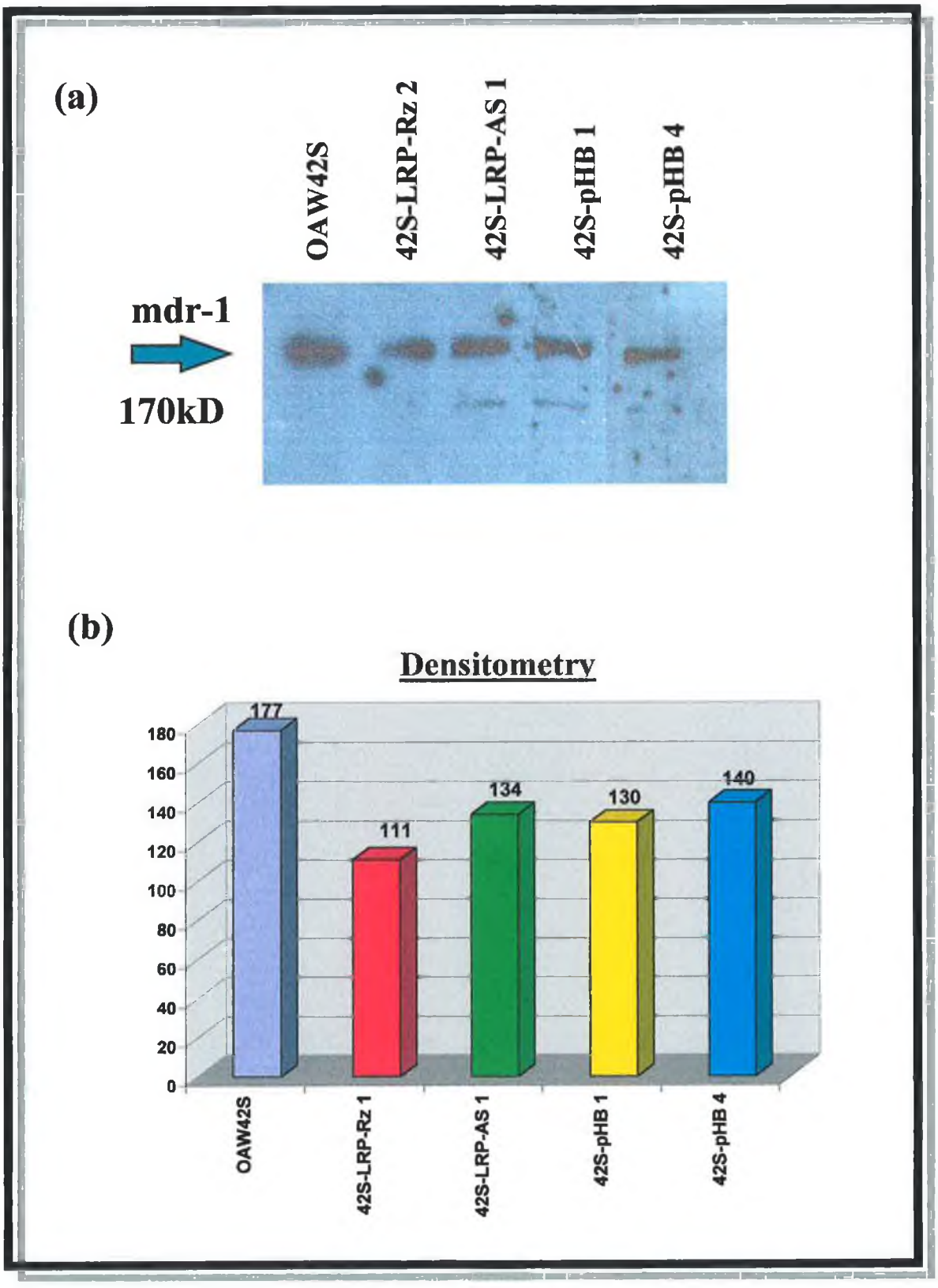


Figure 3.2.6.1 Western Blot and Densitometry analysis of MRP expression in OAW42SR clones

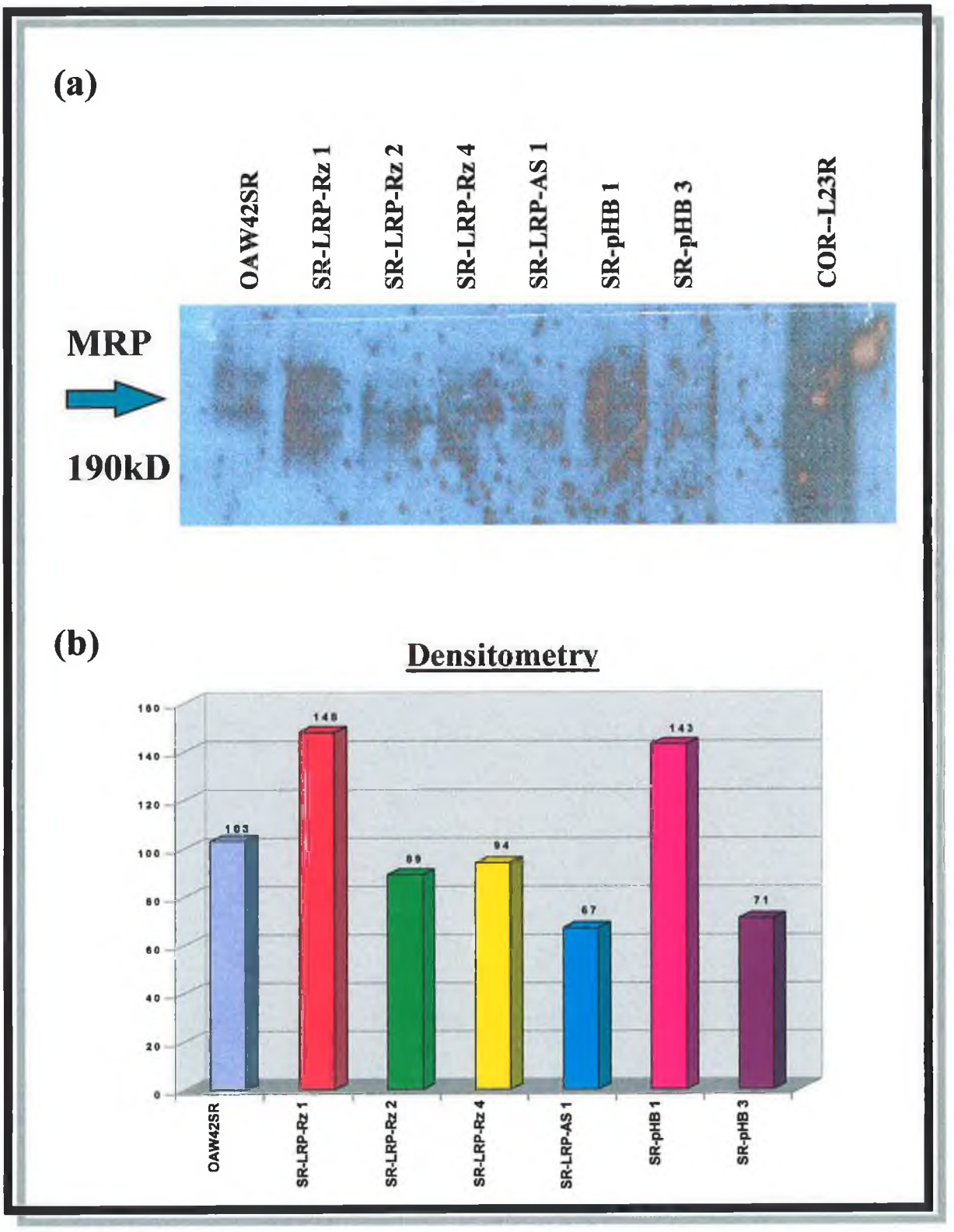
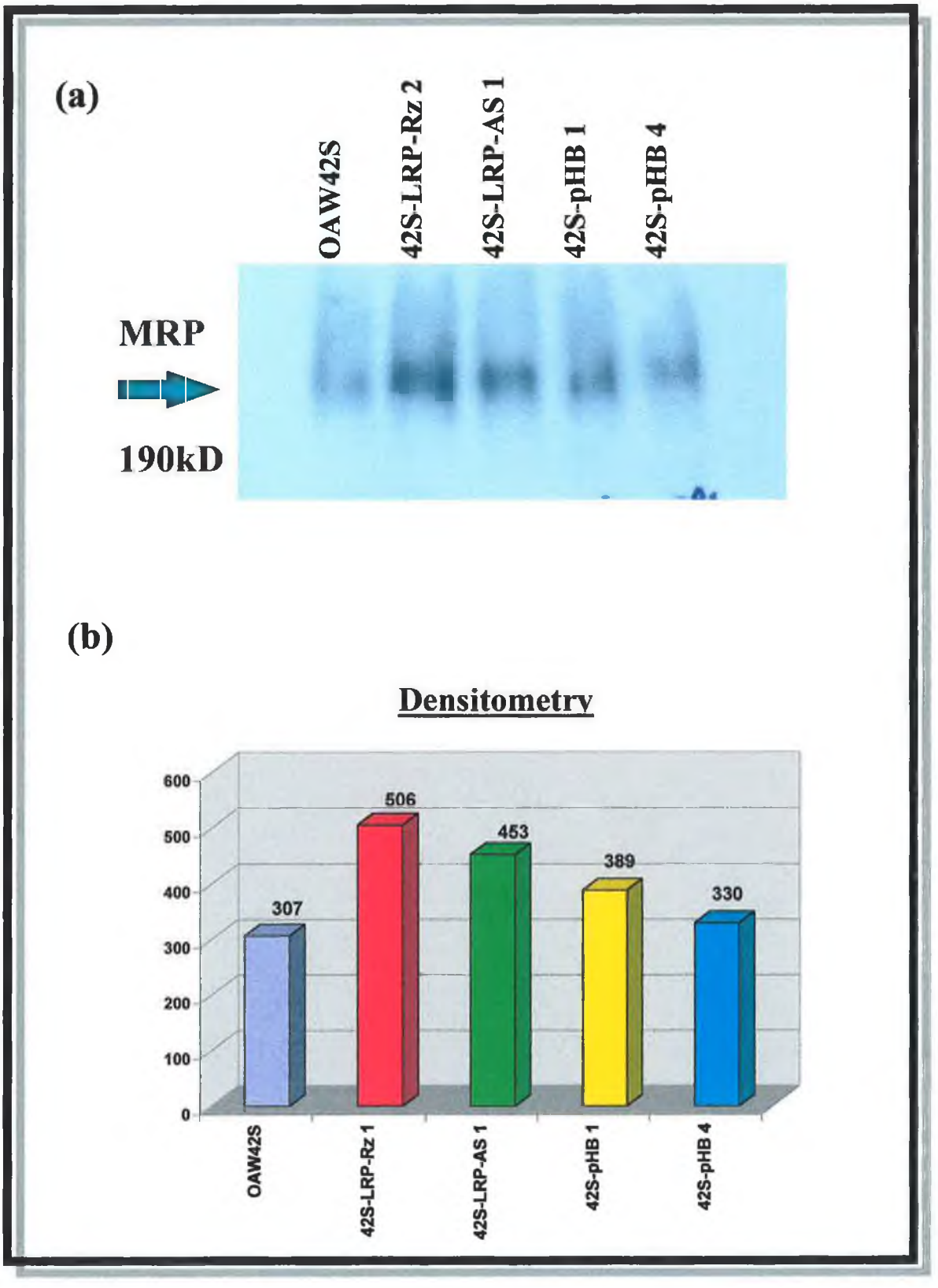


Figure 3.2.6.2 Western Blot and Densitometry analysis of MRP expression in OAW42S clones



expression, as reflected in the densitometry values. However, only the SR-LRP-Rz 1 and SR-LRP-AS 1 clones appear to have decreased MDR-1 expression as compared to parental OAW42SRs. The clone SR-LRP-Rz 2, which appeared to have slightly reduced levels of mdr-1 mRNA as determined by RT-PCR (Section 3.2.1.2), appears to have slightly greater MDR-1 protein expression than the OAW42SRs. These results reflect the findings from immunocytochemistry (Section 3.2.3) where no significant decrease in MDR-1 protein levels were observed in the clones as compared to the parental cells. The positive control cell line DLKP-A, which expresses high levels of mdr-1, gave a very strong signal as expected.

Similarly for the OAW42S clones (Figure 3.2.5.2), there appears to be no significant differences in the levels of MDR1 expression between the clones and the parental OAW42S cells.

The results for the western blot with the MRP MAb for OAW42SR and OAW42S clones are shown in Figure 3.2.5.5 and 3.2.5.6 respectively. From Figure 3.2.5.5, it can be seen that there are only slight decreases in MRP expression levels for SR-LRP-AS 1 and SR-pHβ 3, as compared to the parental cells, while there is a slight increase in the clones SR-LRP-Rz 1 and SR-pHβ 1. MRP expression appears unchanged in SR-LRP-Rz 2 and 4. The MRP-positive control cell line COR-L23R, gave an intense band as expected.

Figure 3.2.5.6, shows that all of the OAW42S clones have a slightly higher level of MRP expression compared to the untransfected parents, with 42S-LRP-Rz 2 exhibiting the highest levels.

3.2.6 Assessment of LRP, Pgp and MRP protein expression levels

The above results clearly demonstrate that the clones SR-LRP-Rz 2 and SR-LRP-AS 1 have dramatically reduced levels of LRP protein expression. This highlights the efficacy of the ribozyme and antisense constructs in inhibiting LRP expression. In the first repeat of the immunoprecipitation procedure, the clones SR-LRP-Rz 4 and SR-

pHβ-3 exhibited reductions in LRP expression, as compared to the parental cells. In the second repeat, however, no reduction is evident. The expression level of LRP in the OAW42SR parental cells appears to be reduced in relation to most of the clones in this second repeat. It has been noted that low passage numbers (passage 86) of OAW42SR cells contain low levels of LRP, and that with increasing passage number (over passage 92) an increase in LRP expression levels is paralleled by an increase drug-resistance (Moran *et al.*, 1997). It has also been observed in this laboratory (data not shown) that when the OAW42SR population is passaged a certain number of times (110-115), the LRP expression levels appear to decrease gradually. It may be that, above a certain number of passages, the cells are once again reverting to the original low level of LRP expression. The OAW42SR cells used in the second repeat of the immunoprecipitation procedure were at passage number 112, and may have been exhibiting this lowering of LRP levels. This should be taken into account when comparing the strength of LRP expression. The clone SR-LRP-Rz 1 exhibited little change in LRP expression compared to the parental cells, as measured by immunocytochemistry and the first repeat of the immunoprecipitation. This clone was, therefore, used as a standard against which to compare LRP expression in the second repeat of the immunoprecipitation. In comparison to SR-LRP-Rz 1, both SR-LRP-Rz 4 and SR-pHβ 3 maintain a reduction in LRP expression. SR-pHβ 1 also exhibits a reduction in LRP levels in comparison to SR-LRP-Rz 1. There was little variation in the LRP expression in the OAW42S clones.

The decreased LRP expression in the control vector clones, once again illustrates the clonal variation that exists within the OAW42SR population. It is therefore possible that some of the reductions in LRP seen for SR-LRP-Rz 2 and SR-LRP-AS 1, and indeed all the other clones, are inherent in the cells and are not caused by ribozyme or antisense expression. Although this clonal variation masks, somewhat, the efficacy of the ribozyme and antisense, it nonetheless provides a number of clones with varying levels of LRP expression with which to correlate drug resistance. The clonal variation is also highlighted by the small variations in Pgp and MRP expression between the clones.

The level of LRP protein expression in the OAW42SR ribozyme transfectants appears

to correlate quite well with the levels of LRP mRNA, as measured by northern blot (Section 3.2.2). SR-LRP-Rz 1 exhibits high LRP mRNA and protein levels while SR-LRP-Rz 2 exhibits low mRNA and protein expression. SR-LRP-Rz 4 displayed both mRNA and protein levels that were intermediary between the above two. SR-pHβ 3 displays similar LRP mRNA and protein levels to SR-LRP-Rz 4, while SR-pHβ 1 exhibit similar protein but slightly lower mRNA levels. The only clone for which LRP mRNA and protein levels do not correlate is SR-LRP-AS 1. This clone exhibits minimal reduction in LRP mRNA, but an almost total elimination of protein expression. This is thought to be due to the fact that antisense RNA functions mainly through steric inhibition of translation rather than cleavage of target RNA.

3.2.7 Analysis of Drug toxicity profile of LRP protein.

3.2.7.1 Comparison of IC50 values for SR-LRP-Rz 1 and SR-LRP-Rz 2

In an attempt to establish which, if any, cytotoxic drugs have their efficacy altered by LRP expression, a range of drugs was tested on the clones SR-LRP-Rz 1 and SR-LRP-Rz 2. It appears from results shown above (Sections 3.2.1-3.2.5) that LRP expression is down-regulated at both the RNA and protein level in SR-LRP-Rz 2, while being largely unaltered in SR-LRP-Rz 1. In addition, the results also demonstrate only slight differences in mdr-1 and MRP expression. These two clones were therefore deemed appropriate for examination of any role LRP might play in drug resistance.

Table 3.2.7.1 shows the IC50 values of a range of drugs, averaged over three repeats, for the two clones. From this, it can be seen that the drugs to which SR-LRP-Rz 1 displays a higher level of resistance to are: adriamycin, vincristine, VP-16, taxotere, daunorubicin, taxol and epirubicin. The fold differences in resistance between the two clones vary from a maximum of 37-fold for Vincristine, to a minimum of over 3-fold for VP-16. There is no significant difference in the IC50 values of either clone for 5-Fluoro-Uracil, Melphalan and carboplatin. It therefore appears that 5-Fluoro-Uracil, Melphalan and carboplatin are largely unaffected by the expression of LRP.

Table 3.2.7.1 Toxicity Assay drug IC_{50} values (μg/ml) for SR-LRP-Rz1 and SR-LRP-Rz2

DRUG	SR-LRP-Rz 1	SR-LRP-Rz 2	FOLD CHANGE IN RESISTANCE
ADRIAMYCIN	0.165 ± 0.007	0.0165 ± 0.0007	10
VINCRISTINE	0.16 ± 0.0	0.00425 ± 0.00007	37.697
VP-16	0.729 ± 0.238	0.198 ± 0.055	3.682
5-FLUOR0-URACIL	1.647 ± 0.261	1.56 ± 0.297	1.056
TAXOTERE	0.00169 ± 0.00023	0.000065 ± 0.0	26.000
MELPHALAN	2.237 ± 0.087	1.865 ± 0.457	1.199
CARBOPLATIN	6.067 ± 0.911	6.512 ± 2.157	0.932
DAUNORUBICIN	0.100 ± 0.012	0.01095 ± 0.00184	9.132
TAXOL	0.0205 ± 0.0052	0.00167 ± 0.00079	12.275
EPIRUBICIN	0.111 ± 0.028	0.00768 ± 0.00031	14.435

Values are given in μg/ml and are averaged over three repeats.

3.2.7.2 Drug Toxicity profiles for OAW42SR and OAW42S clones.

As an additional study, all the OAW42SR and OAW42S clones, were treated with a range of drugs, to test if the level of drug resistance correlated with LRP expression, and if the profile of drugs affected by LRP was the same as shown in Table 3.2.7.1. Tables 3.2.7.2 (a) and 3.2.7.3 (a) show the IC50 values and of various classical MDR and non-MDR drugs for the OAW42SR and OAW42S clones respectively. Tables 3.2.7.2 (b) and 3.2.7.3 (b) give the fold changes in IC50 values between the parental cells, given a value of 1, and the clones. Graphs 3.2.7.1 to 3.2.7.7 also illustrate these results.

As can be seen from Tables 3.2.7.2 (a) and (b) and Graphs 3.2.7.1 to 3.2.7.7, the pattern of drug resistance among the OAW42SR clones was very similar to the one found previously for adriamycin and vinblastine/vincristine alone. The SR-LRP-Rz 2, SR-LRP-Rz 4 and SR-LRP-AS 1 all showed significant decreases in IC50 values compared to the parent cells. However, the resistance of the SR-pHβ 3 clone, which had been included in the assays as a control clone, with apparently unaltered levels of LRP expression, decreased dramatically, down to the level of the SR-LRP-Rz 2 and SR-LRP-AS 1 clones. This is an unusual result, as it previously had displayed a resistance profile similar to that of the parental cells and SR-LRP-Rz 1. The other clones were largely invariant in their resistance to adriamycin and vincristine.

Once again, there appears to be very little difference between the resistance of the clones and the parental cells to 5-fluoro-uracil, carboplatin and melphalan. While only SR-LRP-Rz 2 and 4 display slightly reduced levels of resistance to VP16 as compared to the parental cells, (Tables 3.2.7.2(a) and (b)), all of the clones display a reduction in VP16 resistance in relation to that of SR-LRP-Rz 1.

Table 3.2.7.2 (a) IC_{50} values (μg/ml) for several classical MDR and non-MDR drugs, averaged over three repeats, for the OAW42SR parent and clones.

DRUG	OAW42SR	SR-LRP-Rz1	SR-LRP-Rz2	SR-LRP-Rz4	SR-LRP-AS1	SR-pHβ1	SR-pHβ3
ADRIAMYCIN	0.256 ± 0.0	0.1496 ± 0.0006	0.0234 ± 0.0006	0.0247 ± 0.0012	0.0246 ± 0.0061	0.0305 ± 0.012	0.028 ± 0.004
VINCRISTINE	0.147 ± 0.0	0.145 ± 0.006	0.0021 ± 0.0002	0.0029 ± 0.0004	0.0033 ± 0.0009	0.0017 ± 0.0004	0.0043 ± 0.0011
VP-16	0.128 ± 0.02	0.303 ± 0.168	0.103 ± 0.055	0.083 ± 0.024	0.159 ± 0.087	0.141 ± 0.093	0.107 ± 0.041
5-FLUORO-URACIL	1.464 ± 0.0	1.220 ± 0.444	1.609 ± 0.213	1.524 ± 0.595	1.756 ± 0.308	0.954 ± 0.144	1.146 ±0.689
MELPHALAN	0.51 ± 0.038	1.731 ± 0.0	1.245 ± 0.65	0.565 ± 0.244	0.836 ± 0.595	0.476 ± 0.433	0.586 ± 0.384
TAXOL	0.064 ±0.0	0.062 ± 0.0	0.00090 ± 0.0001	0.0010 ± 0.0003	0.00094 ± 0.00007	0.00129 ± 0.00061	0.00115 ± 0.00005
CARBOPLATIN	2.88 ± 0.0	4.165 ± 0.997	4.66 ± 1.98	4.692 ± 1.041	4.877 ± 0.748	2.284 ± 1.097	3.44 ± 1.194

Table 3.2.7.2 (b) Fold changes in IC50 values between OAW42SR parental cells and clones.

DRUG	OAW42SR	SR-LRP-Rz1	SR-LRP-Rz2	SR-LRP-Rz4	SR-LRP-AS1	SR-pHβ1	SR-pHβ3
ADRIAMYCIN	1	-1.71	-10.94	-10.36	-10.41	-8.39	-9.14
VINCRISTINE	1	-1.01	-70.00	-50.68	-44.55	-86.47	-34.18
VP-16	1	+2.37	-1.24	-1.54	+1.24	+1.10	-1.20
5-FLUORO-URACIL	1	-1.20	+1.10	+1.04	+1.20	-1.53	-1.28
MELPHALAN	1	+3.39	+2.24	+1.11	+1.63	-1.07	+1.15
TAXOL	1	-1.03	-71.11	-64.00	-68.09	-49.61	-55.65
CARBOPLATIN	1	+1.45	+1.62	+1.63	+1.69	-1.26	+1.19

\- indicates a fold decrease in IC50 value
\+ indicates a fold increase in IC50 value

Table 3.2.7.3 (a) IC_{50} values (μg/ml) for several classical MDR and non-MDR drugs, averaged over three repeats, for the OAW42S parental cell line and clones.

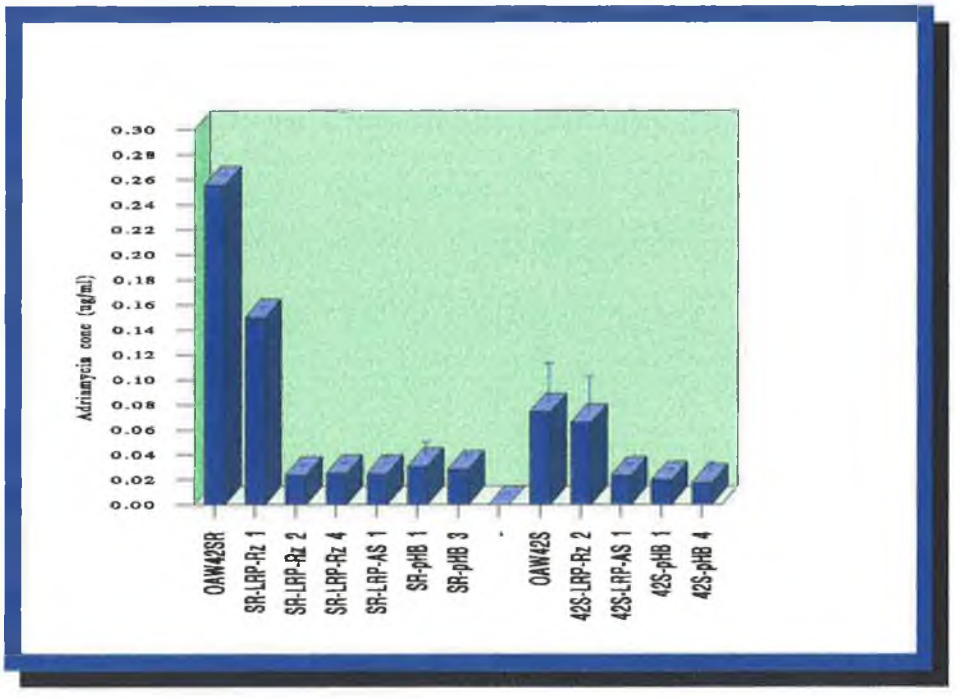
DRUG	OAW42S	42S-LRP-Rz2	42S-LRP-AS1	42S-pHβ1	42S-pHβ4
ADRIAMYCIN	0.0741 ± 0.031	0.0657 ± 0.0289	0.02345 ± 0.0021	0.0198 ± 0.0001	0.175 ± 0.0082
VINCRISTINE	0.00583 ± 0.00181	0.00395 ± 0.00085	0.00304 ± 0.0003	0.00254 ± 0.00068	0.003475 ± 0.00177
VP-16	0.363 ± 0.177	0.207 ± 0.102	0.158 ± 0.030	0.213 ± 0.126	0.147 ± 0.04
5-FLUORO-URACIL	1.866 ± 0.605	1.03 ± 0.587	1.921 ± 0.347	1.424 ±0.748	0.559 ± 0.083
MELPHALAN	2.117 ± 0.270	0.873 ± 0.449	0.709 ± 0.250	1.004 ± 0.506	0.762 ± 0.537
TAXOL	0.00279 ± 0.00121	0.00211 ± 0.0003	0.00168 ± 0.00055	0.00154 ± 0.00096	0.00215 ± 0.00123
CARBOPLATIN	5.259 ± 2.77	5.404 ± 2.39	4.869 ± 3.554	5.121 ± 2.818	4.428 ± 3.482

Table 3.2.7.3 (b) Fold changes in IC50 values between OAW42S parental cells and clones.

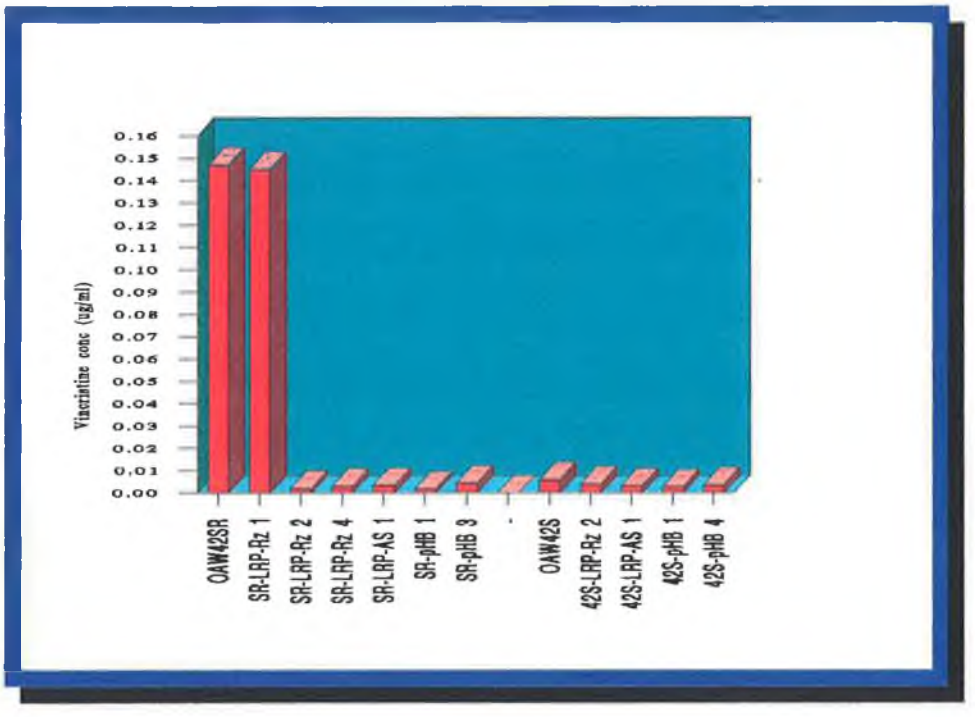
DRUG	OAW42S	42S-LRP-Rz2	42S-LRP-AS1	42S-pHβ1	42S-pHβ4
ADRIAMYCIN	1	-1.13	-3.15	-3.74	+2.36
VINCRISTINE	1	-1.48	-1.92	-2.30	-1.68
VP-16	1	-1.75	-2.30	-1.70	-2.47
5-FLUORO-URACIL	1	-1.81	+1.03	-1.31	-3.12
MELPHALAN	1	-2.42	-2.99	-2.11	-2.78
TAXOL	1	-1.32	-1.66	-1.81	-1.30
CARBOPLATIN	1	+1.03	-1.08	-1.03	-1.19

- indicates a fold decrease in IC50 value
+ indicates a fold increase in IC50 value

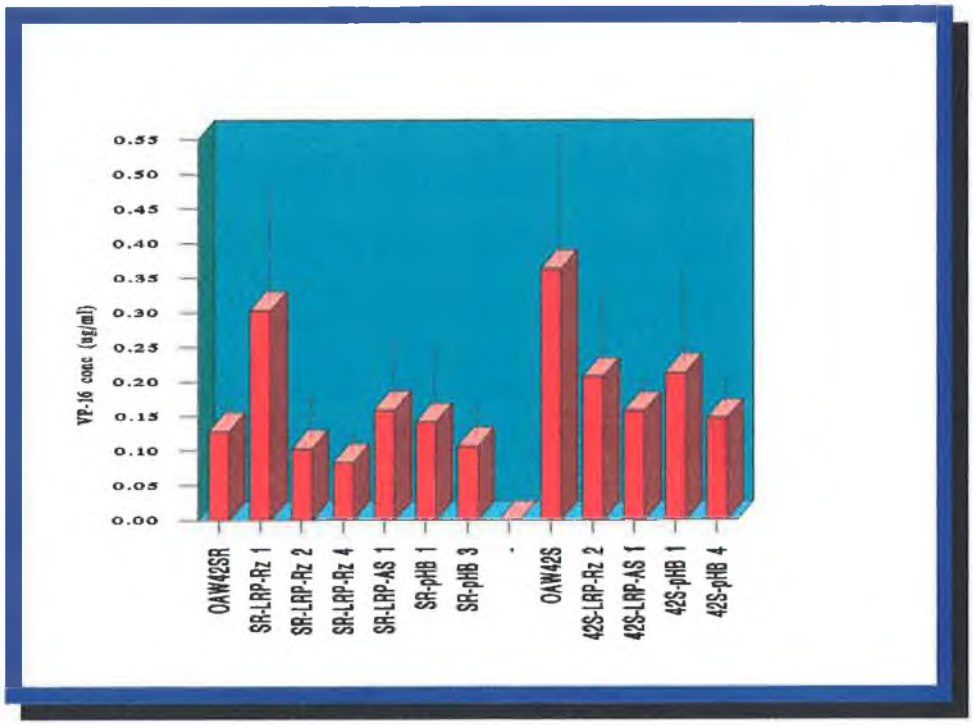
Graph 3.2.7.1 Adriamycin IC_{50} values (μg/ml) for OAW42SR and OAW42S clones



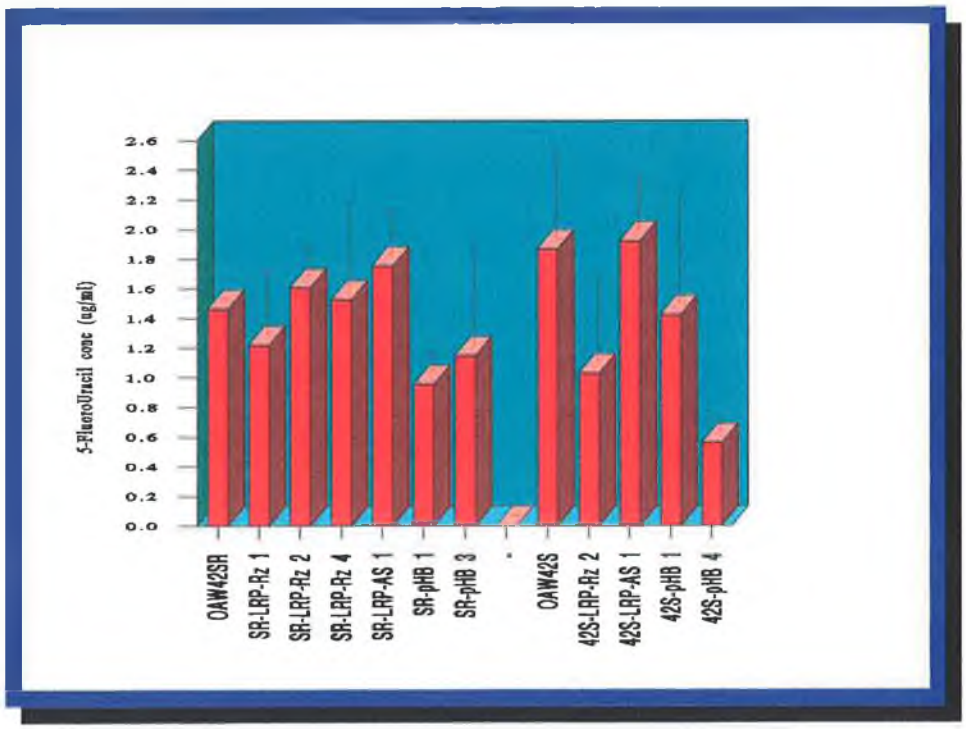
Graph 3.2.7.2 Vincristine IC_{50} values (μg/ml) for OAW42SR and OAW42S clones



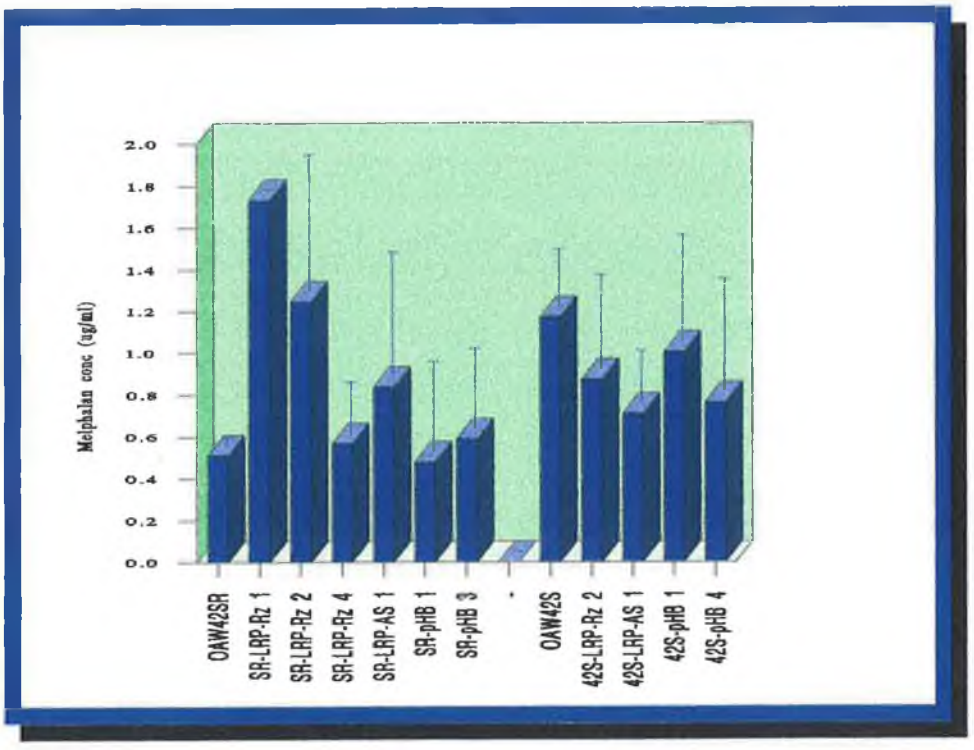
Graph 3.2.7.3 VP-16 IC_{50} values (μg/ml) for OAW42SR and OAW42S clones



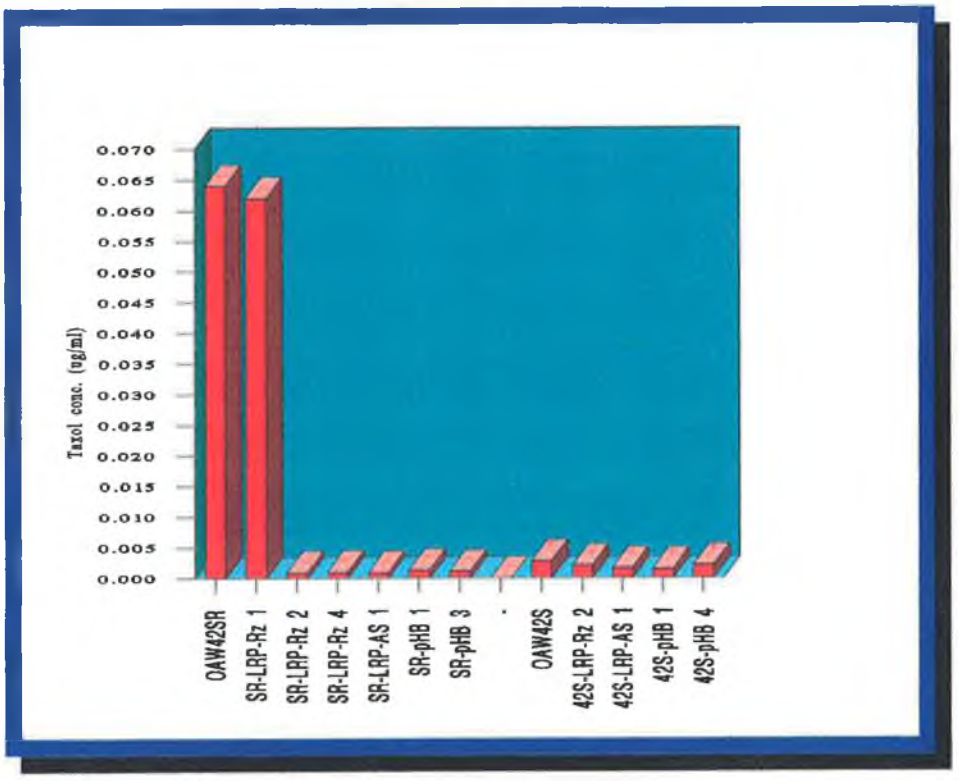
Graph 3.2.7.4 5-Fluoro-Uracil IC_{50} values (μg/ml) for OAW42SR and OAW42S clones



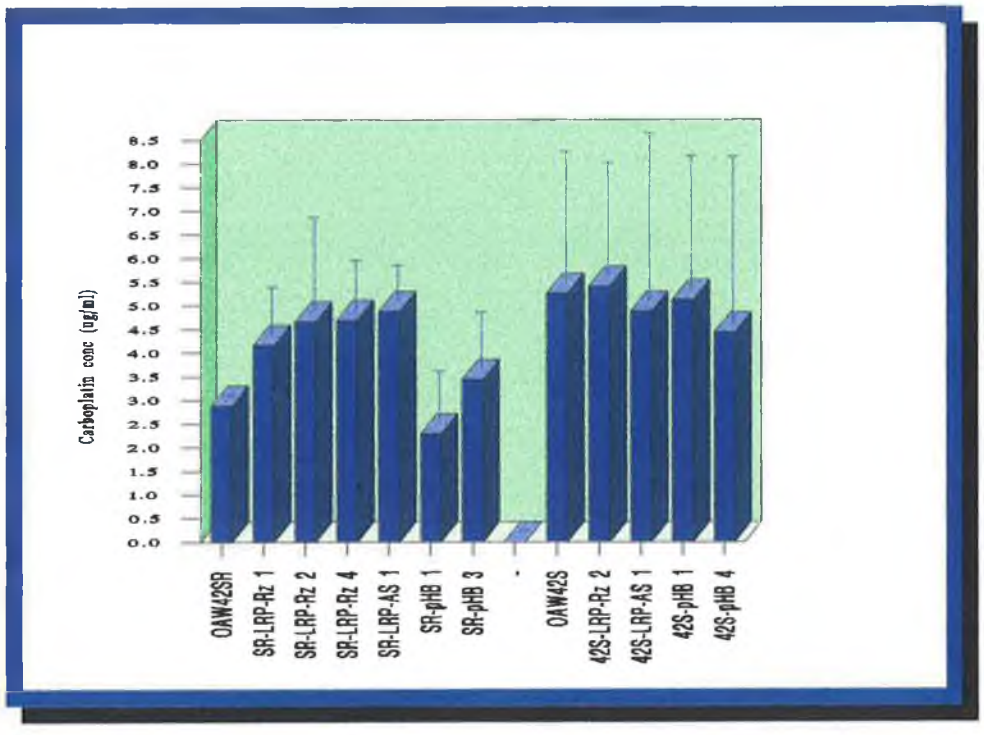
Graph 3.2.7.5 Melphalan IC_{50} values (μg/ml) for OAW42SR and OAW42S clones



Graph 3.2.7.6 Taxol IC_{50} values (μg/ml) for OAW42SR and OAW42S clones



Graph 3.2.7.7 Carboplatin IC_{50} values (μg/ml) for OAW42SR and OAW42S clones



From Tables 3.2.7.3 (a) and (b), and Figure 3.2.7.1 to 3.2.7.7, it can be seen that there is very little variation in the IC50 values between any of the clones and the parental cells. 42S-LRP-AS1 and 42S-pHβ 1 show a 3-fold reduction in the resistance to Adriamycin, but only a 2-fold reduction in the resistance to Vincristine. All the clones show greater than a 2-fold reduction in the resistance to Melphalan.

3.2.8 Assessment of drug toxicity assays

All of the OAW42SR clones, with the sole exception of SR-LRP-Rz 1, exhibit a reduction in resistance to adriamycin and vincristine. The reductions in drug resistance are not confined to the ribozyme and antisense transfected cells, with the pHβ transfectants exhibiting similar IC_{50} values. The clone SR-pHβ 3 exhibits a much reduced level of drug resistance as compared with earlier toxicity assays (Section 3.1.2). The drugs to which a number of clones show reduced resistance, fit the classic profile of drugs transported by Pgp. There is little variation in the IC50 values displayed by the OAW42S clones, as compared to the parental cells.

3.2.9 Correlation between LRP expression and drug resistance

The above results have shown a large reduction in LRP mRNA for SR-LRP-Rz 2, with a smaller reduction evident for SR-LRP-Rz 4 and SR-pHβ 1. However, at the protein level, SR-LRP-Rz 2 and SR-LRP-AS 1 both exhibit marked reductions in LRP, with smaller decreases in SR-LRP-Rz 4, SR-pHβ 1 and SR-pHβ 3. However, as regards adriamycin and vincristine IC_{50} values, SR-pHβ 1 exhibits a greater reduction in adriamycin resistance than SR-LRP-Rz 2 or SR-LRP-AS 1. Additionally, SR-LRP-Rz 4 exhibits an equal, or greater, reduction in adriamycin and vincristine resistance as SR-LRP-AS 1. Consequently, there appears to be no correlation between LRP expression and drug resistance.

3.3 Transfection of OAW42SR cells without cloning

The OAW42SR cell line is a heterogeneous population, highlighted by the fact that from this population the more sensitive cell line OAW42S was originally cloned. Thus, there are many individual cell populations within the cell line, which would differ phenotypically from each other if cloned. Transfecting foreign DNA into cells, by its very nature, perturbs the genetic make up of the cells. As there is no control over the site of integration of the foreign DNA into the host genome, it is quite possible that the transfection may alter the genetic make up of the cells in some small way other than simply the introduction of the foreign DNA. Therefore clones which are isolated after transfection may differ from each other and from the original population in some small, often imperceptible, way.

Due to this possibility of clonal variation, and due to the high degree of variability seen in the clones previously isolated from the OAW42SR population, the OAW42SR cell line was once again transfected with the anti-LRP ribozyme, anti-LRP antisense and control pHβ plasmids (Section 2.10.3.1). On this occasion, however, the transfectants were not cloned into individual colonies during selection with Geneticin. Rather all the transfectants were pooled and the population examined in bulk in an effort to eliminate clone to clone variation that was not due to different levels of LRP expression.

After transfection with the plasmids, the cells were selected with increasing concentrations of Geneticin. After four weeks, cell stocks were made and stored in liquid Nitrogen. Subsequently, adriamycin and vincristine toxicity assays were performed, along with northern blots, Immunoprecipitation and RT-PCR, to see if the reduction of LRP expression in the bulk population had the same effect on drug resistance as individual clones. The bulk populations were simply termed SR-LRP-Rz, SR-LRP-AS and SR-pHβ for the LRP-ribozyme, LRP-antisense and control plasmid transfectants respectively.

3.3.1 Adriamycin and Vincristine Toxicity assays on Uncloned OAW42SR transfectants.

Toxicity assays were carried out in 96-well plates with both adriamycin and vincristine as described earlier (Section 2.3). The assays were repeated four times for each of the clones. The results are given in terms of IC50 values and are shown in Figure 3.3.1.1 and Figure 3.3.1.2 for adriamycin and vincristine respectively. The averages of the IC50 values for each clone are depicted in Figures 3.3.1.3. and 3.3.1.4

From Figures 3.3.1.1 to 3.3.1.4, it can be seen that for the SR-LRP-Rz and SR-LRP-AS populations, there were decreases in the levels of resistance to adriamycin and vincristine as compared to the parental population. The magnitude of this decrease was only approximately 2-fold for both drugs. The SR-pHβ population, transfected with only the control plasmid, however showed a marginally greater increase in sensitivity to both drugs. There was a high degree of standard deviation in the results for the parental OAW42SR population, which detracts from the significance of these results.

Figure 3.3.1.1 Adriamycin toxicity assys on uncloned OAW42SR trasnfectants : IC50 values (μg/ml)

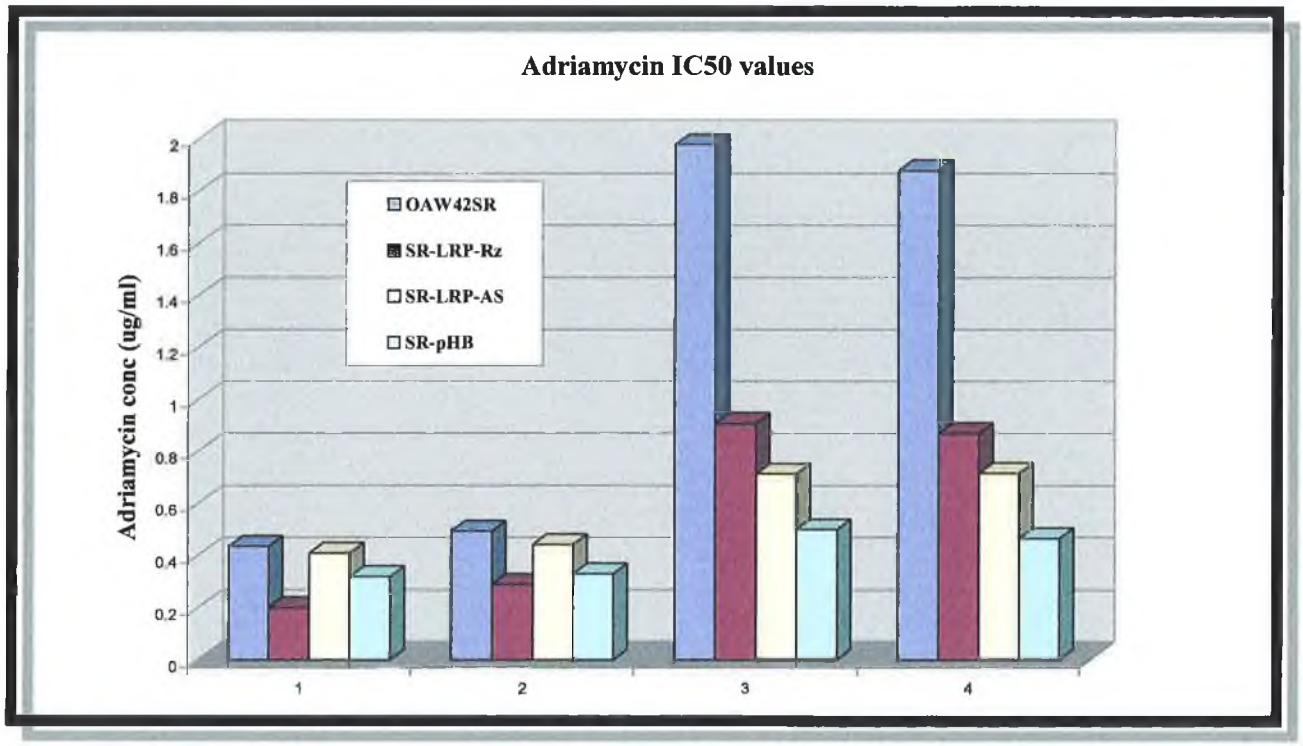


Figure 3.3.1.2 Vincristine toxicity assys on uncloned OAW42SR trasnfectants : IC50 values (μg/ml)

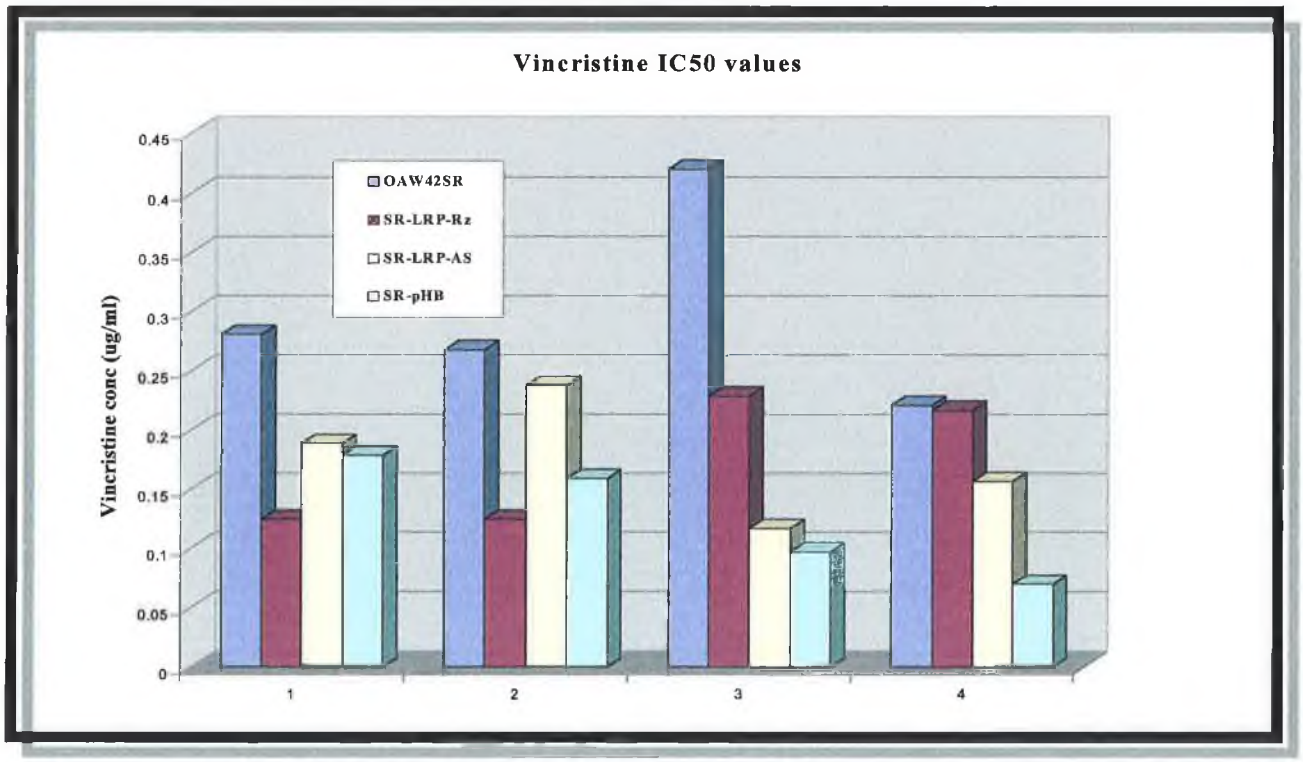


Figure 3.3.1.3 Adriamycin toxicity assys on uncloned OAW42SR transfectants : Average IC50 values (μg/ml)

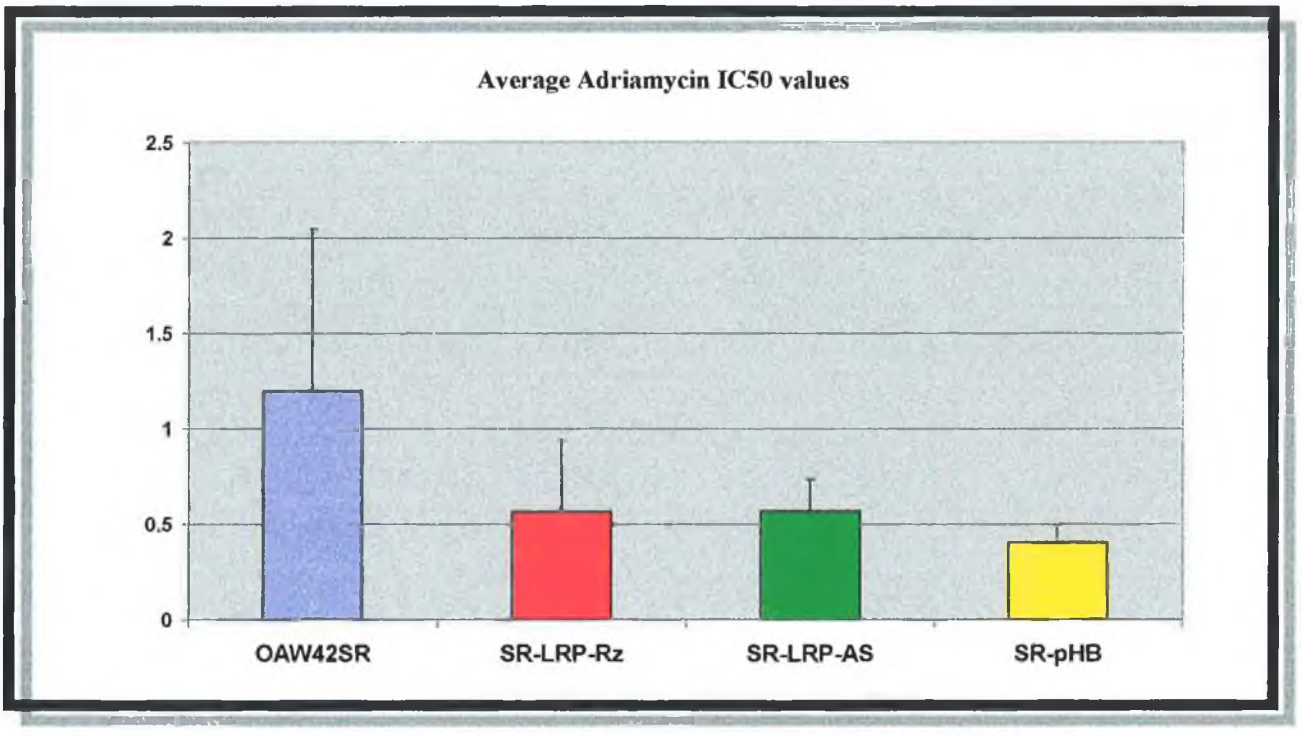
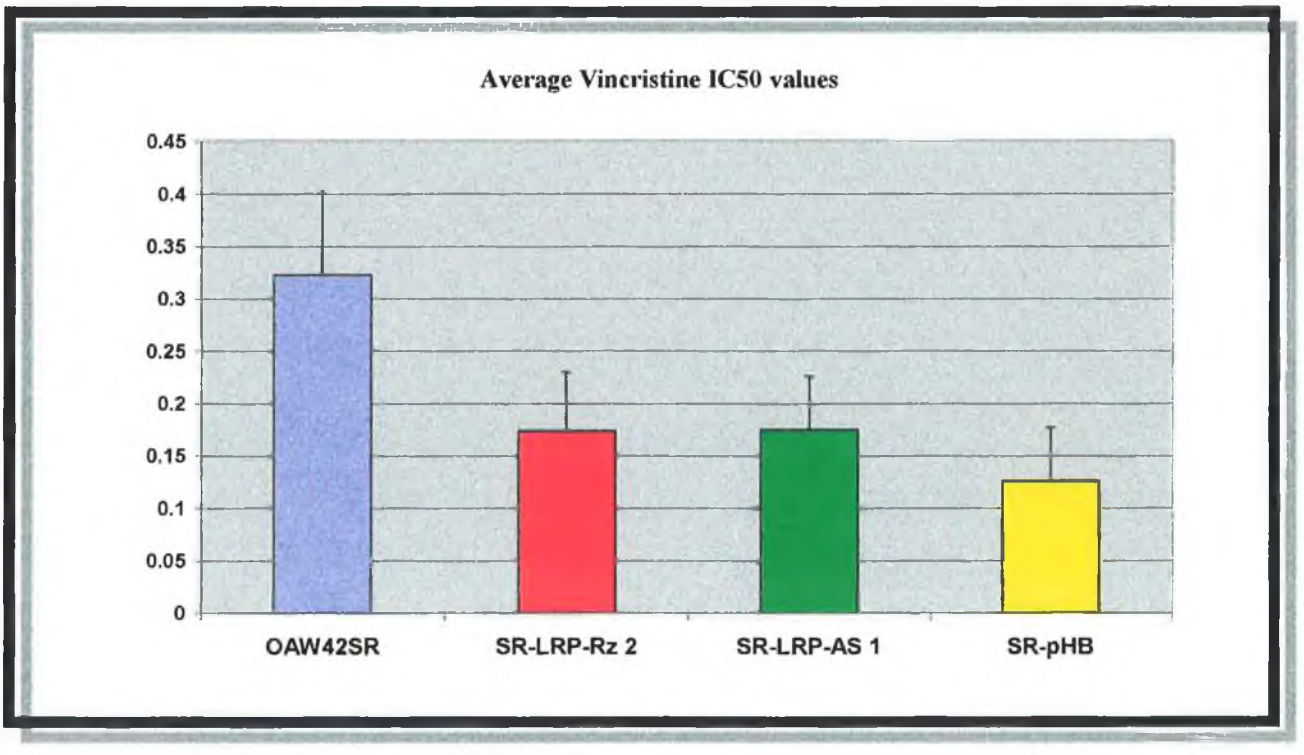


Figure 3.3.1.4 Vincristine toxicity assys on uncloned OAW42SR transfectants : Average IC50 values (μg/ml)



3.3.2 Northern blot analysis of LRP expression in uncloned OAW42SR transfectants.

Northern blots of 2 μg of mRNA isolated from each of the transfected populations were prepared as previously described (Section 2.8). The blots were hybridised with an LRP riboprobe, stripped and rehybridised with a GAPDH probe. This procedure was carried out on two separate occasions, with different preparations of Poly A+ RNA. The X-rays developed from the northern blots are shown in Figure 3.3.2.1 and Figure 3.3.2.2, along with densitometry analysis of the signal strength.

From Figure 3.3.2.1 (a), it is clearly evident that there is a reduction in the LRP band intensity for both SR-LRP-Rz and SR- pHβ, while there appears to be a slight increase in the signal strength for the SR-LRP-AS population, as compared to the parental OAW42SR population. However, the GAPDH bands (Figure 3.3.2.1 (b)) show that the there was unequal loading of mRNA samples. When the values for LRP strength from densitometry are normalised to GAPDH values (Figure 3.3.2.1 (c)), it can be seen that there is very little variation in LRP expression between SR-LRP-Rz and the parental cells. The SR-LRP-Rz population shows a slight decrease in LRP expression. However the SR-LRP-AS cells show a significant increase in LRP mRNA levels. Only the SR- pHβ population shows a substantial decrease in LRP expression.

The pattern of signal strength in the repeat of this procedure (Figure 3.3.2.2) is very similar to that above. SR-LRP-Rz cells show a slight reduction in LRP mRNA expression. The SR-LRP-AS population shows a significant, if somewhat smaller than previously found, increase in LRP signal strength, while SR- pHβ once again exhibits a marked reduction in LRP mRNA levels compared to the parental population.

Figure 3.3.2.1 Northern Blot and densitometry analysis of LRP expression in uncloned OAW42SR transfectants : Repeat 1

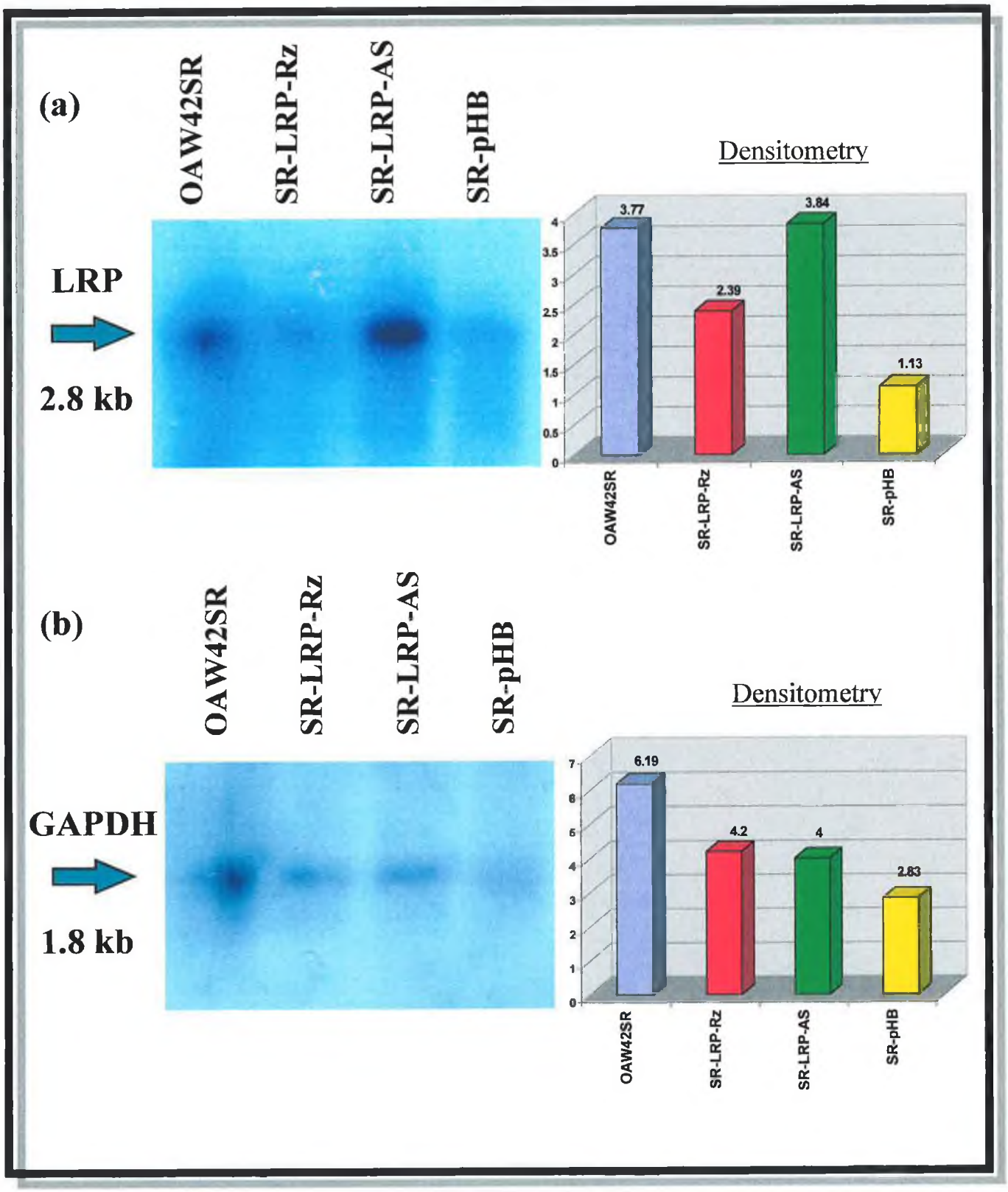


Figure 3.3.2.1 (cont'd)Northern Blot and densitometry analysis of LRP expression in uncloned OAW42SR transfectants : Repeat 1

(C) LRP densitometry values normalised to GAPDH

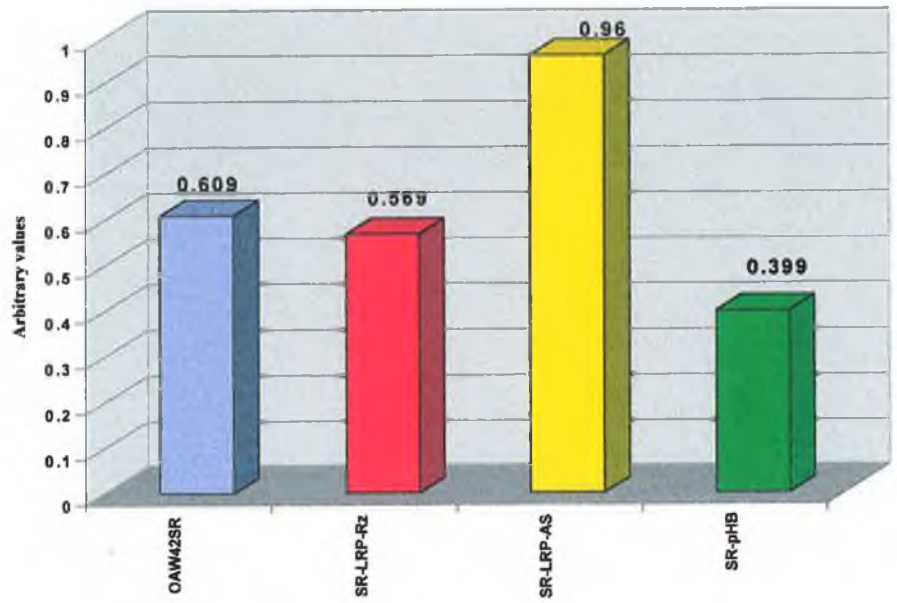


Figure 3.3.2.2 Northern Blot and densitometry analysis of LRP expression in uncloned OAW42SR transfectants : Repeat 2

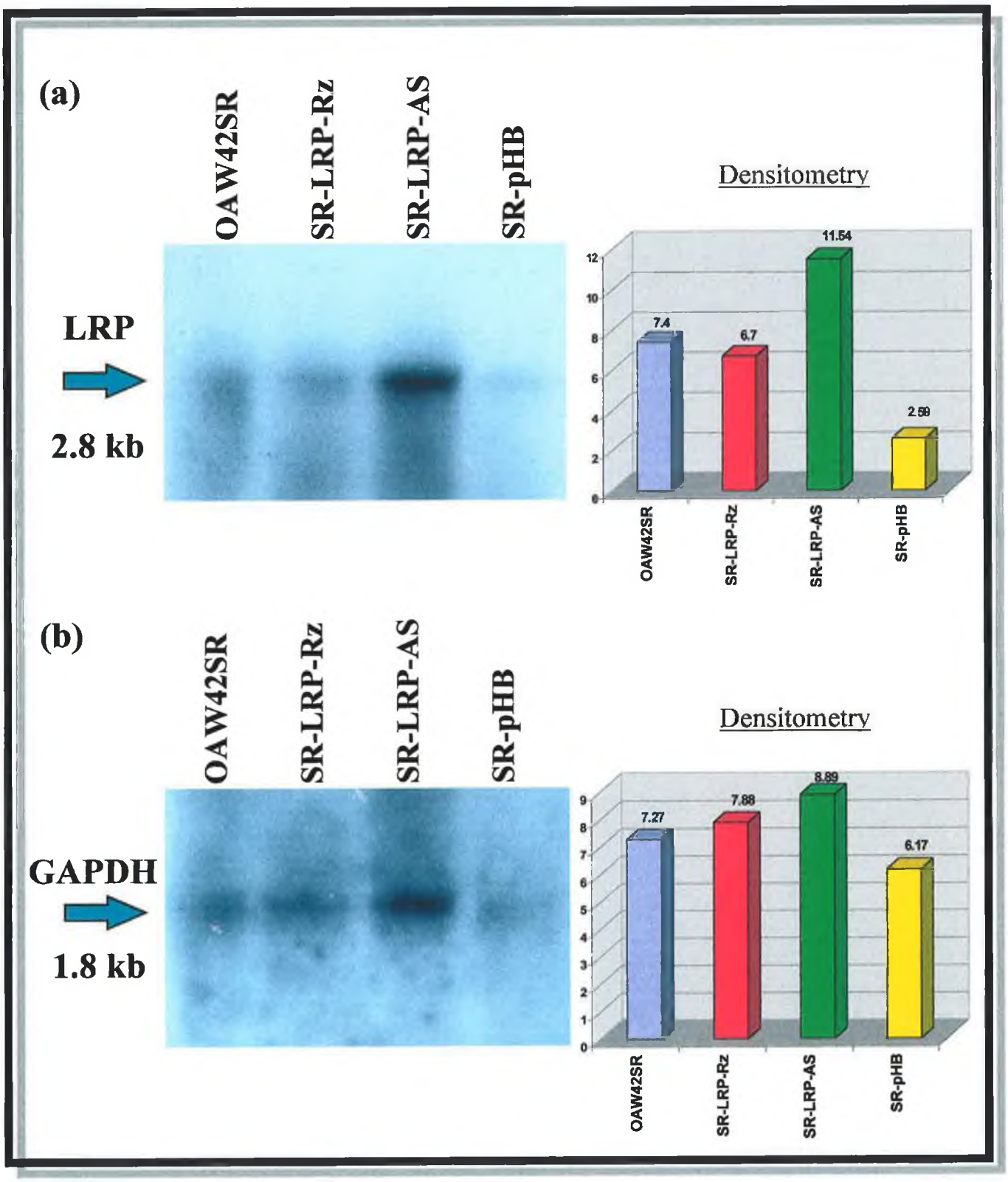
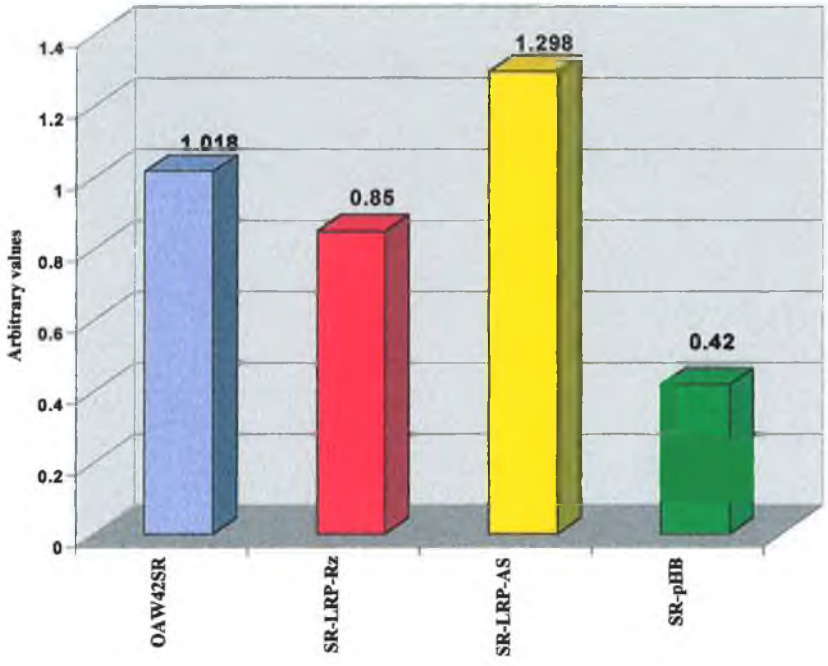


Figure 3.3.2.2 (cont'd) Northern Blot and densitometry analysis of LRP expression in uncloned OAW42SR transfectants: Repeat 2

(C) LRP densitometry values normalised to GAPDH



3.3.3 RT-PCR analysis of mRNA expression levels in Uncloned OAW42SR transfectants.

RT-PCR was carried out on total RNA samples from each of the transfected populations as previously described (Sections 2.7.5-2.7.6 and 3.2.1). The results of RT-PCR using primers for ribozyme/antisense expression, β-actin, MRP and mdr-1 are shown in Figures 3.3.3.1, 3.3.3.2, 3.3.3.3 and 3.3.3.4 respectively. Duplicate samples from each population were amplified in each reaction.

Figure 3.3.3.1 shows the results of RT-PCR with the ribozyme or antisense expression primers. It can be clearly seen from this that both the ribozyme and antisense are being expressed in the appropriate transfectants, and the pHβ plasmid is present in the SR- pHβ population. There are no bands present for the OAW42SR parental cells, as expected. No β-actin control primers were included in this PCR, as they interfere with the amplification of the ribozyme/antisense expression bands. However, β-actin RT-PCR was carried out on duplicate samples simultaneously, and the result of this is shown in Figure 3.3.3.2. From this there appears to be no variation in the β-actin bands, and thus equal loading of RNA samples can be assumed.

Figures 3.3.3.3 and 3.3.3.4 show the result of RT-PCR with primers for MRP and mdr-1 respectively. These results show that there is no significant variation in the levels of MRP or mdr-1 expression between any of the populations.

Figure 3.3.3.1 RT-PCR analysis of ribozyme/antisense expression levels in uncloned OAW42SR transfectants.

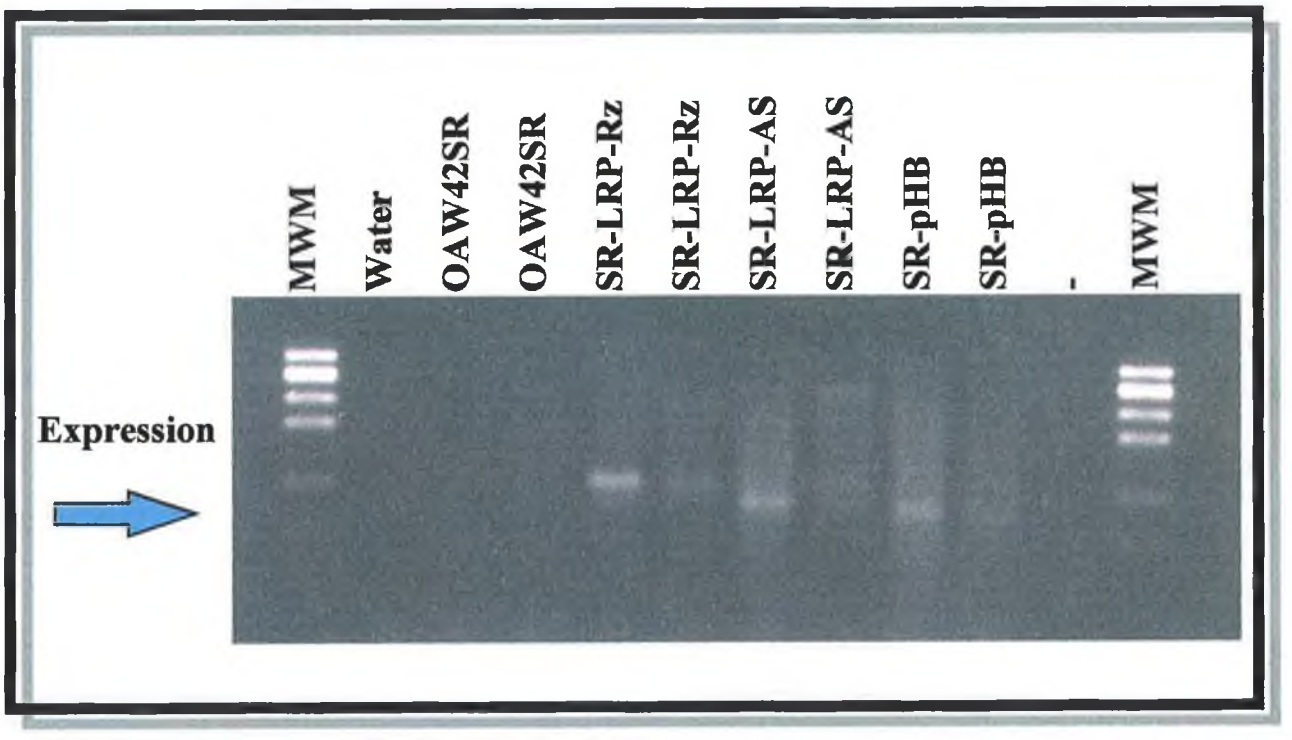


Figure 3.3.3.2 RT-PCR analysis of β-actin expression levels in uncloned OAW42SR transfectants

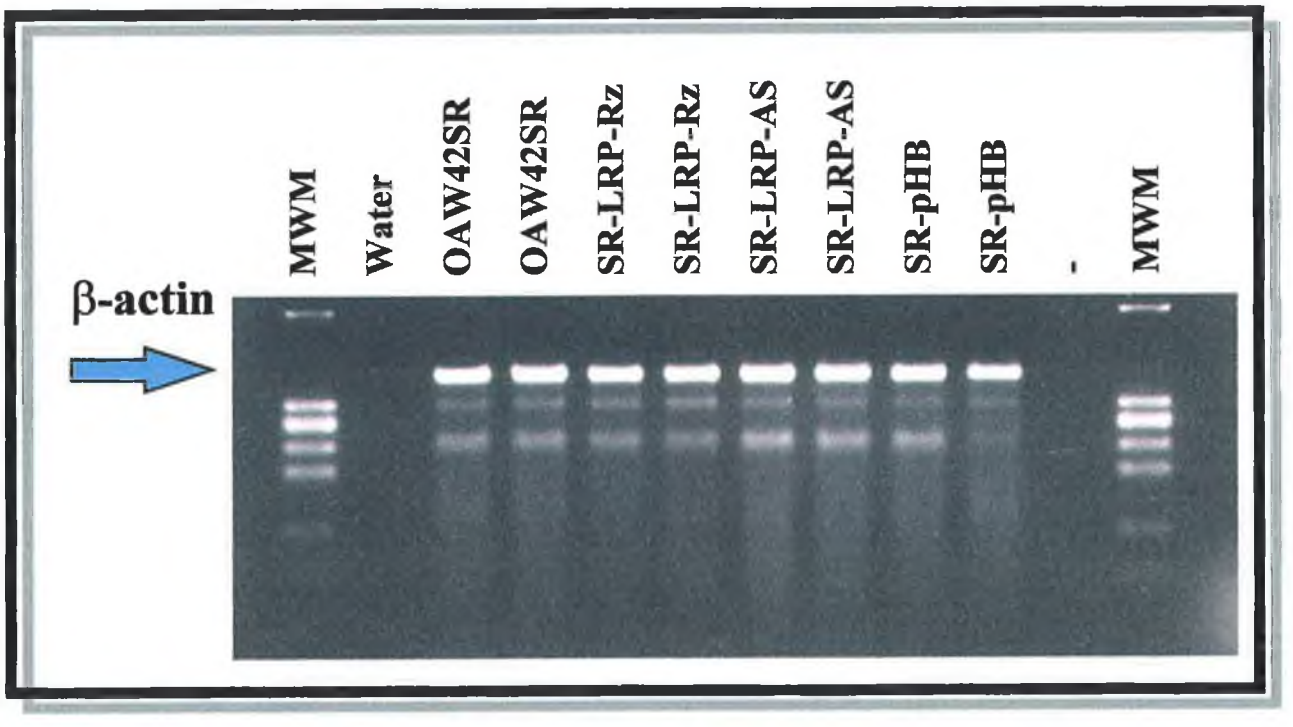


Figure 3.3.3.3 RT-PCR analysis of MRP expression levels in uncloned OAW42SR transfectants

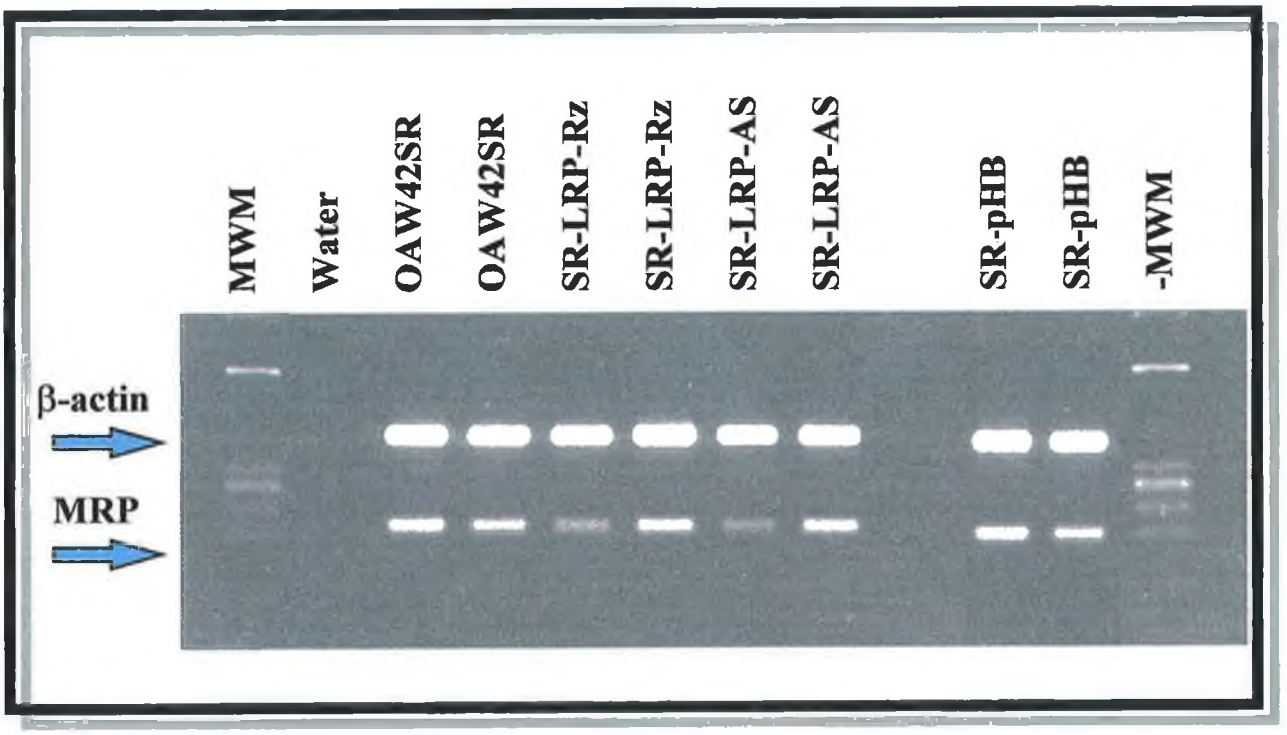
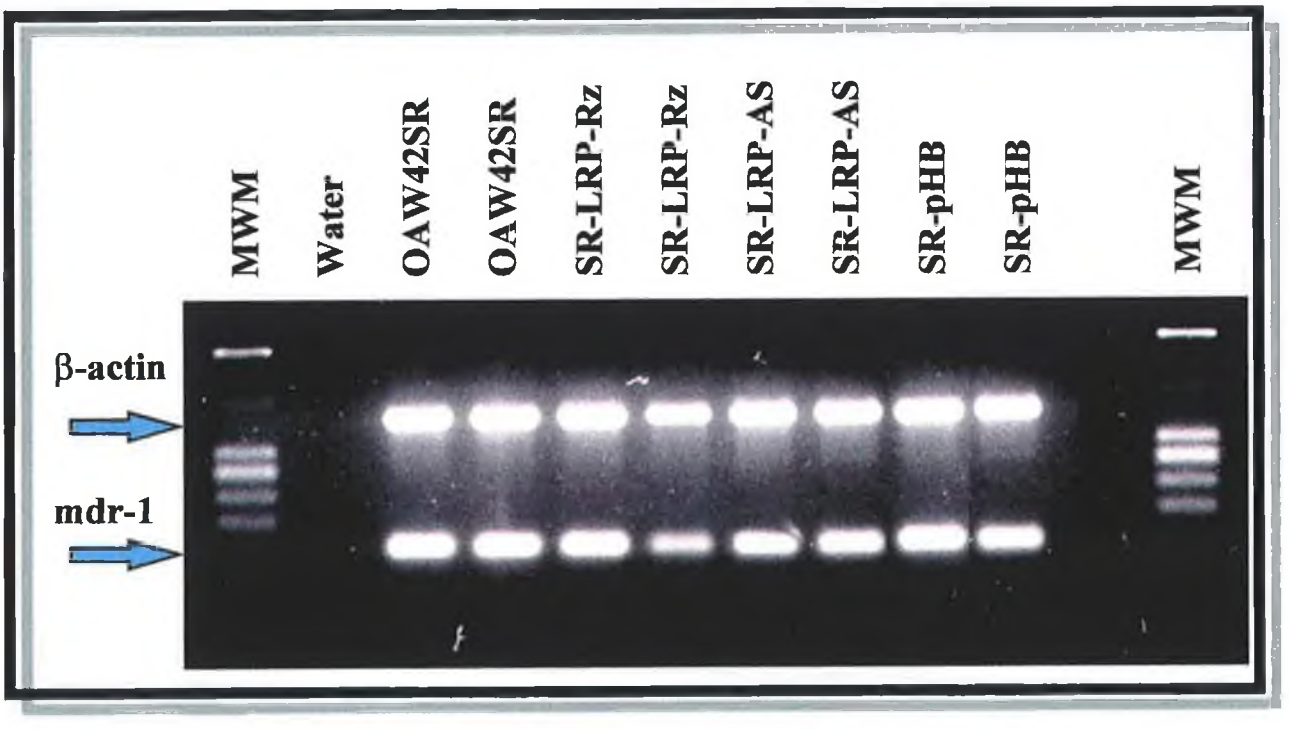


Figure 3.3.3.4 RT-PCR analysis of mdr-1expression levels in uncloned OAW42SR transfectants



3.3.4 Correlation of LRP expression and drug resistance

The above results demonstrate a reduction in LRP mRNA expression in the ribozyme and pHβ transfectants, while no reduction was observed in the antisense transfectants. As there was no conclusive immunoprecipitation data, the levels of mRNA and protein cannot be correlated. No correlation can be drawn from the levels of mRNA expression and drug resistance in these cells.

3.4 Antisense oligonucleotide treatment of OAW42SR and 2R120 cells

3.4.1 Antisense sequences

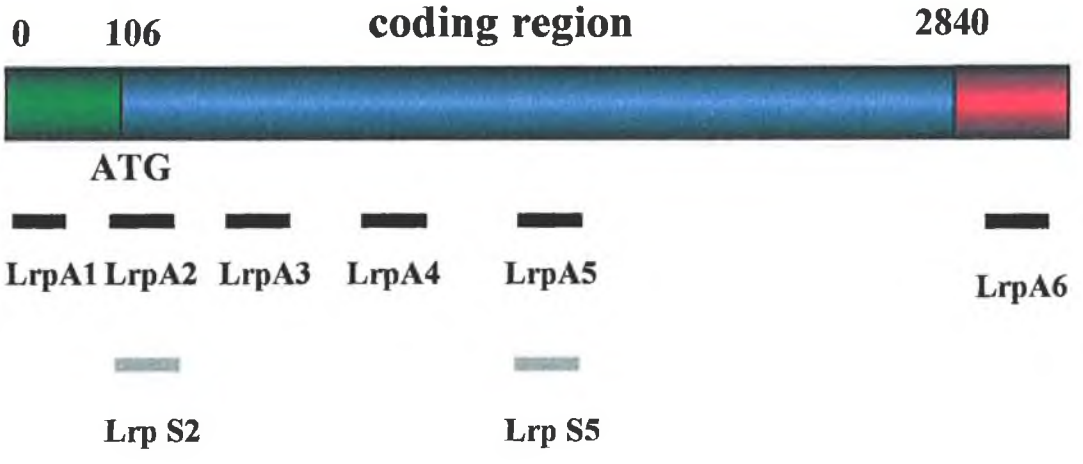
50 antisense sequences, ranging in size from 16 to 20bp, were designed from the LRP cDNA sequence using the criteria normally associated with picking PCR primers, while also avoiding sequences known to cause non-sequence specific antisense effects (e.g. G-quartets and palindromes; see Section 1.5). These sequences were tested for compatibility to known human gene sequences on the National Centre for BioInformatics (NCBI) Blast Internet server. 6 sequences (LRP A1 to 6, see Table 3.4.1) were then chosen on the basis of least compatibility to human gene sequences. In initial experiments, the sense sequence corresponding to LRP AS 5 (LRP S5) was chosen as the control. In later experiments, the corresponding sense sequence to LRP AS 2 (LRP S5) was used. A scrambled version of LRP AS 2 (SCR 2) and a nonsense sequence (NON 1) were also included in later experiments. To check the effectiveness of the transfection protocol and the methods for detecting antisense efficacy, antisense (mdr A1 and A2) and sense (mdr S1 and S2) oligonucleotides against the mdr-1 gene were also synthesised.

Table 3.4.1 Sequences of anti-LRP Antisense, Sense and control oligonucleotides.

Name	Sequence	Position on cDNA*
LRP A1	CAA CGT CGA TGG ACG TGA	- 60 to - 42
LRP A2	GAA TCC TCA GTG GTA CCG	-12 to + 6
LRP A3	CTC AAG TAG TAG GCG TAG	+12 to + 30
LRP A4	TGG TAG TAG TCC GTC TTG GT	+ 495 to + 514
LRP A5	CTC TTC CAG AGT GTG G	+ 1038 to + 1054
LRP A6	AAT TAT GTT ACC TTC AAA GA	+ 2686 to + 2706
mdr A1	CTC CAC CAC TAC CTC	-9 to +6
mdr A2	GTC CCC TTC AAG ATC CAT	+1 to +18
LRP S2	CTT AGG AGT CAC CAT GGC	+ 6 to -12
LRP S5	GAG AAG GTC TCA CAC C	+ 1054 to + 1038
NON	AGC GAT CCA GTA TTA GCG	
SCR	ACT GCC ATA GGC TCT GCG	
mdr S1	GAG GTG GTG ATG GAG	+6 to -9
mdr S2	ATG GAT CTC GAA GGG GAC	+18 to +1

* in all sequences, +1 is the position assigned to the A residue in the ATG initiation codon

Figure 3.4.1 Position of antisense sequences on LRP target



3.4.2 Treatment of OAW42SR and 2R120 cells with Antisense Oligonucleotides

Treatments were carried out in either 25 or 75 cm^2 flasks or 96 well plates. 24 h prior to treatment cells were seeded at a density of $1x10^5$/ml for the 25 cm^2 flasks, 1 or $2x10^5$/ml for the 75cm^2 flasks, and $1x10^4$/ml in the 96 well plates. 25 cm^2 flasks were used for the isolation of total RNA (for RT-PCR) and for treating cells for cytospins. 75cm^2 flasks were used for treating cells for immunoprecipitation and isolation of Poly A+ RNA. 96-well plates were used for the treatment of cells prior to toxicity assays.

Cells were initially exposed to the antisense for 24h at a concentration of 1μM combined with Lipofectin at a concentration of 10μL/3mls media. After 24h, the media containing the antisense was removed and fresh media containing fresh antisense and lipofectin at the same concentration was added to the cells. After an additional 24 h (48h total treatment time) cells were taken down for either total or Poly A+ RNA isolation. After 72h cytospins were made for immunocytochemistry or cell pellets made for immunoprecipitation. Cells treated in 96-well plates were exposed to cytotoxic drugs, in fresh antisense-free media, for the toxicity assays after 72h and left for a further 96 h.

3.4.3 Immunocytochemistry analysis of LRP expression

For each antisense treatment, four cytospins were made as described earlier (2.9.7.1). The immunocytochemical staining was carried out on three separate occasions for each set of cytospins. The results are shown in Tables 3.4.3.1 to 3.4.3.3 and are given in terms of staining intensity. Representative staining patterns are also shown in Figure 3.4.3.1. The staining in these photographs relates to those in Table 3.4.3.1-treatment 1, repeat 1.

The first treatment (Table 3.4.3.1 and Figure 3.4.3.1) shows that all of LRP antisense sequences appeared to decrease LRP expression levels. Treatment 2 appeared not to be quite as effective, or as consistent. The results for Treatment 3 indicated that LRP

Table 3.4.3.1 Immunocytochemistry Staining intensity on OAW42SR cytospins with LRP-56 MAb

	Treatment 1			Treatment 2			Treatment 3		
	1	2	3	1	2	3	1	2	3
LRPA1	0	0/+	?	+	+ (?)	-	0	0/+	+
LRPA2	0/+	0/+	0/+	++	0/+	-	0/+	0/+	+/++
LRPA3	0/+	0/+	+	++	0/+	-	+	0	+/++
LRPA4	0/+	0/+	0/+	+	+/++	-	+	+	++/++ +
LRPA5	0/+	+	0/+	++	+ (?)	-	+	?	++/++ +
LRPA6	+ (?)	0/+	+	+	+ (?)	-	+	+	+
LRP S 5/2 *	++	+/++	++	+	++	-	+	+	+++
Lipo	+/++	0/+	0/+	++	++	-	+/++	+/++	++
Control	++	+	++	++	+++	-	++	++	+++
LRPA2 1μM							0	0/+	+/++
LRPA2 2μM							0/+	0/+	+/++
LRPA2 5μM							0/+	0/0/+	+

Staining intensity : 0 - No staining, + - low staining, ++ - medium level staining, +++ - very intense staining

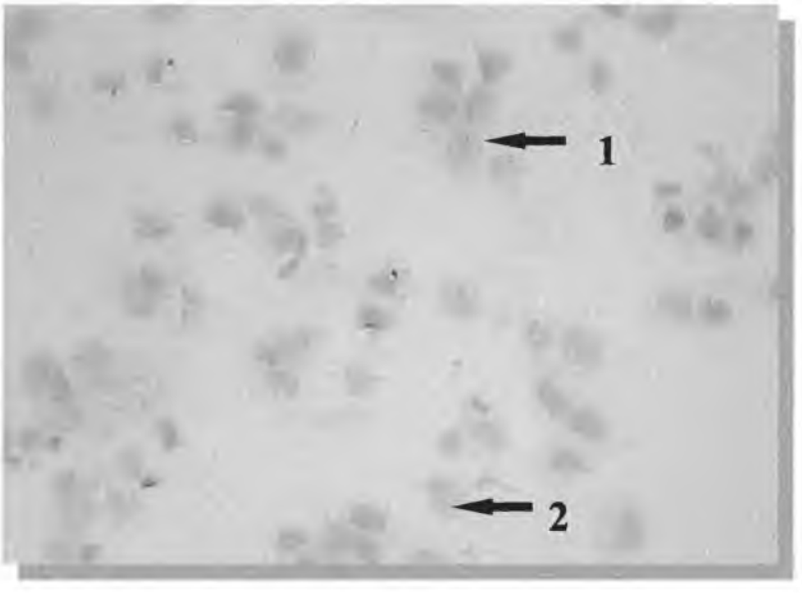
/ - indicates a level of staining in between two of the above categories.

* - LRP S5 was used for treatment 1 only. LRP S2 was used for treatment 2 and 3.

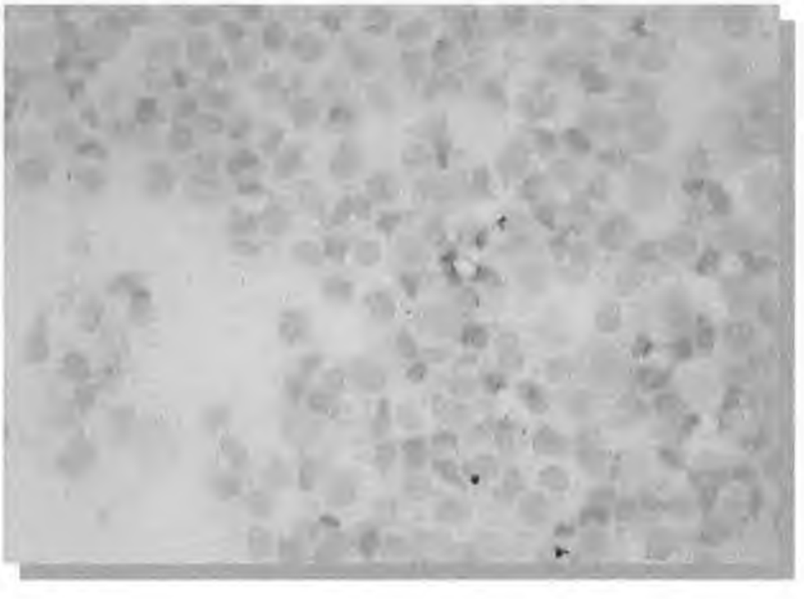
? - Staining uncertain due to low cell number/ stickiness of the antibody

Figure 3.4.3.1 Immunocytochemical staining on LRP antisense treated OAW42SRs with LRP-56 MAb

(a)



(b)

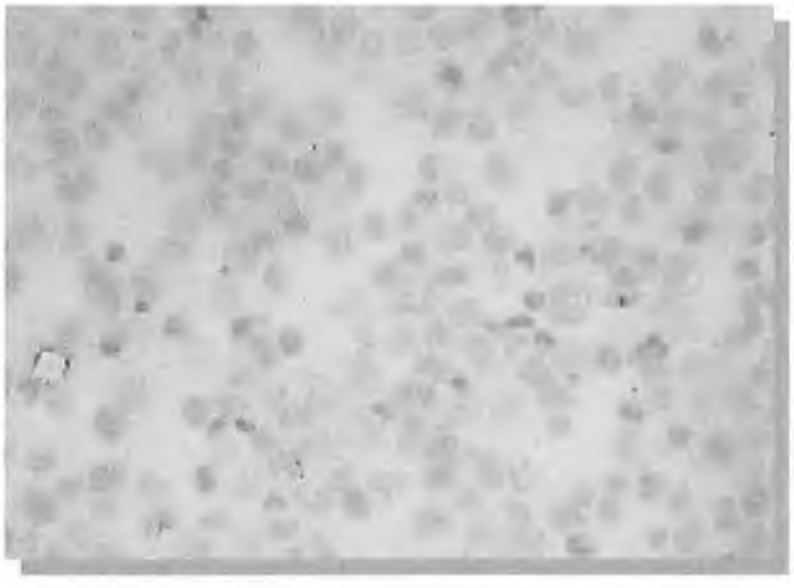


(a) LRP A1 treated; (b) LRP A2 treated

1- Punctate cytoplasmic LRP staining; **2-** Blue nucleus counter stain

Figure 3.4.3.1 (cont'd) Immunocytochemical staining on LRP antisense treated OAW42SRs with LRP-56 MAb

(C)

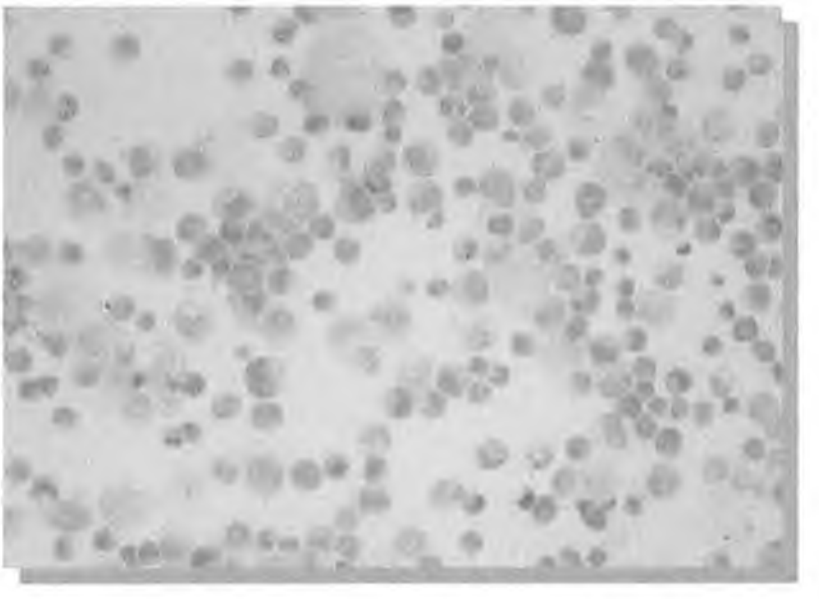


(d)

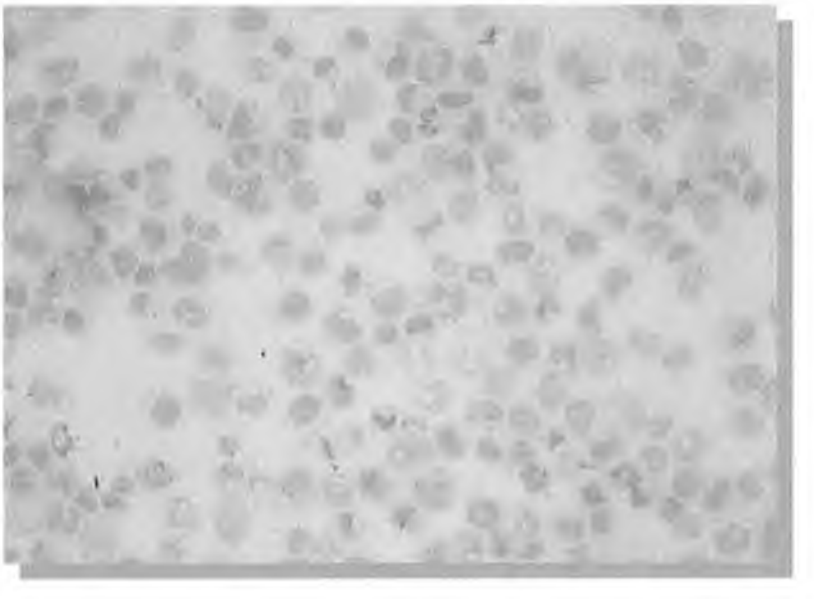
(C) LRP A3 treated; (d) LRP A4 treated

Figure 3.4.3.1 (cont'd) Immunocytochemical staining on LRP antisense treated OAW42SRs with LRP-56 MAb

(e)



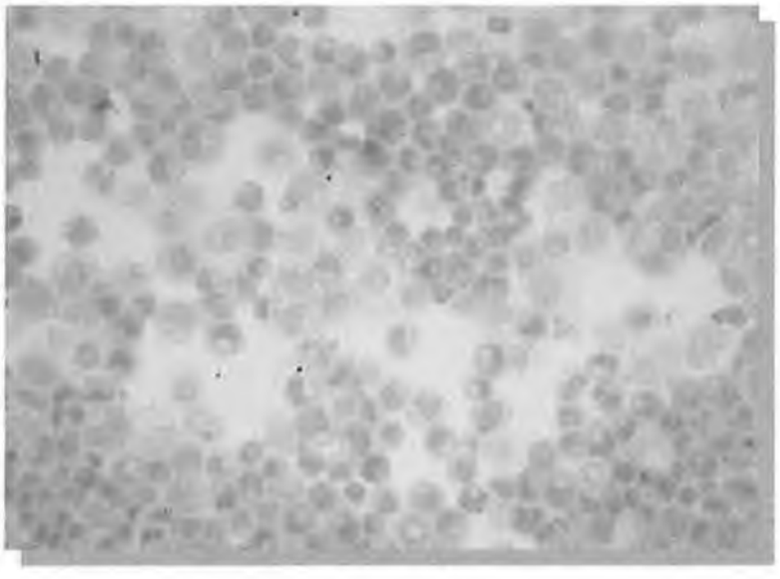
(f)



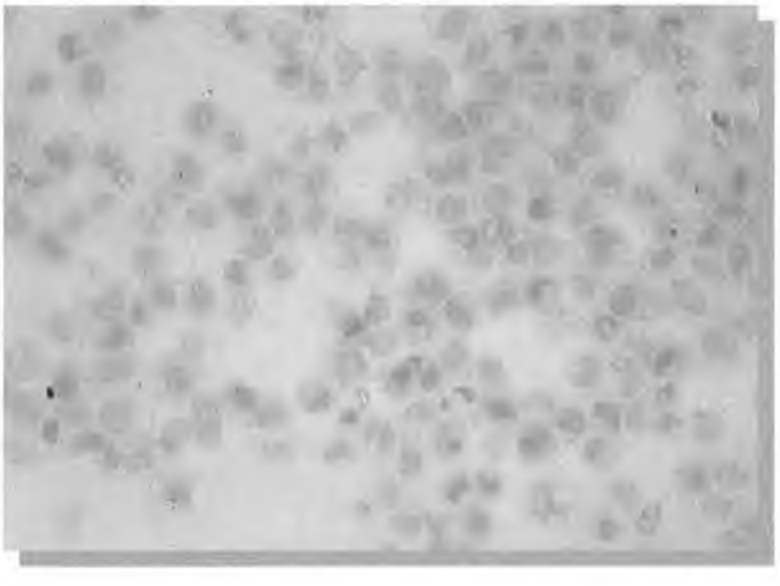
(e) LRP A5 treated; (f) LRP A6 treated

Figure 3.4.3.1 (cont'd) Immunocytochemical staining on LRP antisense treated OAW42SRs with LRP-56 MAb

(g)



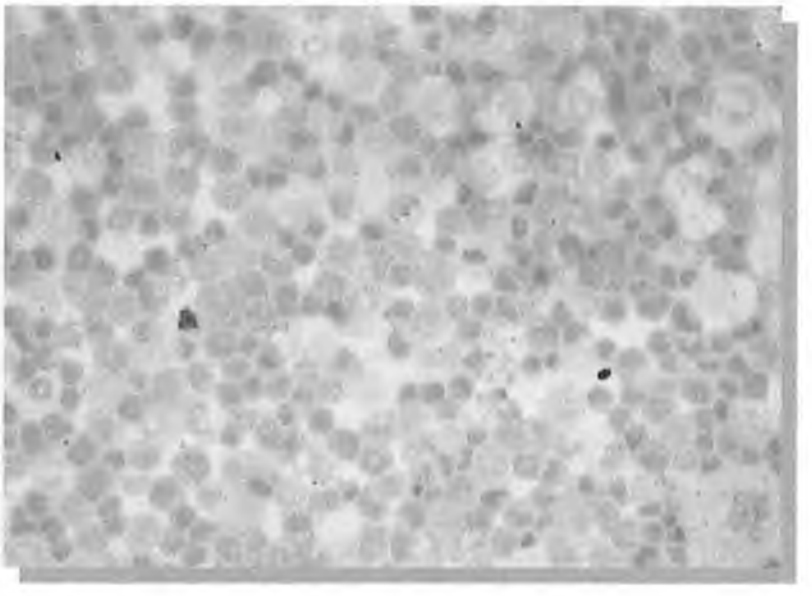
(h)



(g) LRP S2 treated; (h) Lipofectin treated

Figure 3.4.3.1 (cont'd) Immunocytochemical staining on LRP antisense treated OAW42SRs with LRP-56 MAb

(i)



(I) Untreated controls

A1 and A2 were consistently the most effective at down regulating LRP. The sense oligonucleotides, LRP S2 and S5, appeared to have varying levels of effect on LRP expression, but were consistently less inhibitory to LRP expression than LRP A1 or A2. Using higher concentrations of LRP A2 (2 and 5μM) did not have a significantly greater inhibitory effect on LRP expression under these conditions. Table 3.4.3.2 (see also Figure 3.4.3.2) shows that mdr-1 AS 1 or 2 have little effect on the expression of p-glycoprotein compared to the sense or lipofectin treated or untreated cells. The cells in these cytospins were consistently in bad condition, possibly due to the harsh staining protocol, and so accurate evaluation was difficult.

Table 3.4.3.3a shows immunocytochemistry results of treatments with just LRP A1 and A2, along with LRP S2 and nonsense and scrambled controls (NON and SCR). 2R120 cells were included in this set of treatments (Table 3.4.3.3a). The results show that on this occasion only LRP A1 consistently down-regulated LRP in the OAW42SRs, while in the 2R120 cells, LRP A2 appeared to be more effective. The control oligonucleotides appeared to have little effect on LRP expression. The difference in effectiveness of LRP A1 and A2 in the OAW42SRs and 2R120s, could indicate that the site for LRP A1 is more accessible in OAW42SRs than 2R120s, and vice versa for LRP A2. This could possibly be due to different post-transcriptional modifications of the LRP mRNA in the OAW42SRs and 2R120s.

Table 3.4.3.3b appears to contradict this trend since in this set of treatments, neither antisense oligonucleotide appears to have any effect on LRP levels in 2R120 cells. However, both LRP A1 and A2 appear to have varying effect on LRP expression in the OAW42SR. The immunocytochemical staining technique used to obtain these results is known to be susceptible to extremes of heat or cold, often resulting in inaccurate staining patterns when the ambient temperature is above normal. This may go some way to explain the variations observed. Once again, though, the control oligonucleotides appear to have little, if any effect on LRP expression levels compared to that of the control untreated cells.

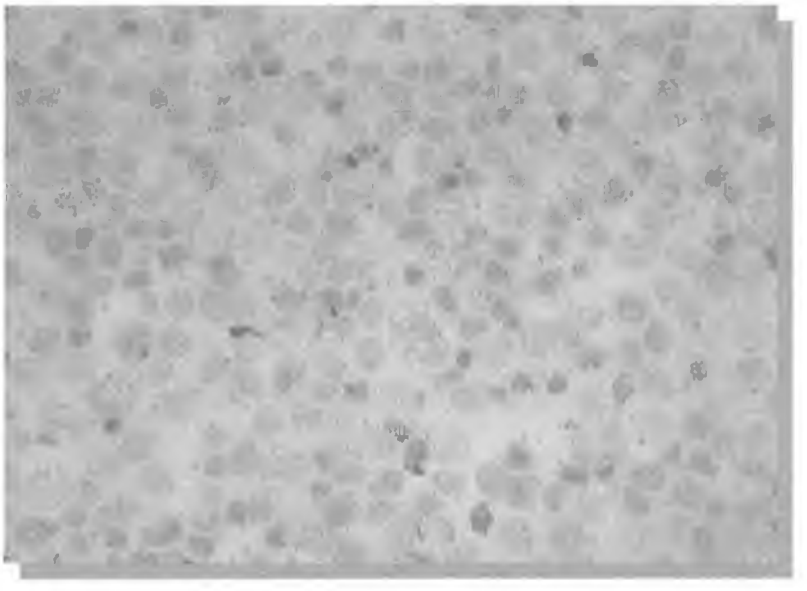
Table 3.4.3.2 Immunocytochemistry Staining intensity on OAW42SR cytospins with mdr-1 MAb

	1	2	3
mdrAS 1/2 *	0/+	+/++	+
mdrS 1/2 *	+	+/++	++
Lipo	++	+	+
Control	++	+	+

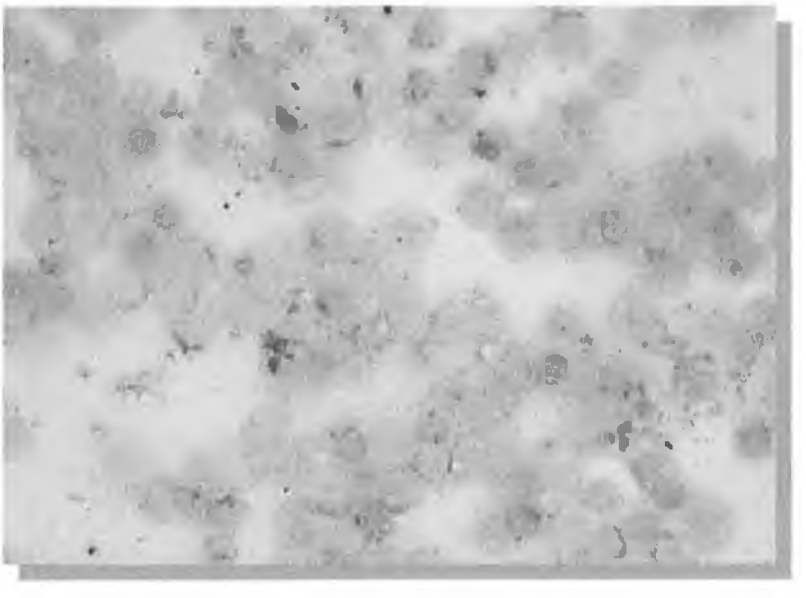
Staining intensity : 0 - No staining, + - low staining, ++ - medium level staining, +++ - very intense staining
/ - indicates a level of staining in between two of the above categories.
* - mdr AS1 and S1 were used in repeat 1, and mdr AS2 and S2 were used in repeats 2 and 3

Figure 3.4.3.2 Immunocytochemical staining on mdr-1 antisense and sense treated OAW42SRs with mouse ascites mdr-1 MAb

(a)



(b)



(a) mdr A1 treated; (b) mdr S1treated

Table 3.4.3.3a Immunocytochemical staining on OAW42SR and 2R120 cytospins with the LRP-56 MAb - treatment 1

	OAW42SR			2R120		
	1	2	3	1	2	3
LRP A1	+	0/+	+	+	0/+	+++
LRP A2	++	+/++	++	+	0/+ *	0 *
LRP S2	++	+/++	++	++	0/+ *	+++
NON	++/+++	+/++	++	+++	+++	+++
SCR	+++	+/++	+/++	++	++/+++	+++
Lipofectin	+++	++	++	++/+++	++/+++	++
Control	+++	++	++	++	+++	+++

Staining intensity : 0 - No staining, + - low staining, ++ - medium level staining, +++ - very intense staining
/ - indicates a level of staining in between two of the above categories.
* - indicates low cell numbers on cytospin, so staining intensity difficult to evaluate accurately.

Table 3.4.3.3b Immunocytochemistry staining on OAW42SR and 2R120 cells with the LRP-56 MAb - treatment 2

	OAW42SR			2R120		
	1	2	3	1	2	3
LRP A1	++	0/+	+++	++	++	+++
LRP A2	0/+	++	+/++*	+++	+++	+/++**
LRP S2	++	++/+++	+/++	++/+++	++/+++	++/+++
NON	++/+++	++	+++	+++	+++	+++
SCR	++	++	++	+++	+++	++
Lipofectin	++	++	+++	++/+++	0/+	++
Control	++	++	+++	++	+++	+++

Staining intensity : 0 - No staining, + - low staining, ++ - medium level staining, +++ - very intense staining
/ - indicates a level of staining in between two of the above categories.
* - cells packed very tightly, accurate determination difficult
** - cells dried up, accurate determination difficult

3.4.4 LRP RT-PCR analysis

Total RNA was isolated from cells after 48h of treatment with the antisense oligonucleotides and RT-PCR was performed with primers for LRP, giving a band size of 300bp. 2 sets of β-Actin primers, which were used as internal controls, gave band sizes of 142 or 353bp. Mdr-1 primers were used for RT-PCR on RNA extracted from mdr-1 antisense and sense treated cells, and gave a band size of 157bp. The RT-PCR procedure is outlined in Sections 2.7.5 and 2.7.6.

Figure 3.4.4.1 shows representative results of RT-PCR from the first three sets of antisense treatments on the OAW42SRs. There was no obvious consistent reduction in the LRP bands for any of the antisense oligonucleotides as compared to the controls (LRP S5, Lipofectin and untreated Control). The samples from LRP A5, LRP A6, LRP S5, Lipofectin and control treated cells actually appear to have reduced levels of LRP expression as compared to the other antisense treated cells. However, the β–actin control bands are also reduced, indicating simply reduced loading of these samples. Figure 3.4.4.2 shows the results of mdr-1 RT-PCR on mdr-1 antisense and sense treated cells. As can be seen, the mdr-1 band for mdr A1 treated cells was less than for mdr S1 or lipofectin treated or control cells, while the β-actin bands are all of equal intensity. This indicated that the antisense transfection and detection methods were functioning properly, as the mdr-1 antisense appeared to be able to specifically reduce the levels of mdr-1 mRNA.

In an attempt to see if the inhibition of LRP mRNA levels could be observed over time, cells were treated with LRP A2, with total RNA samples isolated at 0, 4, 24 and 48 h. This procedure was repeated three times and the results are shown in Figure 3.4.4.3 (a), (b) and (c). Figure 3.4.4.3(a), clearly shows a gradual decrease in LRP expression levels from 0 to 48 h. There was a very significant reduction in the amount of LRP present after 4h as compared to 0h, indicating that the antisense acts rapidly to reduce the LRP levels. A repeat of the experiment, Figure 3.4.4.3(b), also shows a great reduction in LRP levels between 0 and 4 h, with less change being visible for the additional time points. It also appears that the levels of LRP maybe increasing at 48h, indicating that the phosphorothioate oligonucleotides may be being degraded by

Figure 3.4.4.1 RT-PCR analysis of LRP mRNA expression in LRP antisense treated OAW42SRs

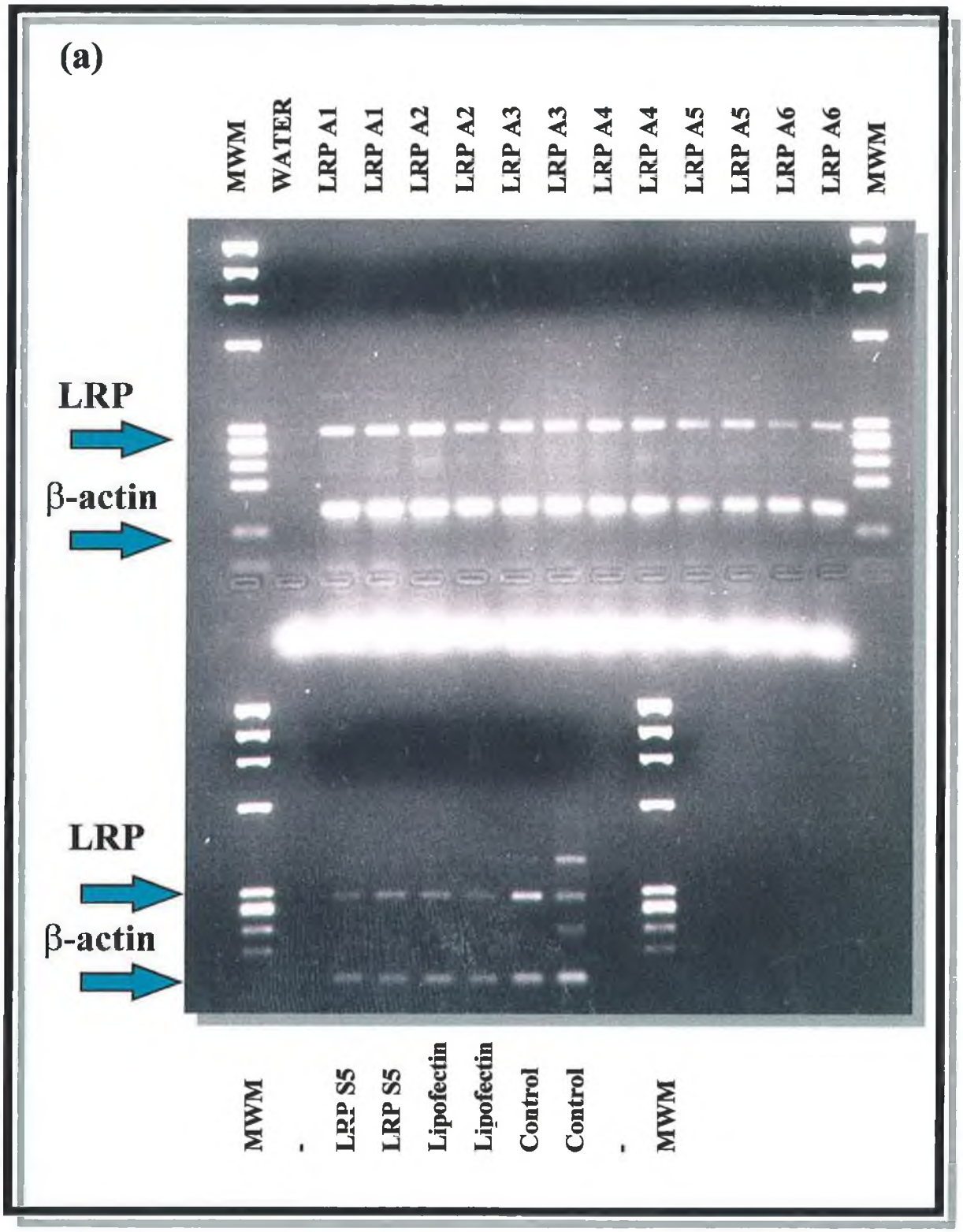


Figure 3.4.4.2 RT-PCR analysis of mdr-1 mRNA expression in mdr-1 antisense treated OAW42SRs

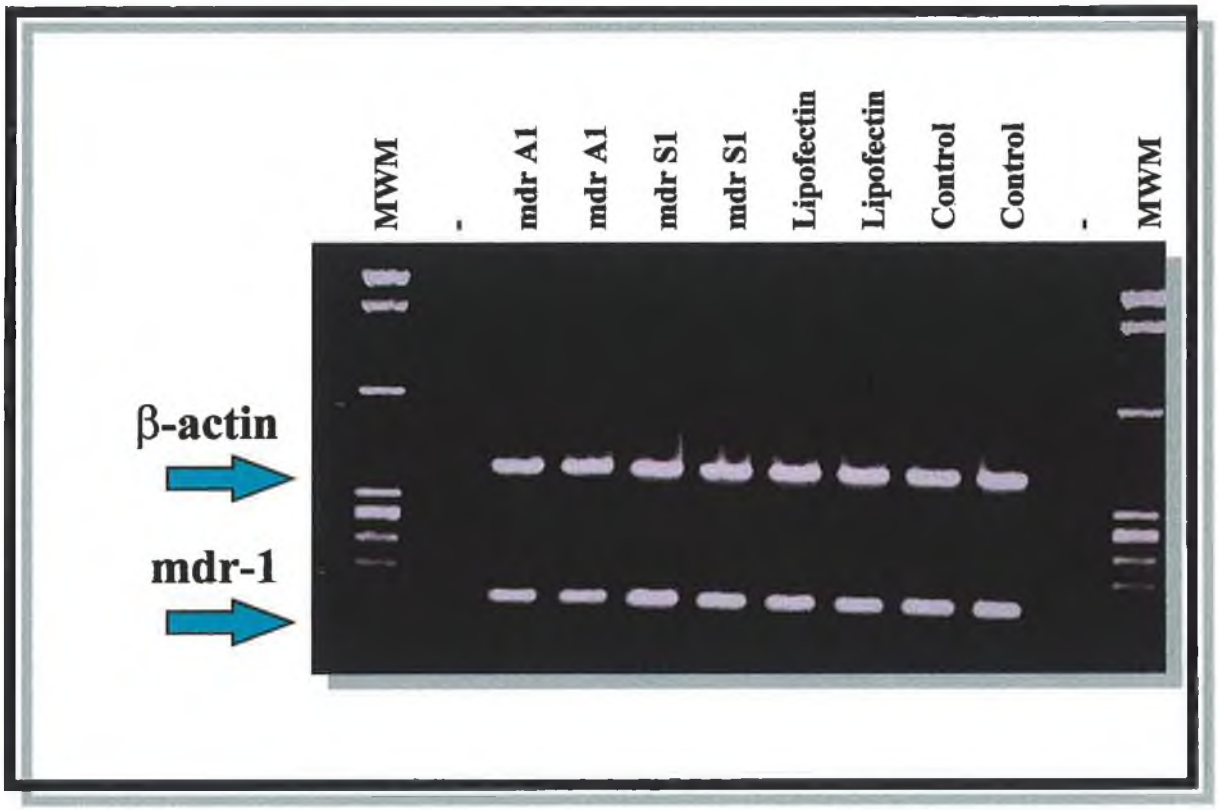


Figure 3.4.4.3 RT-PCR analysis of time scale of LRP down regulation in LRP antisense treated OAW42SRs

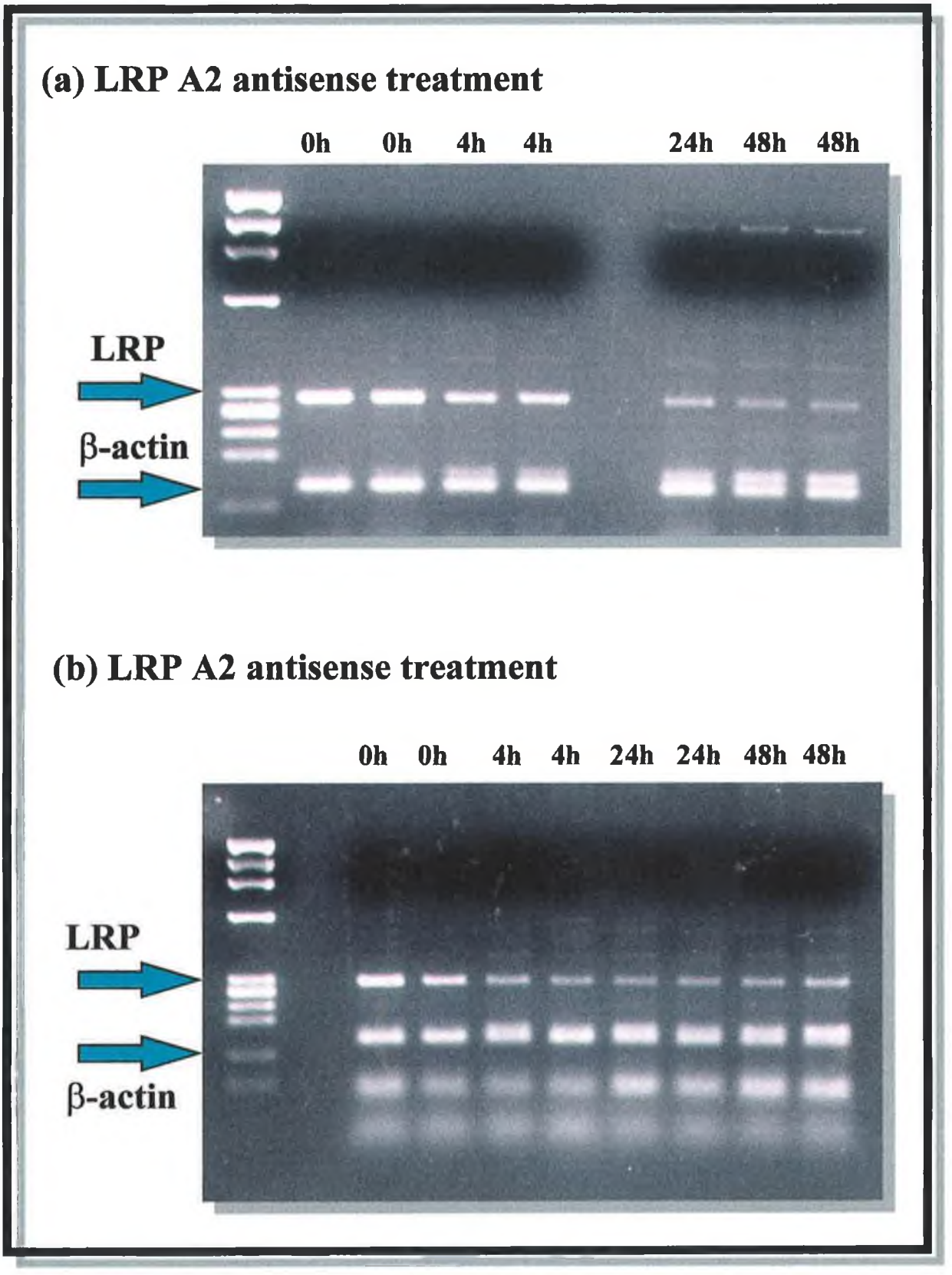


Figure 3.4.4.3 (cont'd) RT-PCR analysis of time scale of LRP down regulation in LRP antisense treated OAW42SRs

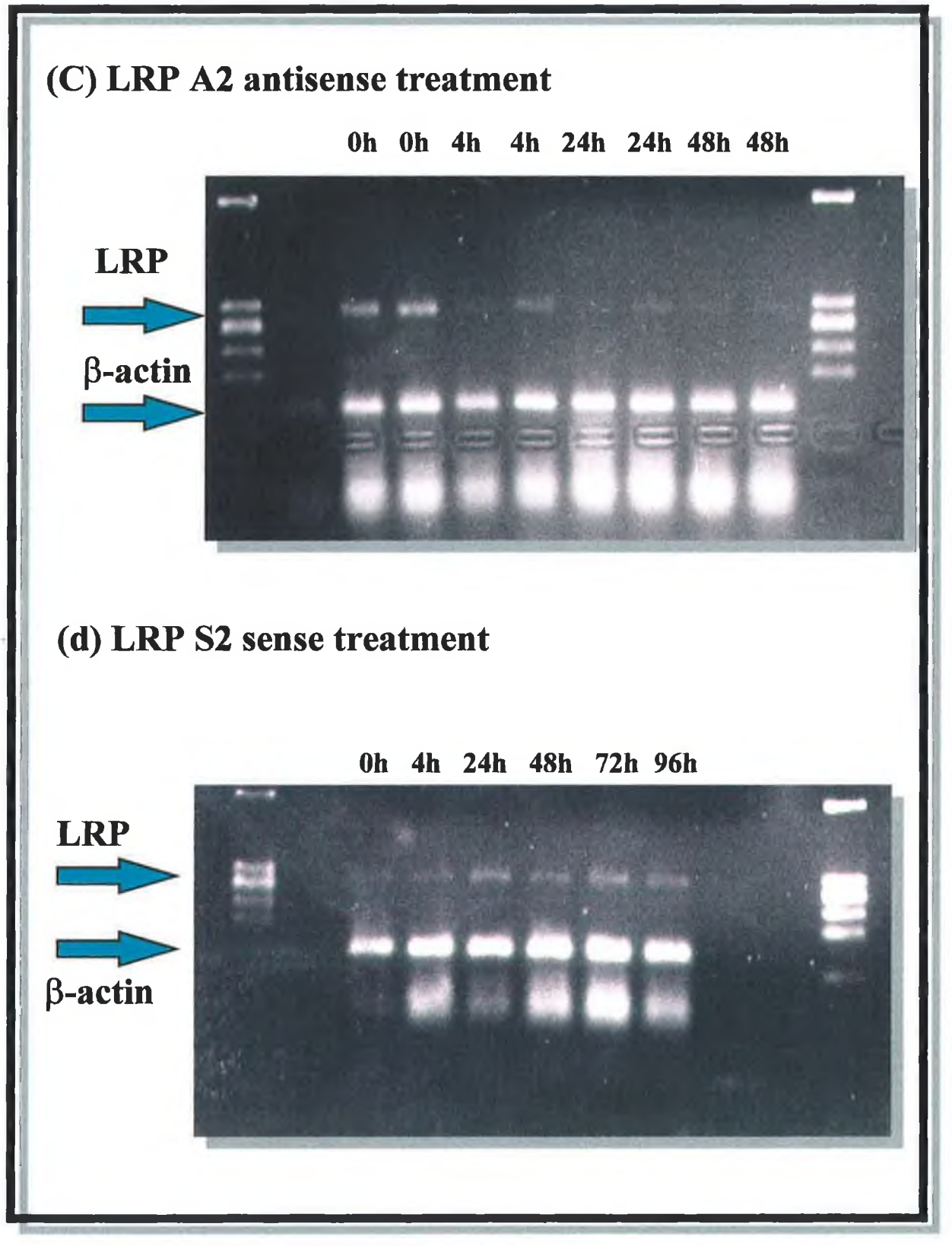
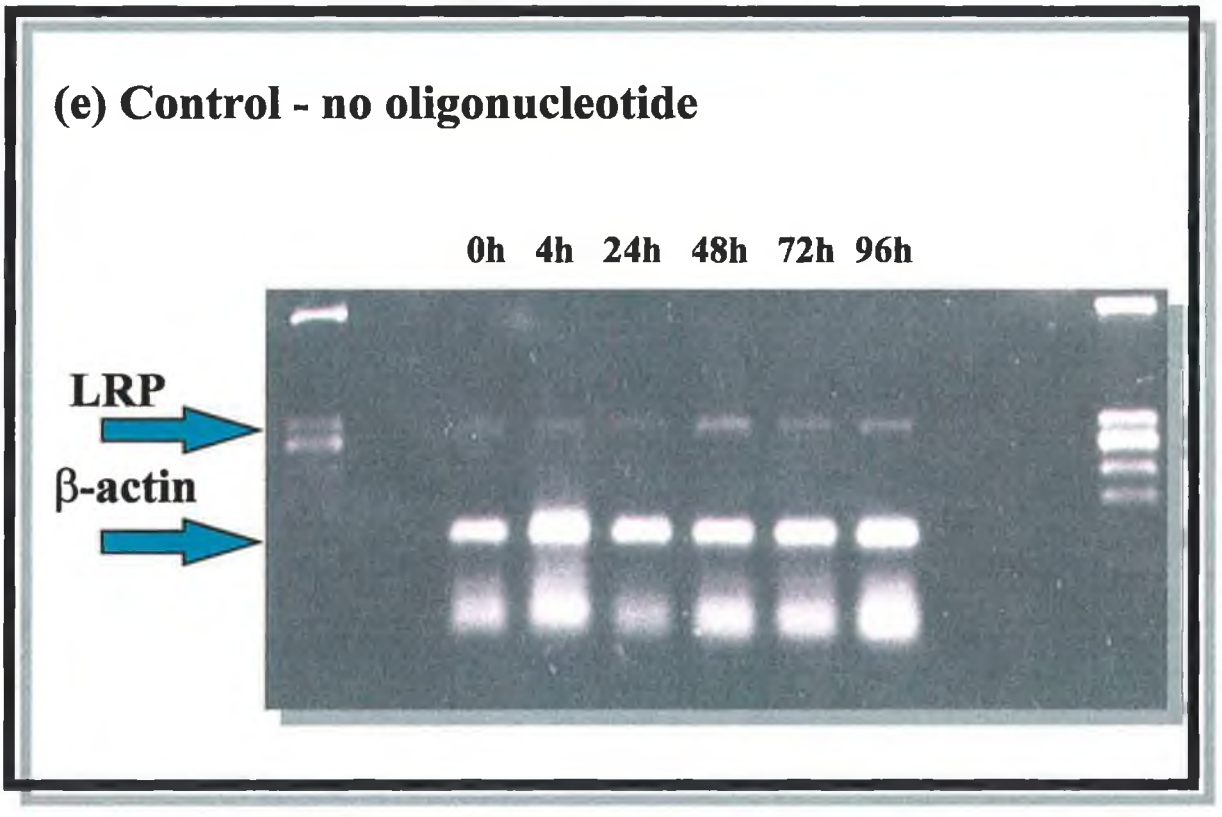


Figure 3.4.4.3 (cont'd) RT-PCR analysis of time scale of LRP down regulation in LRP antisense treated OAW42SRs



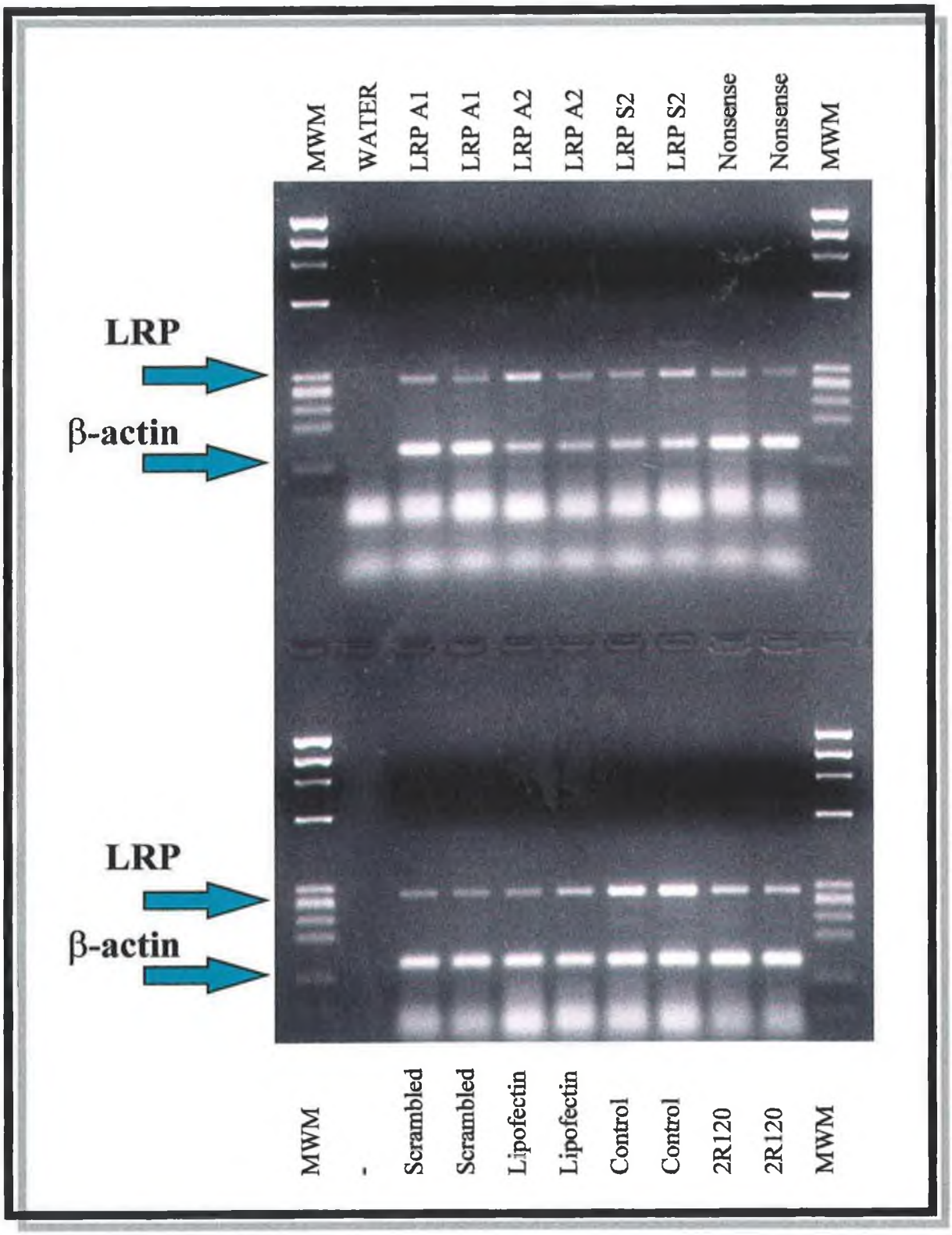
this time, and losing their potency. A third repeat of this procedure yielded an almost total elimination of LRP RNA by 24h post initiation of treatment, which was even further reduced by 48h.

Figures 3.4.4.3 (d) and (e) show the result RT-PCR on RNA extracts from a time scale treatment of OAW42SR cells with the sense oligonucleotide corresponding to LRP A1 (LRP S1), and untreated cells at various time points. These figures clearly show no reduction in LRP levels at any time point compared to time point 0h, even up to 96h, for either untreated or LRP S2 treated cells.

This result, while not being quantitative, clearly demonstrates the efficacy of the LRP A2 oligo in reducing LRP mRNA levels.

Figure 3.4.4.4 shows a representative result of LRP RT-PCR on OAW42SRs treated with LRP A1 and A2 and as well as the control oligonucleotides LRP S2, scrambled (SCR) and nonsense (NON). As with previous treatments (Figure 3.4.4.1), there was no obvious differences between the different treatments, although on this occasion, all treatments appeared to have slightly lower levels of LRP compared to the untreated control.

Figure 3.4.4.4 RT-PCR analysis of LRP mRNA expression in LRP antisense and control oligonucleotide treated OAW42SRs



3.4.5 Adriamycin Toxicity Assays on anti-LRP Antisense treated OAW42SRs

After 72h of treatment with oligonucleotides in 96 well plates, cells were treated with various concentrations of Adriamycin for a further 4 days. In an effort to negate the effects of the endogenous MRP and PGP expression in OAW42SRs on the resistance of the cells, indomethacin and cyclosporin A, which block MRP and PGP function respectively, were included in some treatments. The IC50 value for each treatment was calculated and is shown below (Tables 3.4.5.1 and 3.4.5.2).

Table 3.4.5.1 shows the results from the first 4 sets of treatments. It can be seen that in treatments 1 to 3, all the antisense oligos appeared to increase the sensitivity of the cells to Adriamycin as compared to the control and lipofectin only treated cells. The magnitude of this change was greater when indomethacin and cyclosporin A were not included in the treatments (Table 3.4.5.1, Treatment 2, normal vs. indomethacin + cyclosporin A). However the sense oligonucleotides also had a considerable sensitising effect, being comparable to many of the antisense oligonucleotides.

The cells treated with mdr-1 antisense were generally more sensitive than those treated with mdr-1 sense. However, the margin was never greater than two-fold, and both were significantly more sensitive than the control or lipofectin only treated cells.

Increasing the concentration of the oligonucleotides appeared to have only a marginal, if any, effect on resistance. Increasing the concentration of LRP A2 from 1 to 5 μM only decreased the IC50 value from 0.020 to 0.015 μg/ml and 0.032 to 0.018 μg/ml in treatments 3 and 4 respectively with indomethacin and cyclosporin A included. However, when exposed to Adriamycin alone, the IC50 appeared to increase from 0.302 to 0.878 μg/ml for 1 and 5 μM LRP A2 respectively.

Table 3.4.5.2 shows the IC50 values for OAW42SR and 2R120 cells treated with LRP A1, A2, S2 and additional controls, SCR (scrambled) and NON (nonsense), as well as control and lipofectin only treated cells. There were no significant reductions in the drug sensitivity between the antisense treated, control oligonucleotide and the control cells for treatments 1 and 2. Indeed, in treatment 1-(Indomethacin + cyclosporin A)

Table 3.4.5.1 Adriamycin IC_{50} values (μg/ml) for LRP and mdr Antisense oligo treated OAW42SR cells with or without the addition of cyclosporin and indomethacin (5μg/ml each)

	Indomethacin + Cyclosporin A				Normal	
	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 2	Treatment 4
LRP A1	0.108	0.037	0.026	0.037	0.313	0.849
LRP A2	0.064	0.019	0.020	0.032	0.154	0.302
LRP A3	0.200	0.025	0.018	0.011	0.502	0.531
LRP A4	0.127	0.056	0.034	0.026	1.482	0.544
LRP A5	0.077	0.034	0.018	0.019	0.630	0.299
LRP A6	0.060	0.022	0.029	0.026	0.455	0.314
LRP S5/2*	0.117	0.021	0.070	0.028	0.413	0.311
Lipofectin	0.382	0.101	0.169	0.029	5.000	0.950
Control	0.389	0.201	0.244	0.048	5.000	3.027
Mdr AS 1/2 **		0.010	0.022	0.023	0.118	0.540
Mdr S 1/2 **		0.020	0.028	0.018	0.145	0.940
LRPA2 1μm			0.020	0.032		0.302
LRPA2 2μm			0.018	0.023		0.698
LRPA2 5μm			0.015	0.018		0.878

* - LRP S5 was used for repeats 1 and 2, while LRP S2 was used for repeats 3 and 4
** - mdr AS1 and S1 were used for repeat 2, while mdr AS2 and S2 were used for repeats 3 and 4

Table 3.4.5.2 Adriamycin IC_{50} values (μg/ml) for LRP antisense and control oligo treated OAW42SR and 2R120 cells

	Indomethacin + Cyclosporin A			Normal	
	Treatment 1	Treatment 2	Treatment3*	Treatment 2	Treatment3*
LRP A1	2.560	1.120	1.622	0.541	0.578
LRP A2	1.919	1.280	1.761	0.310	0.408
LRP S2	1.530	0.256	0.472	0.569	0.523
NON	1.724	0.869	1.372	0.330	0.480
SCR	1.674	0.876	0.542	-	0.691
Lipofectin	1.887	4.250	5.500	0.275	0.514
Control	1.924	1.656	5.200	0.272	0.500

- - Cells in repeat 3 were incubated for 18h pre drug addition in antisense free media.

Table 3.4.5.3 Adriamycin IC_{50} values (μg/ml) for LRP antisense pulse-treated OAW42SR cells in 25 cm^2 flasks

	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Average
LRP A1	- *	- *	- *	- *	-
LRP A2	3.65	0.6	3.03	- *	2.43 ± 1.61
LRP S2	- *	- *	5	2.5	3.75 ± 1.77
NONSENSE	13.89	4.8	22.91	8.51	12.53± 7.86
CONTROL	5.18	5.0	3.85	2.3	4.08 ± 1.33

* - Too few cells for accurate readings.

and treatment 2-normal, LRP A1 treated cells appear to have increased resistance to Adriamycin. Only in treatment 3, with indomethacin and cyclosporin A addition, do the antisense treated cells exhibit a reduction in Adriamycin resistance as compared to untreated cells. However, in this case all of the control oligonucleotides caused greater sensitivity to the drug than LRP A1 or A2. This indicates that the initial reductions observed in drug resistance (Table 3.4.5.1) may be due to phosphorothioate oligonucleotides in general, possibly because of some inherent toxicity, as it does not appear to be an antisense specific effect.

To ensure that the apparent lack of effect of the LRP antisense oligonucleotides in modulating resistance to Adriamycin was not due to the treatment conditions, cells were treated with antisense in $25cm^2$ flasks as opposed to 96-well plates used in the previous experiments, prior to addition of drug. All the cells treated for the analysis of LRP protein and RNA expression were treated in flasks, which creates a slightly different cellular environment than in 96-well plates. It was also decided to pulse the cells after antisense treatment, for just 2 hours with a higher concentration of drug. Normal toxicity assay conditions require that the cells be exposed to drug continuously over 7 days. Any initial effect of the antisense molecules of the cells at the start of treatment would have worn off by the end of the drug treatment. Therefore, by pulsing the cells, it was postulated that the cells would be "hit" with the drug at a time when the antisense was at its most potent. After pulsing, cells were incubated for a further 7 days to allow any drug effect to be expressed.

Table 3.4.5.3 shows the result of four treatments with LRP A1, LRP A2, LRP S2, Nonsense and untreated control and their subsequent pulsing with Adriamycin. No results were obtained for treatments with LRP A1, as this oligonucleotide, along with LRP S5 appeared to have toxic non-specific side-effects, preventing sufficient cells to grow to enable accurate readings. However, it can be seen that the average IC50 value for treatment with LRP A2 is lower than for LRP S2, Nonsense treated and untreated cells. However, this reduction is only of the magnitude of 1.7. Given the large standard deviations, this result is probably not significant.

3.4.6 Immunoprecipitation analysis of LRP expression

After 72 h of treatment with the various oligonucleotides, cells were trypsinised, washed three times, pelleted and frozen at -80°C until ready to use. Each pellet contained $1x10^6$ cells. Protein was precipitated with the LRP-56 MAb and run on a 7.5% polyacrylamide gel, as described earlier (Section 2.94-2.9.6). Band intensity was measured by densitometry to give relative levels of protein expression.

Figure 3.4.6.1 (a) shows the results of the first repeat of the immunoprecipitation procedure. It can be clearly seen that the LRP band for LRP A1 treated OAW42SRs is virtually eliminated. The LRP band for SCR treated cells is also significantly reduced, but this is probably due to under loading of that sample, judging by the lack of a secondary band (IgG band) on the gel. LRP A2 did not appear to have any affect on LRP protein levels, nor did the controls LRP S2, NON and Lipofectin, all being similar to the control cell samples. The relative quantities of protein present, as measured by densitometry, are shown in Figure 3.4.6.1 (b).

The results for the second repeat of antisense treatment and immunoprecipitation procedure are shown in Figure 3.4.6.2. From Figure 3.4.6.2 (a) it can be seen that both LRP A1 and LRP A2 caused a significant decrease in LRP expression levels in the OAW42SR cells, as compared to the controls. This is also reflected in the densitometry measurements (Figure 3.4.6.2 (b)), which show the band intensity for LRP A1 and A2 clearly reduced. The discrepancy between bands for LRP A2 in repeats 1 and 2 may be due to the degradation of the oligonucleotide used for repeat 1. A fresh batch of antisense was used for the second repeat, possibly explaining the greater efficacy.

Figure 3.4.6.1 Immunoprecipitation and densitometry analysis of LRP antisense and control oligo treated OAW42SR cells : Repeat 1

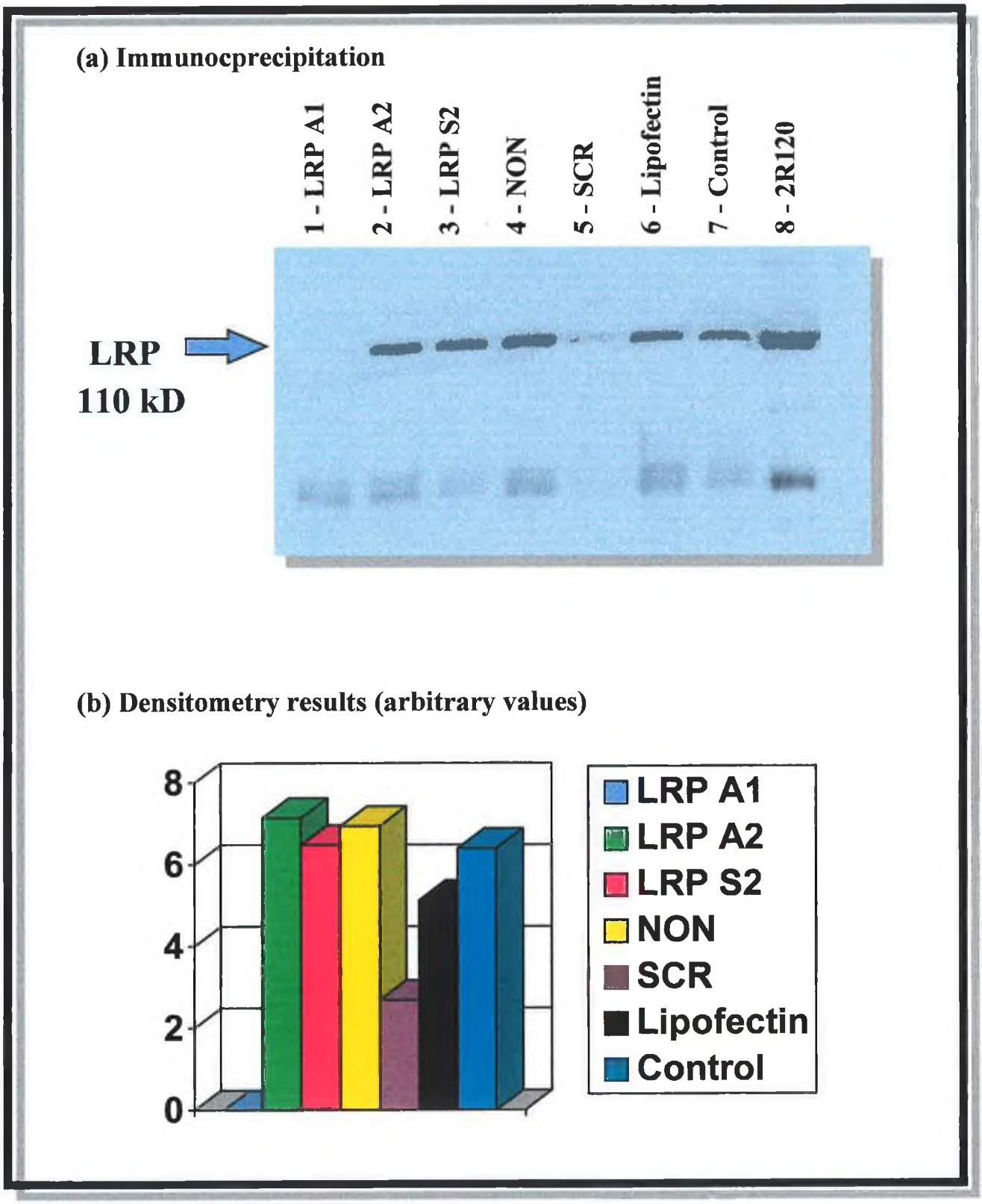
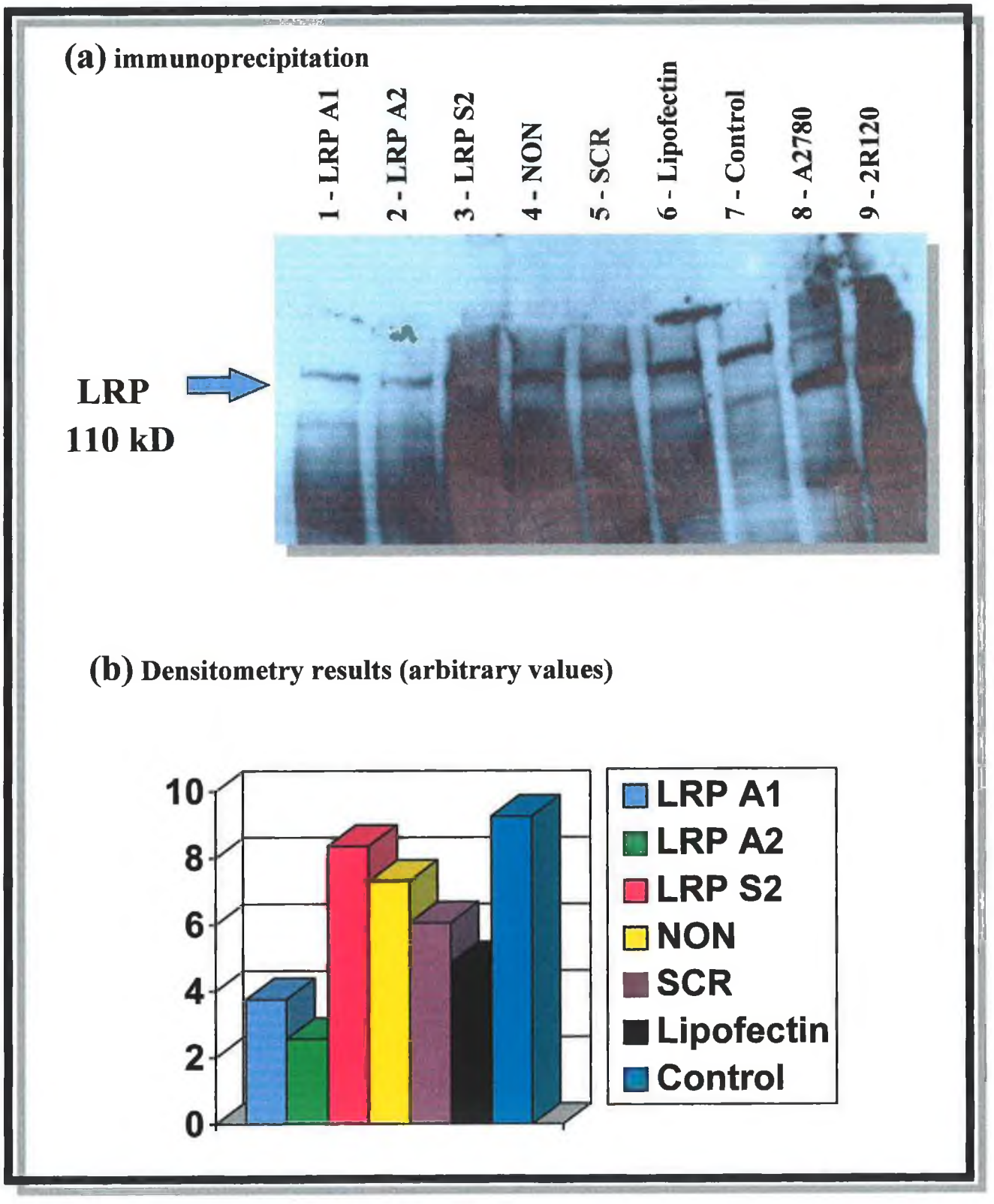


Figure 3.4.6.2 Immunoprecipitation and densitometry analysis of LRP antisense and control oligo treated OAW42SR cells : Repeat 2



3.4.7 Northern blot analysis of LRP expression

Poly A+ RNA was isolated from antisense and control oligonucleotide treated OAW42SRs, 48h after initiation of the treatment. 1µg of Poly A+ RNA from each sample was run on a denaturing RNA gel and electroblotted onto Hybond nylon membrane. The membranes were then hybridised with an LRP-Riboprobe and, after stripping, a GAPDH probe, both labelled with P^{32}. After hybridisation and washing the membranes, X-ray film was exposed for varying lengths and developed. Figure 3.4.7 (a) and (b) shows a representative result from hybridising with an LRP and a GAPDH probe respectively. Corresponding densitometry readings are also given, and Figure 3.4.7 (c) shows the LRP bands densitometry values normalised to the GAPDH bands densitometry readings.

From Figure 3.4.7 (a), it can be seen that there appeared to be little difference in the levels of LRP expression between the antisense treated or control oligonucleotide treated cells. However, the untreated control cells appeared to have reduced levels of LRP expression. As expected, the 2R120 cells gave a strong positive signal for LRP.

Figure 3.4.7 (b) shows the result of the GAPDH hybridisation with the same membrane as above. It is clearly evident that the samples from LRP A1 and LRP A2 treated OAW42SRs contain far more Poly A+ RNA than any of the other samples. The untreated control cell sample shows the least amount of RNA loaded.

When the values for LRP expression are normalised to the GAPDH values (Figure 3.4.7 (c)), there is clearly a significant reduction in the levels of LRP mRNA expression for both the LRP A1 and the LRP A2 treated OAW42SR cells. LRP A1 treated cells show over a 3-fold reduction in the levels of LRP mRNA expression, as compared to the untreated control cells, while LRP A2 treated cells exhibit almost a 2-fold decrease. Unusually, the Nonsense and Scrambled oligonucleotide controls appeared to have increased the level of LRP mRNA expression in this treatment. The positive control cell line, 2R120, has the highest level of normalised LRP expression.

Figure 3.4.7 Northen Blot and densitometry analysis of LRP expression in LRP antisense treated OAW42SRs

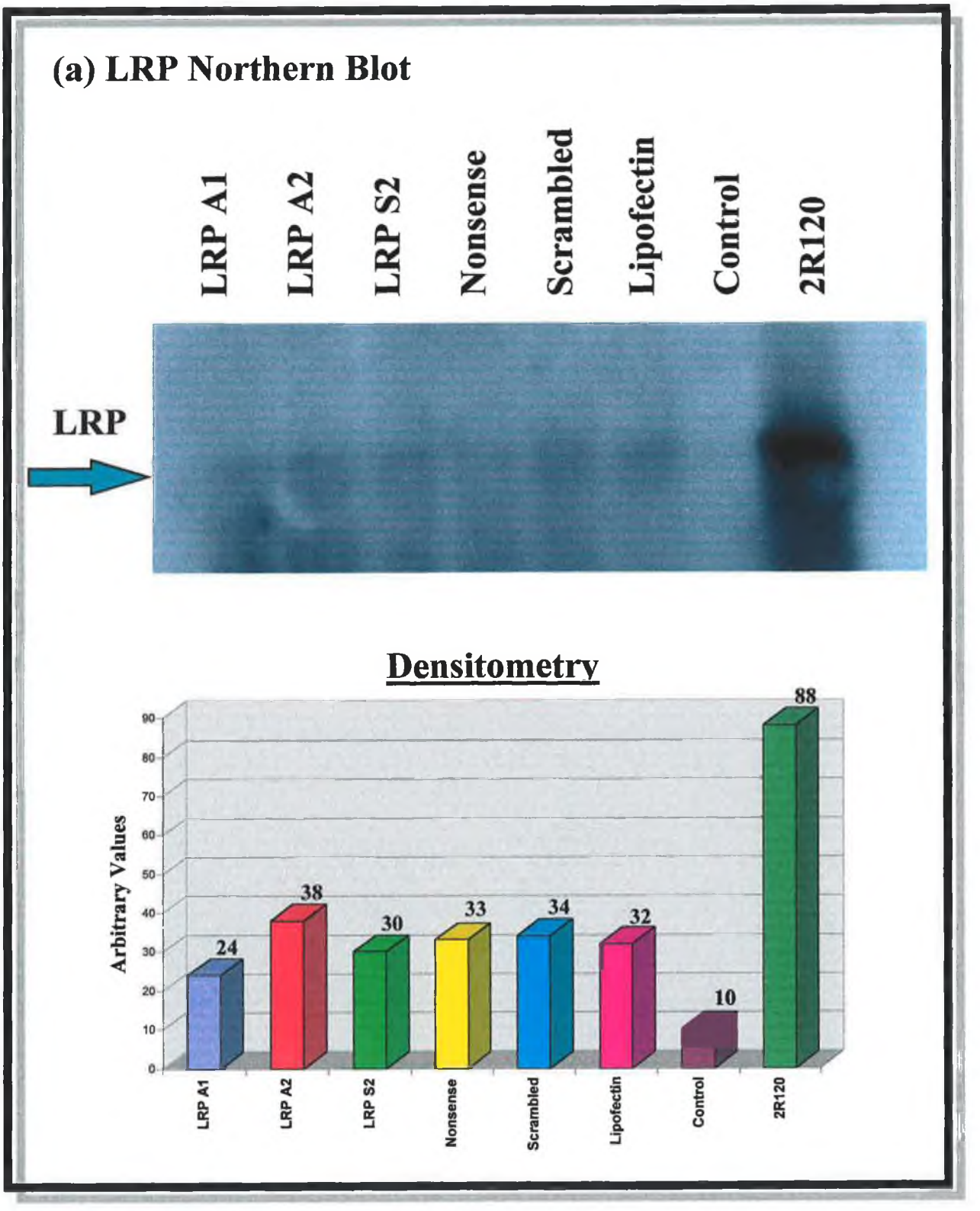


Figure 3.4.7 (cont'd) Northen Blot and densitometry analysis of LRP expression in LRP antisense treated OAW42SRs

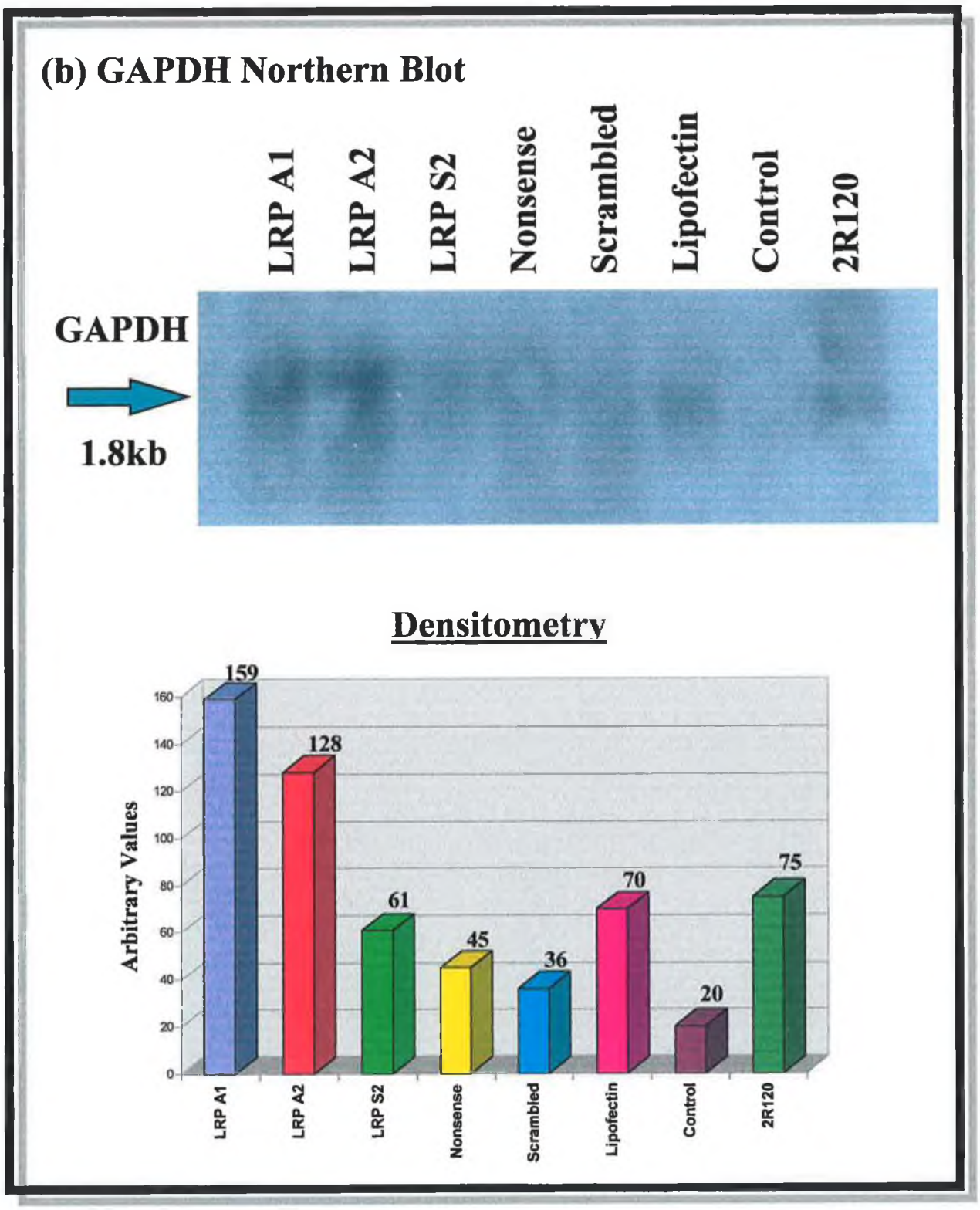
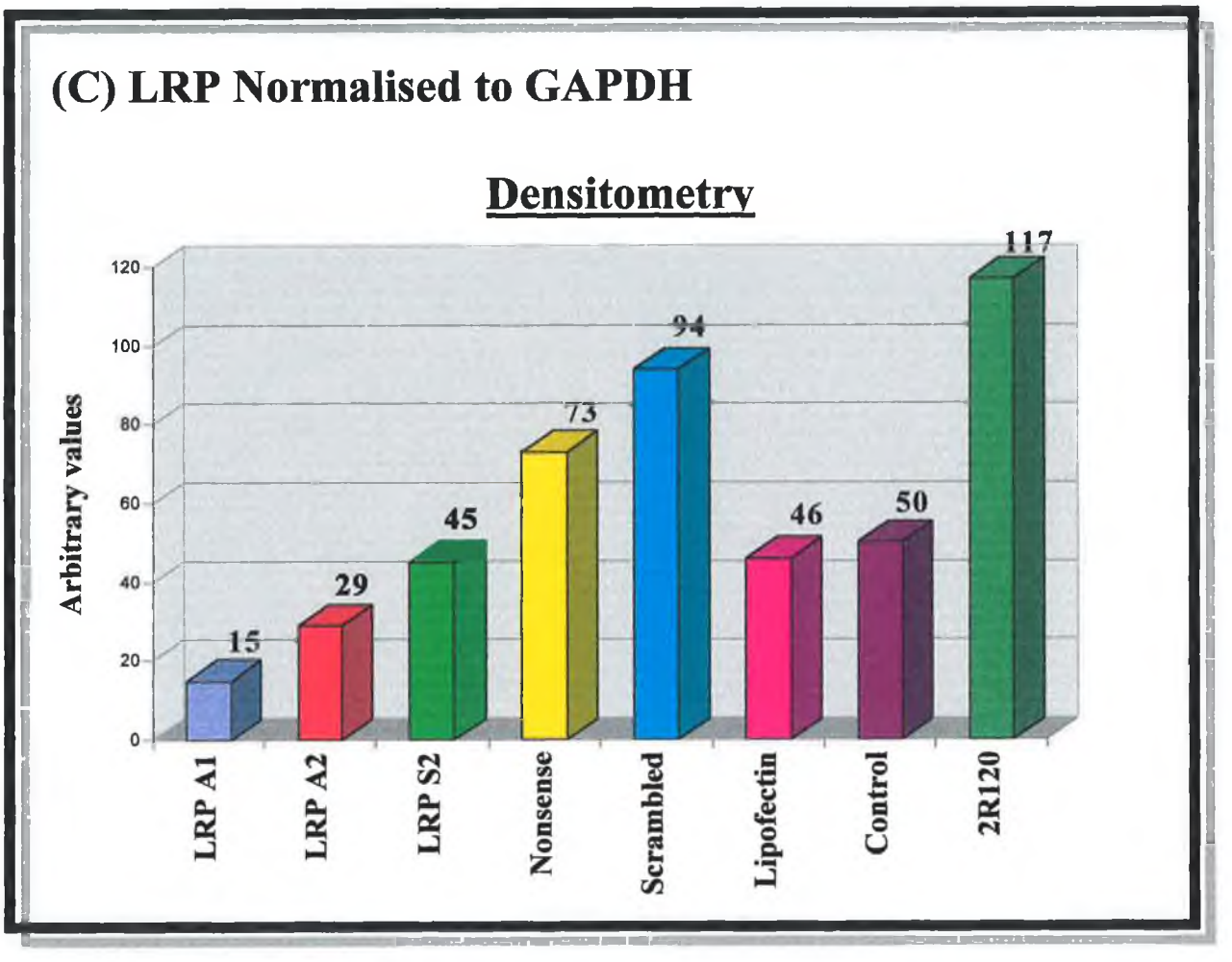


Figure 3.4.7 (cont'd) Northen Blot and densitometry analysis of LRP expression in LRP antisense treated OAW42SRs



3.4.8 Correlation of LRP expression and drug resistance

RT-PCR analysis demonstrated the ability of the antisense oligonucleotides to inhibit LRP mRNA expression maximally between 4 and 48h after initiation of treatment. Inhibition of LRP mRNA expression by LRP A1 and A2 was also clearly demonstrated by northern blot analysis. This inhibition of LRP mRNA expression was reflected by a marked reduction in the LRP protein levels in cells treated with these two antisense oligonucleotides. The control oligonucleotides had no obvious effect on LRP protein levels and little effect on mRNA levels. However, the adriamycin toxicity assays demonstrated no difference in drug resistance in cells treated with LRP A1 or A2 and the control oligonucleotides. This indicates that LRP does not directly mediate MDR in the OAW42SR cell line.

4. DISCUSSION

4.1 Analysis of LRP-ribozyme and antisense RNA transfectants

4.1.1 Initial selection of clones

The OAW42SR and OAW42S cells were transfected with either the LRP-ribozyme or –antisense RNA plasmids, or the control vector pHβ. The ribozyme construct that was employed was designed using the recommendations in the literature to give optimum cleavage efficiency. The ribozyme targeting LRP was deigned to cleave a GUC triplet at base 1152 in the middle of the coding region of LRP. GUC triplets are the optimal cleavage sites (Perriman *et al.*, 1992). The antisense flanking arms were 12 bases long in total, which represents the most efficient length for flanking sequences (Bertrand *et al.*, 1994). The ribozyme was inserted into the pHβApr-1-neo (pHβ) expression vector, containing the β-actin promoter. This vector has previously been used for effective delivery of ribozyme constructs elsewhere (Kashani-Sabet and Scanlon, 1995) and in our laboratory (Daly *et al.*, 1996). Transfection was achieved with a liposomal transfection reagent, which has been shown to achieve high transfection efficiency. The antisense RNA expression plasmid targets exactly the same region in the LRP sequence, bases 1147 to 1160 as the LRP ribozyme.

The clones were selected with geneticin to a concentration of 400 μg/ml. The geneticin IC_{50} value in the untransfected cells had been determined to be around 150 μg /ml (data not shown). A large number of clones were selected, as can be seen from Table 3.1. These clones were screened for reductions in LRP expression at both the mRNA level, by RT-PCR, and the protein level, by immunocytochemistry. Both of these methods have been widely used by other researchers when examining mdr-1 down-regulation (Holm *et al.*, 1994; Kobayashi *et al.*, 1994; Scanlon *et al.*, 1994; Bertram *et al.*, 1995; see also Section 1.5). In order to examine if any of the selected clones exhibited changes in resistance to cytotoxic drugs, miniaturised *in vitro* toxicity assays were performed on all clones.

4.1.1.1 Analysis of LRP mRNA expression by RT-PCR

LRP expression levels were first measured at the mRNA level by RT-PCR. There were no significant decreases in LRP expression apparent in any of the OAW42SR clones as compared to the parental cells (Figure 3.1.1.1). There were some alterations in LRP expression evident in the OAW42S clones (Tables 3.1.1.2 and 3.1.1.3). There was an absence of an LRP band for the clone 42S-LRP-Rz 7. However, the internal control β-actin band for this clone was much weaker than for any of the other clones. The clones 42S-LRP-AS 1, 9 and 10, and 42S-pHβ 2 exhibited reduced LRP mRNA levels, without a reduction in control β–actin levels. However, as this form of RT-PCR was not quantitative, these results acted only as an early indicator of LRP expression.

4.1.1.2 Analysis of Drug toxicity assay IC_{50} values

LRP overexpressing cell lines have previously been shown to be cross-resistant to the drugs selected for use in these preliminary toxicity assays (Scheper *et al.*, 1993; Moran *et al.*, 1997; see also Section 1.2.1). The average adriamycin IC50 values from three repeats of toxicity assays (Figure 3.1.2.5) show that there were a number of OAW42SR clones which exhibited a marked reduction in resistance to this drug. (In Figures 3.1.2.5 and 3.1.2.6, the IC50 values presented are relative to that of the parental cells OAW42SR, which were given a value of 1). This was due to a high degree of variation between repeats of the toxicity assays on a day-to-day basis (all higher on some days, all lower on other days. Therefore normalisation was required). The resistance of the clones relative to the parental cells did not display such a large degree of variation, and was thought to give a better indication of the resistance of the clones. The clones SR-LRP-Rz 2, SR-LRP-Rz 4, SR-LRP-AS 1 and SR-LRP-AS 4 exhibited between a 5 and 10-fold reduction in adriamycin IC50 values relative to the parental cells. The clones SR-LRP-AS 7, SR-LRP-AS 8, SR-pHβ 1, SR-pHβ 6, and SR-pHβ 7, also displayed significant, if not as substantial, reductions in resistance

relative to the parental cells. SR-LRP-Rz 1, SR-LRP-AS 2, SR-LRP-AS 6 and SR-pHβ 3 showed little if any change in adriamycin resistance levels.

The reduction in resistance for 3 out of 4 of the control vector transfected clones was quite unexpected. The pHβ plasmid contains no genetic element known to be capable of mediating a reduction in drug resistance and has had no effect on resistance when transfected into other cells in this laboratory. The only possible means of perturbation of the cellular machinery is the random integration of the plasmid into the cell's genome. This could be expected to perhaps interfere with some drug resistance related gene, in a very small fraction of clones, but not in such a high proportion. The only logical explanation that one could envisage is the presence of a significant degree of clonal variation, inherent in individual cells within the heterogeneous OAW42SR population. It remained to be seen whether levels of LRP expression varied between the clones and the parental cells.

The vinblastine resistance levels followed the same trend for the OAW42SR clones (Figure 3.1.2.6) with one exception. The clone SR-LRP-AS 2 displayed minor (approx. 2-fold) reductions in resistance to vinblastine, whereas no changes in resistance to adriamycin was evident for these clones. The magnitude of reduction in IC_{50} values for all clones was greater for vinblastine than for adriamycin. For example, SR-LRP-Rz 2 showed around a 10-fold reduction in adriamycin resistance, but over a 20-fold reduction in vinblastine resistance. Therefore, any sensitisation to drugs appears to be amplified with the use of vinblastine. The appearance of reduced drug resistance in the clone SR-LRP-AS 2 in the vinblastine toxicity assay may be explained by this amplification effect.

The margin of sensitisation to adriamycin was not as great for clones of OAW42S (Figure 3.1.2.7) as for OAW42SR clones. However, the reductions in resistance were still quite significant for some clones. The clones 42S-LRP-Rz 2, 3, 4, 7 and 10 and 42S-LRP-AS 1, 5, 10 and 13 all displayed a 2 to 3-fold reduction in IC_{50} values for adriamycin. Once again, however, some of the control vector transfectants, namely 42S-pHβ 3 and 4, also exhibited a lowering of resistance to adriamycin. This was especially unexpected, as the OAW42S is a cloned population, derived from a single

cell. Cell to cell variation within a cloned population has, however, previously been observed (Hanchett *et al.*, 1994; see also Section 1.4).

The reductions in vincristine IC_{50} values for the OAW42S clones (Figure 3.1.2.8) followed the same pattern as for adriamycin. In the case of these OAW42S clones, the proportion of pHβ plasmid transfectants exhibiting reductions in resistance levels, is lower than for the OAW42SR clones, but it is still intriguingly high. It was deemed important, therefore, to examine if any of these control clones exhibited a detectable down-regulation of any related MDR gene (Sections 3.2.1, 3.2.2, 3.2.3 and 3.2.5).

4.1.1.3 Analysis of LRP protein expression by immunocytochemistry with the LRP-56 MAb

Using the LRP-56 monoclonal antibody to stain cytospins of clones via immunocytochemistry, marked reductions in LRP expression levels were evident for a number of clones (Table 3.1.3.1 and Figure 3.1.3.1). The LRP-56 MAb was the antibody originally used to isolate LRP and the staining was punctate and cytoplasmic as has been observed in all LRP-overexpressing cell lines (Scheper *et al.*, 1993; Izquierdo *et. al.*, 1995, Izquierdo *et. al.*, 1996a, 1996b, Scheffer *et. al.*, 1995). The clones SR-LRP-Rz 2, SR-LRP-AS 1, 6 and 7 and SR-pHβ 1 all exhibited little or no staining for LRP. The parental cells stained intensely, as did the clones SR-LRP-Rz 1, SR-pHβ 6 and the SW1573-2R120 positive control cells (data not shown). Smaller reductions in LRP protein levels were evident for SR-LRP-Rz 4, SR-LRP-AS 2 and 4. The clones SR-pHβ 3 and 7 also exhibited a marginal decrease in staining. It appeared, therefore, that the ribozyme and antisense constructs were effective in reducing LRP expression. However, the full extent of the inhibition of LRP expression is masked somewhat by the extent of clonal variation. The reduction in LRP expression in the pHβ transfectants demonstrates that clonal variation was also evident in the levels of LRP.

As regards the OAW42S clones, there were large decreases in LRP staining evident for 42S-LRP-Rz 2, 42S-LRP-AS 1 and 42S-pHβ 1. All the other clones expressed LRP at a similar level to the parental cells.

From these preliminary results, there appeared to be a number of clones where the extent of reduction in drug resistance corresponded with a decrease in LRP protein expression. The clones SR-LRP-Rz 2, SR-LRP-AS 1, 42S-LRP-Rz 2 and 42S-LRP-AS 1 all exhibited very low LRP expression and significant reductions in drug resistance. Some of the clones with a slightly higher level of LRP expression (i.e. SR-LRP-Rz 4, SR-LRP-AS 7 and 8) displayed a correspondingly higher level of resistance. Similarly, the clone SR-LRP-Rz 1, with expression levels of LRP similar to the parental cells, exhibited a matching drug resistance profile.

Likewise, however, there was a collection of clones for which no correlation between LRP expression and sensitivity to cytotoxic drugs could be found. The clones SR-LRP-AS 6 and 42S-pHβ 1 displayed low LRP expression but high IC_{50} values. Conversely, SR-pHβ 6 and 42S-LRP-Rz 4, exhibited high levels of LRP, yet low IC_{50} values. In general, it seemed as if a greater number of clones supported a relationship between LRP expression and resistance, and it is possible that other factors are dominant in determining resistance in the clones where a correlation is not seen. Nevertheless, no definite conclusions could be drawn from these preliminary and contradictory results. It was, therefore, necessary to narrow down the number of clones being analysed, and employ more thorough assays for LRP mRNA and protein expression. Additionally, the expression levels of mdr-1 and MRP were examined, as alterations in these proteins could equally affect the levels of drug resistance observed.

The clones which were selected for further analysis were chosen to give a representative cross-section of the population of clones, reflecting low, medium and high levels of LRP expression and drug resistance. These clones are shown in Table 3.1.4.

4.1.2 Analysis of LRP, mdr-1 and MRP mRNA expression

4.1.2.1 mRNA analysis by RT-PCR

The only OAW42SR clone to exhibit a consistent reduction in LRP expression as measured by LRP RT-PCR (Figure 3.2.1.1.1) was SR-LRP-Rz 2. All the other clones maintained a level of LRP similar to the parental cells. None of the OAW42S clones exhibited visible changes in LRP (Figure 3.2.1.1.2).

Mdr-1 RT-PCR showed that none of the OAW42SR clones displayed a lower level of mdr-1 expression than the parental cells (Figure 3.2.1.2.1). Indeed, all except SR-LRP-Rz 2 appeared to have slightly increased levels, compared to the parent OAW42SRs. Similarly, with MRP RT-PCR, no reductions in MRP expression for any of the OAW42SR or OAW42S clones was evident (Figures 3.2.1.3.1 and 3.2.1.3.2).

Ribozyme expression was detected for all the ribozyme clones (SR-LRP-Rz 1, 2 and 4, 42S-LRP-Rz 2) (Figures 3.2.1.4.1 and 3.2.1.4.2). However, the level of expression for SR-LRP-Rz 4 was much weaker than any of the other ribozyme transfected clones. This lower level of expression may, however, be sufficient to cause a small reduction in LRP mRNA levels. The high level of ribozyme expression in SR-LRP-Rz 1 is unexpected, since no reductions in LRP mRNA or protein levels had been detected for this clone. For some unknown reason, the ribozyme appears not to be functioning in this clone.

As regards expression of the antisense construct, expression was detected for SR-LRP-AS 1, but not for the 42S-LRP-AS 1 clone. The bands that appeared in the lane for this clone in Figure 3.2.1.4.2, are primer dimers, which also appear in the lane for the negative control OAW42S cells. The pHβ plasmid is present in the clones SR-pHβ 1, 3 and 42S-pHβ 1 and 4.

As valuable as RT-PCR is, it is not quantitative and gives only an approximate guide to mRNA expression levels.

4.1.2.2 Northern and slot blot analysis of mRNA expression

Northern blotting of RNA samples and hybridising with a radioactive probe has been widely used to analyse the expression of both mdr-1 mRNA and other cancer related genes (Holm *et al.*, 1994; Kobayashi *et al.*, 1994; Scanlon *et al.*, 1994). However, there have been no previous reports of its use in detecting LRP mRNA expression. A probe to detect LRP was developed during the course of this thesis. Comparison of the size of the bands with standardised RNA size markers supported the hypothesis that the band detected was indeed LRP mRNA

The first LRP northern blot (Figure 3.2.2.1) shows that there was a significant reduction in LRP mRNA levels in the SR-LRP-Rz 2 clone, as well as the SR-pHβ 1 clone. Slight reductions were also visible for the SR-LRP-Rz 4, SR-LRP-AS 1 and SR-pHβ 3 clones. SR-LRP-Rz 1 appeared to show an increase in LRP expression over the parental cells. The procedure was repeated using a Riboprobe (Figure 3.2.2.3), which gives much stronger signals as RNA-RNA hybridisation is much stronger than RNA-DNA hybridisation. In this case SR-LRP-Rz 2 still exhibited a marked decrease in LRP mRNA. The extent of LRP reduction was not as great for SR-pHβ 1 in this repeat. SR-LRP-Rz 4 was the only other clone to demonstrate a reduction in LRP mRNA levels.

SR-LRP-Rz 2 appears to be the only one of the chosen clones, therefore, that consistently demonstrates a large reduction in LRP mRNA. SR-LRP-Rz 4 and SR-pHβ 1 also demonstrate a consistent, if much smaller reduction. This decrease in LRP mRNA for SR-LRP-Rz 2 correlates with the RT-PCR results. The reductions observed for SR-LRP-Rz 4 and SR-pHβ 1 may have been too minor to be observed by RT-PCR. It seems, also, that there was no reduction in LRP mRNA in the SR-LRP-AS 1 clone. This is not entirely unexpected, as antisense RNA cannot elicit RNase H activity (Branch, 1996), and may act through steric inhibition, translation arrest or inhibition of splicing molecules (Neckers *et al.*, 1992; Sharma and Narayanan, 1995; Crooke and Bennett, 1996). The reduction in LRP mRNA in SR-pHβ 1 demonstrates that the clonal variation is displayed at the transcriptional level, and not just in levels of protein expression. This once again masks the degree of

inhibition caused by the ribozyme, as it is unknown how much of the reduction is caused by the ribozyme, and how much is inherent in the cells.

Analysis of mdr-1 expression using Slot-blot preparation of membranes (Figure 3.2.2.2) reveals no marked reductions in mdr-1 mRNA levels in any of the clones as compared to the parental OAW42SR cells. There may be a slight reduction in the SR-LRP-AS 1 clone, but it is difficult to discern, due to faintness of the signals. There was a high degree of background signal over a number of the bands, which gives the impression of stronger signals. MRP mRNA levels were also analysed using Slot-blots. However, no signals were observed for any of the clones or the parents. These low levels of mdr-1 and MRP expression in the OAW42SR cell line have been previously observed (Moran *et al.*, 1997).

The analysis of mRNA expression levels in the OAW42S clones failed to yield any clear-cut results, despite numerous repeats. This was due mainly to high radioactive background on the northern blot membranes. This can occur through partial drying of the membrane during hybridisation. As a result, any bands present can become obscured. Figure 3.2.2.4, is an example of a northern blot with a probe for LRP. The bands for OAW42S and 42S-LRP-Rz2 are missing. There does appear to be a reduction in LRP mRNA in the 42S-LRP-AS 1, as compared to 42S-pHβ 1 and 4. However, as the levels of LRP cannot be compared to the parental cells, no conclusions can be drawn from this result.

In summary, the analysis of mRNA expression shows that there are a number of clones with varying levels of LRP mRNA expression. All of the observed reductions in expression cannot be attributed entirely to the expression of the ribozyme or antisense constructs. Nonetheless, these variations in LRP expression give a good basis to assess the expression of LRP in relation to drug resistance.

4.1.3 Analysis of LRP protein expression

4.1.3.1 Immunocytochemical analysis of LRP expression with the LRP-56 MAb

The staining patterns on the OAW42SR clones with the LRP-56 MAb (Table 3.2.3) followed the same pattern as previous experiments (Table 3.1.3.1). Substantial reductions in LRP expression (approx. 70-80 %) were observed for the clones SR-LRP-Rz 2, SR-LRP-AS 1 and SR-pHβ 1. A smaller decrease (approx. 40-50%) was once again observed for SR-LRP-Rz 4, while no significant changes were evident for SR-LRP-Rz 1 and SR-pHβ 3, as compared to parental OAW42SR cells. LRP staining patterns of the OAW42S clones (data not shown) was also as previously observed (Table 3.1.3.2).

Staining with a mdr-1 monoclonal antibody (Table 3.2.3 and Figure 3.2.3.2) revealed no variations in Pgp expression between any of the clones. MRP expression was not analysed by this method due to the stickiness of the MAb to the OAW42 cells during the staining procedure. This makes accurate evaluation of staining intensity difficult.

4.1.3.2 Analysis of LRP expression by immunoprecipitation

The two repeats of the immunoprecipitation procedure (Figures 3.2.4.1 and 3.2.4.2) highlight a consistent and marked decrease in LRP expression in the clones SR-LRP-Rz 2 and SR-LRP-AS 1, as compared to OAW42SR cells. (The repeated presence of bands under the 110 kD LRP bands has been noted previously (Izquierdo *et al.*, 1996a). They are the result of the precipitation of the immunoglobulins present in every sample). Indeed, there is an almost total elimination of LRP protein in these clones. These results correlate with the immunocytochemistry results, which also demonstrated a large reduction in LRP for both of these clones.

In Figure 3.2.4.1, reductions in LRP expression can be observed for the clones SR-LRP-Rz 4 and SR-pHβ 3. However, upon repetition of the procedure (Figure 3.2.4.2),

these reductions were no longer evident. The expression level of LRP in the OAW42SR parental cells appears to be reduced in relation to most of the clones in this second repeat. It has recently been observed in this laboratory that when the OAW42SR population is passaged a certain high number of times (110-115), the LRP expression levels appear to decrease gradually. It has been noted that low passage numbers (passage 86) of OAW42SR cells contain low levels of LRP, and that with increasing passage number (over passage 92) the LRP expression levels and drug-resistance increase (Moran *et al.*, 1997). While these results are, at first sight, contradictory, it may be that above a certain number of passages, the cells are once again reverting to the original low level of LRP expression. The OAW42SR cells used in the second repeat of the immunoprecipitation procedure were at passage number 112, and may have been exhibiting this lowering of LRP levels. This should be taken into account when comparing the strength of LRP expression. In comparison to SR-LRP-Rz 1, both SR-LRP-Rz 4 and SR-pHβ 3 maintain a reduction in LRP expression. This moderate reduction in LRP correlates with the immunocytochemistry results for SR-LRP-Rz 4, but not however for SR-pHβ 3.

The clone SR-pHβ 1 appears to display an over-expression of LRP in Figure 3.2.4.1, with a much lower level of expression in Figure 3.2.4.2. However, despite the fact that equal cell numbers of each clone are used for the preparation of samples, the SR-pHβ 1 lane appears to be overloaded. This is highlighted by the strength of the secondary immunoglobulin band, which is much stronger for SR-pHβ 1 than any other sample. In Figure 3.2.4.2, this clone appears to have a lower level of LRP expression than SR-LRP-Rz 1, SR-LRP-Rz 4 and SR-pHβ 3. This is in line with the previous findings by immunocytochemistry, where SR-pHβ 1 exhibited lower levels of LRP than these other three clones.

LRP expression levels appear to be similar for OAW42S, and the clones 42S-LRP-Rz 2, 42S-LRP-AS 1 and 42S-pHβ 4 (Figure 3.2.4.3 and 3.2.4.4). However, no LRP expression could be observed for the 42S-pHβ 1 clone. This correlates with the immunocytochemistry results. However, the two sets of results do not correlate for the clones 42S-LRP-Rz 2 and 42S-LRP-AS 1, as they were found to have reduced LRP expression by immunocytochemistry.

4.1.3.3 Analysis of Pgp and MRP expression by Western blotting

Figure 3.2.5.1 shows that both SR-LRP-Rz 1 and SR-LRP-AS 1 appear to have significant reductions in Pgp levels as compared to the OAW42SR parental cells. SR-LRP-Rz 4 and SR-pHβ 1 exhibited a smaller decrease in Pgp. These reductions contradict the earlier immunocytochemistry results, where no reduction in Pgp expression was observed in any clone. No significant differences were observed between the OAW42S clones and the parental cells.

Only SR-LRP-AS 1 and SR-pHβ 3 appear to show a reduction (approx. 35% and 30% respectively) in MRP levels, as compared to OAW42SR cells (Figure 3.2.6.1). The variations observed are most likely due to the inherent variations in individual cells within the heterogeneous OAW42SR population. The OAW42S clones appear to have increased levels of MRP, when compared to the parental cells (Figure 3.2.6.2).

4.1.4 Correlation of LRP mRNA and protein expression levels

Table 4.1.4. shows the approximate levels of LRP expression at the mRNA and protein level of all the clones relative to their parental cells. This is to facilitate an understanding of all the above results and enable correlation's to be drawn. The values shown are averaged from the results previously discussed, and are meant as only rough guides to the trends in expression levels.

For SR-LRP-Rz 1, there is a strong correlation between the mRNA and protein expression levels, both of which are largely unchanged from the parental cells. SR-LRP-Rz 2 shows a drop in LRP mRNA levels of around 50 to 70%, and this is reflected by a drop in LRP protein levels to between 5 and 20 % of the parental levels. SR-LRP-Rz 4 exhibits a smaller drop in LRP mRNA levels (~ 20%), and this is reflected in a smaller reduction in LRP protein levels of around 50%. The SR-LRP-AS 1 clone shows a very minimal, if any, reduction in LRP mRNA. However, there is a drop in protein expression in this clone to between 5 and 20% of the parental cell

levels. This discrepancy between protein and RNA levels highlights the fact that cleavage of the target RNA is not strictly necessary in order to block translation of the target protein. As mentioned previously, antisense RNA is incapable of eliciting RNase H cleavage, and probably acts mainly through steric hindrance of the cellular translational machinery. (Branch, 1996).

The relatively high level (~45%) of downregulation of LRP at the mRNA level in SR-pHβ 1, is reflected by approx. a 20% reduction as measured by immunocytochemistry, but only around a 50 % reduction at the protein level by immunoprecipitation. The two methods for assessing LRP protein expression in the SR-pHβ 3 clone do not, however, correlate. While there is a moderate (~15%) reduction in LRP mRNA levels, no protein reduction was observed by immunocytochemistry. However, immunoprecipitation indicated over a 2-fold reduction in protein levels. The analysis by immunoprecipitation and immunocytochemistry was separated by a gap of several months. It may be that the SR-pHβ 3, undergoes the same reversion in LRP levels with increasing passage number as has been by the OAW42SR parental cells. It has been shown that even within a clonal population, individual cells can maintain different expression levels of certain proteins (Hanchett *et al.*, 1994). A low LRP expressing sub-population may have dominated the growth in the SR-pHβ 3 cells, resulting in a reduction of observed LRP levels.

The immunocytochemistry and immunoprecipitation results for the clones of OAW42S also show a high degree of variation. However, since the only measure of LRP mRNA expression is the non-quantitative RT-PCR results, no conclusions can be made regarding a correlation of LRP mRNA and protein levels in these cells.

The results show that these clones represent a wide range of LRP expression levels, which allows an accurate analysis of LRPs role in drug resistance. The observed changes may not be strictly due to the action of the ribozyme or antisense, as highlighted by the reductions observed in pHβ clones. However, the indications are that in SR-LRP-Rz 2 and SR-LRP-As 1 the ribozyme and antisense constructs

respectively are functioning properly, as the margin of LRP reduction is much greater than for either pHβ clone.

Table 4.1.4 Expression levels of LRP mRNA and protein relative to the parental cells (Approximate values only)

	RNA		Protein	
Clone	RT-PCR	Northern Blot	Immnocyto-chemistry	Immuno-Precipitation
OAW42SR	100	100	100	100
SR-LRP-Rz 1	100	135	100	135
SR-LRP-Rz 2	50	30	20	5
SR-LRP-Rz 4	100	80	50	50
SR-LRP-AS 1	100	95	20	5
SR-pHβ 1	100	55	20	50*
SR-pHβ 3	100	85	100	45
OAW42S	100	-	100	100
42S-LRP-Rz 2	100	-	30	140
42S-LRP-AS 1	100	-	20	195
42S-pHβ 1	100	-	0	20
42S-pHβ 4	100	-	100	120

Values are averages from at least two repeats, and are given as a rough percentage of the parental cell value (either OAW42SR or OAW42S).

* Estimated from only one repeat of immunocytochemistry through comparison with SR-LRP-Rz 1

4.1.5 Toxicity Assays and LRP drug profile

4.1.5.1 Toxicity Assays on SR-LRP-Rz 1 and SR-LRP-Rz 2

Extensive toxicity assays, using a wide range of cytotoxic drugs, were initially carried out on the clones SR-LRP-Rz 1 and SR-LRP-Rz 2. The reasoning behind this was to establish which drugs were affected by expression of the LRP protein. The two clones above were chosen, as one exhibits consistently high LRP expression levels, while the other exhibits almost total elimination of LRP protein expression. The drugs used in this assay were both classical MDR-related drugs and non-classical MDR drugs. There have been a number of reports in which both classical and non-classical MDR drugs appear to be modulated by the overexpression of LRP (Scheper *et al.*, 1993; Ikeda *et al.*, 1997; Komarov *et al.*, 1997; Moran *et al.*, 1997; Parker *et al.*, 1997). These drugs include adriamycin (doxorubicin), mitoxantrone, etoposide (VP16), vincristine, cytarabine, methotrexate and cisplatin. These drugs fall into following categories: Anthracyclines (adriamycin); Epipodophyllotoxins (etoposide (VP-16)); *Vinca* alkaloids (vincristine); Antimetabolites (methotrexate and cytarabine); Covalent DNA-binding drugs (cisplatin); and Non-covalent DNA-binding drugs (mitoxantrone) (Pratt *et al.*, 1994).

The drugs employed in this assay were selected from five of the aforementioned drug categories. From Table 3.2.6.1, it can be seen that SR-LRP-Rz 2 shows a decrease in resistance to anthracyclines (adriamycin, daunorubicin and epirubicin), epipodophyllotoxins (VP-16) and *Vinca* alkaloids (vincristine, taxol, and taxotere). This is in following with the previous reports mentioned above. However, there was no significant reduction in resistance between SR-LRP-Rz 1 and SR-LRP-Rz 2 for either 5-Fluoro-Uracil (antimetabolites), or the covalent DNA-binding drugs melphalan (alkylating agent) and carboplatin (platinum compound). This contradicts the reports in which cell lines overexpressing LRP have been found to be resistant to both these categories of drugs (Ikeda *et al.*, 1997; Komarov *et al.*, 1997; Parker *et al.*, 1997).

This pattern, however, follows that of a typical mdr-1 overexpressing MDR cell line, which exhibit cross-resistance to anthracyclines, epipodophyllotoxins and *Vinca*

alkaloids, but are unchanged in their resistance to antimetabolites, alkylating agents or platinum compounds (Clynes *et al.* 1993). Due to this striking similarity to an MDR drug-profile, and the fact that small differences in Pgp and MRP expression was observed between the clones SR-LRP-Rz 1 and SR-LRP-Rz 2, it was decided to extend this drug-profile assay, to all the OAW42SR and OAW42S clones. This was done to establish whether the same pattern of drug resistance was to be found between the parental cells and all of the clones.

4.1.5.2 LRP drug profile assay on all OAW42SR and OAW42S clones

Table 3.2.6.2 (a) and (b) shows that all the clones display the same pattern of drug resistance as seen between SR-LRP-Rz 1 and SR-LRP-Rz 2. However, there was only a minimal, if any, reduction in resistance to VP-16 in relation to the parental cells. The magnitude of resistance to VP-16 can be up to 100-fold smaller than for adriamycin and vincristine in MDR cell lines (Redmond, 1991; Heenan, 1991). It cannot be ruled out, therefore, that there is alteration in the resistance to epidophyllotoxins in these cells. Additionally, if the IC_{50} levels of the clones are compared to that of SR-LRP-Rz 1, they all display a reduction in the resistance to VP16. The SR-LRP-Rz 1 clone has been shown to be largely invariant in its expression levels of the various MDR-related genes and drug resistance, while the parental cells have displayed some fluctuations. These results, therefore, again appeared to represent a Pgp mediated MDR drug profile.

An interesting observation is that the clone SR-pHβ 3 appears to have a significantly increased sensitivity to a number of drugs, whereas in previous experiments (Figures 3.1.2.5 and 3.1.2.6) this clone displayed resistance levels on a par with the parental OAW42SR cells. This may possibly be a reflection of the reduction in LRP expression observed in this clone over time (see Section 4.1.3.2).

The changes in IC50 values between the OAW42S clones and the parental cells was very minimal for all the drugs (Table 3.2.6.3 (a) and (b)). The clone 42S-pHβ 1, displays only a 3.7 and 2.4 fold increase in sensitivity to adriamycin and vincristine

respectively, although no LRP expression could be observed for this clone by immunoprecipitation or immunocytochemistry (see Sections 3.1.3 and 3.1.4). This result correlates with the finding that for the OAW42S cells, an increase in adriamycin or vincristine resistance was not concomitant with the original increase in LRP expression (Moran *et al.*, 1997). It was postulated by Moran *et al.*, that the form of LRP expressed in the OAW42S cells was non-functional. The results shown here seem to support this idea and the observed small changes in resistance could be due simply to clonal variation or changes induced by the selection process itself.

4.1.6 Correlation of LRP expression levels and drug resistance

It can be seen from Table 4.1.4 that, as regards LRP expression in the OAW42SR clones, SR-LRP-Rz 2 and SR-LRP-AS 1 have the lowest levels, with almost total elimination of protein expression. They are followed by SR-LRP-Rz 4, SR-pHβ 1 and SR-pHβ 3, all of which exhibit around a 50% reduction in LRP protein expression, as measured by immunoprecipitation. SR-LRP-Rz 1 displays a slight increase in LRP expression levels over the parental cells.

If LRP was directly linked to resistance to anthracyclines and *Vinca* alkaloids, then the magnitude of LRP downregulation should be reflected by a similar drop in the IC_{50} value for these drugs. It would follow, therefore, that SR-LRP-Rz 2 and SR-LRP-AS 2, would show a similar drop of resistance, which should be significantly greater than that for SR-LRP-Rz 4, SR-pHβ 1 and SR-pHβ 3. These clones, in turn, should display a much greater sensitivity than either SR-LRP-Rz 1 or the parental cells.

Table 4.1.6 represents a summary of the toxicity assay results discussed above for adriamycin, vincristine and VP16. These three drugs represent the three classes of drugs (namely anthracyclines, *Vinca* alkaloids, and epipodophyllotoxins) to which the clones have displayed altered resistance. Results are given as a percentage value of the average IC50 value for the parental cells. This is to allow direct comparison with the levels of LRP mRNA and protein expression given in Table 4.1.4

4.1.6 Adriamycin, Vincristine and VP16 toxicity assay IC50 values relative to the parental cells.

	Adriamycin	Vincristine	VP16
OAW42SR	100	100	100
SR-LRP-Rz 1	58	99	240
SR-LRP-Rz 2	9	1.4	80
SR-LRP-Rz 4	10	2	65
SR-LRP-AS 1	10	2.2	124
SR-pHβ 1	12	1.2	110
SR-pHβ 3	11	3	84
OAW42S	100	100	100
42S-LRP-Rz 2	89	68	57
42S-LRP-AS 1	32	52	43
42S-pHβ 1	27	44	59
42S-pHβ 4	236	60	41

All IC50 values are given as a percentage of the average IC50 value for the parental cells.

It can be seen from Table 4.1.6 that all of the OAW42SR clones, with the exception of SR-LRP-Rz 1, display almost identical levels of reduction in resistance to the drugs listed. This is in spite of the fact that SR-LRP-Rz 2 and SR-LRP-AS 1 have both exhibited almost total elimination of LRP protein expression, while SR-LRP-Rz 4 and SR-pHβ 1 and 3 displayed only a 50 % reduction in LRP expression. Indeed, SR-pHβ 1 demonstrates a greater sensitivity to vincristine than any of the other clones. These results clearly indicate that no direct correlation can be drawn between LRP expression levels and resistance to these drugs. However, the fact that reductions in LRP expression, are almost always paralleled by some level of increased sensitivity to drugs, even if not directly correlateable points towards LRP being simply co-expressed with another resistance mechanism. Although decreases in LRP expression are generally associated with some level of reduction in drug resistance for these chosen clones, the initial range of clones illustrated that this was not always the case. From Sections 3.1.2 and 3.1.3 it can be seen that the clones SR-LRP AS 6 and 42S-pHβ 1 both exhibited low levels of LRP expression, yet high levels of resistance to adriamycin and vinblastine/vincristine. In addition low levels of drug resistance were observed in the clones SR-pHβ 6 and 42S-LRP-Rz 4 in the absence of a decrease in LRP expression.

The observation that the drugs to which resistance is altered are the same as those for Pgp overexpressing cell lines, indicates that mdr-1 may be mediating resistance in these cells. The form of mdr-1 being expressed in the low-resistance clones may be somehow less active than in the parental cells or SR-LRP-Rz 1. These alterations may be a result of the clonal variation.

4.2 Analysis of Uncloned OAW42SR transfectants

As the variability of the results for the OAW42SR clones showed, the cloning of transfectants from a heterogeneous population can lead to complications in the analysis of protein expression and drug resistance. It is difficult to assess whether all the changes in LRP mRNA and protein expression which were observed, were actually due to targeting of the gene with the antisense and ribozyme constructs. The

high degree of variability seen in the control vector transfectants highlights the need for an alternative to the cloning out of individual cells from a population.

One method of circumventing this is to simply transfect the OAW42SR cell line with the same constructs and select with geneticin, but to analyse the heterogeneous transfectant population en mass. The levels of expression of the ribozyme and antisense constructs, and thus LRP mRNA and protein, may differ greatly from cell to cell. However, if the population as a whole exhibits a drop in LRP expression, it should be possible to assess whether drug resistance is affected, thus avoiding the additional problems of clonal variation.

In keeping with this proposal, the OAW42SR cell line was once again transfected with the anti-LRP ribozyme and antisense constructs, in addition to the control vector. The transfectants were selected for 4 weeks in increasing concentrations of geneticin. The populations were then analysed for LRP mRNA expression by northern blot, LRP protein expression by immunoprecipitation, and drug resistance by *in vitro* toxicity assays. The expression levels of mdr-1, MRP, ribozyme/antisense constructs and internal control gene β-actin were also assessed using RT-PCR.

4.2.1 Analysis of LRP mRNA expression by northern blot

The northern blot analysis of LRP mRNA expression in the uncloned OAW42SR transfectants (Figures 3.3.2.1 and 3.3.2.2) show that the anti-LRP ribozyme caused only a very minor reduction in LRP expression. LRP expression in the antisense RNA transfectants appears to be increased as compared to the parental cells. In fact, only the control vector transfectants displayed a significant reduction in LRP mRNA levels, of between 40 and 60%. These results were contrary to expected findings.

Due to the substantial reduction in LRP mRNA for the pHβ transfectants, it was important to establish that there was no mix up in plasmids prior to transfection, and that it was indeed the empty control vector pHβ that had been transfected. RT-PCR

analysis confirmed the presence of only the vector in these cells (Figure 3.3.3.1). The band seen for the pHβ transfectants in the ribozyme/antisense expression RT-PCR was slightly smaller than the antisense band, as expected. The ribozyme and antisense expression bands were present in the ribozyme and antisense transfectants samples respectively, confirming that the constructs were being expressed in these cells.

4.2.2 Analysis of LRP expression by immunoprecipitation

The immunoprecipitation procedure was repeated three times on different cell pellet samples, prepared at separate times, from the uncloned transfectants and the parental cells. However, absolutely no LRP expression could be observed in any of the samples on any occasion. Even, the positive control SW1573-2R120 cells failed to produce any hint of LRP expression. This failure of the immunoprecipitation procedure was perplexing. During earlier immunocytochemical analysis, batch variation in the LRP-56 MAb had been observed. On certain occasions, no LRP expression could be detected with certain batches of the antibody, in any of the OAW42SR or OAW42S clones. It may have been that the batch of antibody purchased for this immunoprecipitation analysis was not of adequate standard.

4.2.3 Adriamycin and Vincristine Toxicity assays

The results of the toxicity assays (Figures 3.3.1.1 to 3.3.1.4) revealed that all of the transfected cell populations displayed a reduction in resistance to both adriamycin and vincristine. For both drugs, the greatest decrease was observed for the pHβ transfected cells. However, this decrease was only marginally greater than for the antisense or ribozyme transfected cells.

There appears to be no correlation between the levels of LRP mRNA in the transfected cells and the levels of resistance to adriamycin and vincristine. The antisense RNA expressing cells show a much greater level of LRP mRNA expression

than the ribozyme expressing cells, while both exhibit similar drug resistance levels. However, as seen earlier, LRP mRNA levels in the antisense transfected cells cannot be correlated with LRP protein expression.

The levels of mRNA and protein expression were found to correlate for the ribozyme and control pHβ vector clones described earlier. Therefore, between these two populations a weak correlation may be drawn between LRP expression and drug resistance. However, there is some discrepancy between the magnitude of reduction in LRP mRNA and the magnitude of reduction in IC_{50} values. The pHβ-transfected population exhibits a 2-fold lower level of LRP mRNA expression, and yet exhibits only a 1.3-fold drop in adriamycin and vincristine IC_{50} values.

There is, once again, little evidence to support a direct role for LRP in multidrug resistance.

4.2.4 RT-PCR analysis of mdr-1 and MRP expression

RT-PCR analysis revealed a lack of reduction in either mdr-1 or MRP expression for any of the transfected populations (Figures 3.3.3.3 and 3.3.3.4). These two MDR related proteins appear, therefore, to have no role in the observed reductions in drug resistance, although variations in protein level and activity cannot be ruled out. It may be that the transfection of cells with an expression vector interferes with some other mediator of drug resistance, whose analysis has not been included in this work. Whether this postulated mediator of drug resistance was a known protein, or some, as yet, undiscovered mechanism would require much time and effort.

4.3 Use of antisense oligonucleotides to downregulate LRP expression

Another method of modulating LRP expression without the associated problems of selection of clones and clonal variation is to use antisense oligonucleotides. The use of antisense oligonucleotides is more widespread than the use of ribozymes or antisense RNA to downregulate the expression of a target gene. There are therefore an extensive number of reports and guidelines governing their use, as discussed earlier (Stein, 1994; Branch, 1996; Byrne *et al.*, In press; see also Section 1.5).

Out of 50 potential sequences, only 6 were found to be sufficiently unique in terms of compatibility to the human genome. These 6 sequences spanned the entire LRP gene sequence (see Figure 3.4.1): LRP A1 was targeted to the 5' untranslated region; LRP A2 targeted the initiation codon region; LRP A3 was directed to a site just 3' of the initiation codon; LRP A4 and A5 were targeted to coding region; and LRP A6 was directed against the 3' untranslated region. All of these sites have been successfully targeted by other researchers targeting different genes (see Section 1.5.4 and Table 1.5.4). The sequence targeted by LRP A5 was the same as that targeted by the anti-LRP ribozyme and antisense RNA constructs. The sense sequences corresponding to LRP A2 and A5 (LRP S2 and LRP S5 respectively) were used as controls. In later experiments, a scrambled version of LRP A2 and a nonsense oligonucleotide were used as additional controls. Cells treated with only lipofectin and untreated controls were also used. All the oligonucleotides were phosphorothioates, the most widely used form of antisense (see Section 1.5.3). The oligonucleotides ranged in size from 16 to 20 bp, and thus, were sufficiently long to be unique relative to the entire genome (Branch, 1996). All oligonucleotides were delivered using lipofectin, a liposome based transfection reagent. They were delivered, unless otherwise stated, at a concentration of 1 μM. Cells were treated with a double dose of antisense, separated by 24h.

The total length of treatment was 48h prior to RNA isolation, and 72h before protein analysis and toxicity assays. Work on the downregulation of Pgp expression using antisense oligonucleotides, (Bertram *et al.*, 1995; Li *et al.*, 1997) has demonstrated that treatment length must be sufficient to allow for the full antisense effect on protein

expression to be observed. The length of treatment required depends on the half-life of the target protein. The half-life of the LRP protein is, as yet, unknown. It was thought that 72 h of treatment should allow for downregulation of the protein to be evident. Stewart *et al.*, (1996), demonstrated the increased efficacy of antisense oligonucleotides when employed in a two-hit or double-dose regime, as was employed in this thesis.

In order to test the transfection procedure, and the methods used to detect target gene mRNA and protein expression levels, anti-mdr-1 antisense oligonucleotides were employed (Table 3.4.1). The two antisense sequences used had previously been shown to cause downregulation of mdr-1 mRNA and Pgp expression (see Table 1.5.4). The corresponding sense sequences were used as controls. If the down-regulation of mdr-1 and Pgp could be demonstrated through the use of these antisense oligos, then it could reasonably assumed that the same methods of transfection and detection would work for LRP antisense sequences.

4.3.1 Immunocytochemical staining with LRP-56 MAb on antisense treated OAW42SR cells

Initial immunocytochemical analysis with the LRP-56 MAb (Table 3.4.3.1) showed that all 6 antisense oligonucleotides caused a reduction in LRP expression in relation to sense oligonucleotide treated, lipofectin treated and untreated control cells. There was a degree of variation in staining intensity present, even on different cytospins from the same antisense treatment. It has been noted in this laboratory that the staining can be affected by ambient conditions, leading to day-to-day variations in staining patterns and intensity. It was thought unusual that all of the antisense sequences appeared capable of downregulating LRP. Many researchers have employed only one or two antisense oligonucleotides in their experiments, and based all their work upon these (Vasanthakumar and Ahmed, 1989; Rivoltini *et al.*, 1990; Clynes *et al.*, 1992; Efferth and Volm, 1993; Thierry *et al.*, 1993; Cucco and Calabretta, 1996; Hirtake *et al.*, 1997; Liu *et al.*, 1997; Sola and Colombani, 1997). However, any work which has employed a number of oligonucleotides and a screening process to find the most effective sequence, has found that only a small

fraction of sequences tested will actually exhibit antisense effects (Jaroszewski *et al.*, 1990; Bertram *et al.*, 1995; Alahari *et al.*, 1996; Stewart *et al.*, 1996). The fact that all of the antisense sequences were effective, could indicate that the LRP mRNA has a fairly open structure with low levels of intramolecular bonding and secondary structure formation.

To see if the concentration of oligonucleotide being used (1 μM) was the optimal concentration, the effects of three different concentrations (1, 2 and 5 μM) of LRP A2 on LRP expression were compared. The concentration of antisense oligonucleotide to be used in an experiment appears to vary greatly between authors, with no general consensus being evident. Vasanthakumar and Ahmed, (1989), used a 30 μM concentration of oligo. Efferth and Volm, (1993), found that in their experiments 10 μM was more effective than 1 or 5 μM. Bertram *et al.*, (1995), observed that a concentration of 2 μM gave better results than either 0.2 or 5 μM. When targeting MRP expression, Stewart and colleagues used an oligonucleotide of 0.5 μM, as higher concentrations were found to be slightly toxic to cells. It can be seen from these examples that there are no strict guidelines as to which concentration to employ. It appears to vary between the choice of oligonucleotide and cell line. One definite observation is that increased oligonucleotide concentration does not always lead to increased antisense effects, as the use of too high a concentration of oligonucleotides could be toxic to the cells. In the work presented here (Table 3.4.3.1), only very minimal variations were observed, with all three concentrations causing a marked drop in LRP expression. It was therefore decided that the original concentration of 1 μM was sufficient for the purposes of these experiments.

The treatment of the OAW42SR cells with the anti-mdr-1 antisense and sense oligonucleotides had variable effects (Table 3.4.3.2). In 2 out of 3 repeats, the mdr-1 antisense induced a greater reduction in Pgp levels than the corresponding sense sequence. However, in only 1 out of 3 repeats did mdr-1 antisense treated cells show a reduction in Pgp as compared to lipofectin-only treated or untreated cells.

The use of sense control oligonucleotides alone is not deemed sufficient for proof of antisense effect (Stein, 1994; Wagner, 1994; Branch, 1996). Therefore, the scrambled

control (SCR) and a nonsense oligonucleotide (NON) were employed in further immunocytochemical analysis in addition to the LRP A1 and A2 antisense sequences, and the LRP S2 sense oligonucleotide. 2R120 cells were also treated with these oligonucleotides, to examine if cells with higher expression levels of LRP could also be targeted effectively with the antisense sequences.

In the first set of treatments (Table 3.4.3.3 (a)), the LRP A1 antisense appeared to have a greater impact than the LRP A2 sequences in downregulating LRP expression in the OAW42SR cells. However, in the 2R120 cells, this efficacy was reversed, with LRP A2 being more effective than LRP A1. The results for the second set of treatments (Table 3.4.3.3 (b)) shows a highly variable pattern of staining for both antisense sequences. Both antisense sequences appeared to be largely ineffective in 2R120 cells. Both LRP A1 and A2 had varying success in reducing LRP levels in the OAW42SR cells. LRP A2 caused a decrease in LRP in OAW42SR cells in repeats 1 and 3 of the staining, while LRP A1 reduced LRP levels in repeat 2. All the other oligonucleotides had no visible effect on LRP expression, in comparison to the untreated control cells.

This variability in staining patterns meant that these results in isolation were of no real value in determining whether the downregulation of LRP with the antisense sequences, was a true sequence-specific antisense effect.

4.3.2 Immunoprecipitation analysis of LRP expression in antisense treated OAW42SR cells

All samples for immunoprecipitation analysis were taken 72h post-initiation of treatment. The first repeat of the immunoprecipitation procedure (Figure 3.4.6.1) demonstrated an almost total elimination of LRP expression in the LRP A1 antisense treated OAW42SRs. All the other oligonucleotides appeared ineffective at reducing LRP expression. The reduced LRP band observed for cells treated with the scrambled control (SCR), is probably not that significant. The lack of the immunoglobulin secondary band indicates unequal loading of this sample. However, from this result,

LRP A2 appeared to have no effect on LRP expression, contrary to the findings by immunocytochemistry.

In a second repeat of this procedure (Figure 3.4.6.2), however, both LRP A1 and LRP A2 caused a significant reduction in the level of LRP expression, as compared to control oligonucleotide treated and untreated cells. A possible explanation for the failure of LRP A2 to reduce LRP expression during the first treatment, is the degradation of the oligonucleotide through repeated thawing and freezing of the stock sample, although there have been no such reports in the literature. A freshly manufactured batch of antisense oligonucleotides was used for the second repeat of the immunoprecipitation procedure. From that point onwards, the oligonucleotides were aliquoted upon delivery, to avoid repeated thawing/freezing.

These more unambiguous immunoprecipitation results, in addition to the immunocytochemistry results, clearly indicate the ability of the antisense oligonucleotide to reduce LRP expression at the protein level. This reduction appears to be a sequence-specific antisense effect, as none of the control oligonucleotides caused a similar decrease in LRP expression.

4.3.3 RT-PCR analysis of LRP mRNA expression in antisense treated OAW42SR cells

Initial RT-PCR analysis of total RNA samples extracted from OAW42SR cells after 48h treatment with all 6 antisense oligonucleotides (Figure 3.4.4.1), failed to show up any reduction in LRP levels. It may have been that the level of LRP downregulation by the antisense was not discernible by RT-PCR. As seen with anti-LRP ribozyme transfected OAW42SR cells (see Section 4.4), the magnitude of LRP down-regulation was much greater at the protein level than the mRNA level. It seems that small reductions in the mRNA are being amplified to large reductions at the protein level.

RT-PCR on mdr-1 antisense and sense treated cells revealed a visible drop in mRNA levels for the antisense treated over sense treated or untreated cells (Figure 3.4.4.2).

This demonstrated the efficacy of the mdr-1 antisense, and validated the protocols used for transfection of the antisense.

In an attempt to demonstrate antisense inhibition of LRP over a period of time, total RNA samples were taken from OAW42SR cells treated with LRP A2, at times 0, 4, 24 and 48h post initiation of treatment. As with all treatments, a second dose of antisense was added at 24h to prolong the antisense effect. The first repeat of this experiment showed that at 4h post-initiation of treatment, the level of LRP mRNA is already significantly reduced (Figure 3.4.4.3 (a)). At 24h, the LRP levels appear to have reduced further, with this level of inhibition being maintained until at least 48h. However, the bands for the internal control β-actin gene, are slightly fainter for these last two time points, indicating that maximal inhibition may indeed be reached by 4h. This is borne out by two further repeats of this assay (Figure 3.4.4.3 (b) and (c)), in which LRP reaches its lowest level after just 4h of treatment, and this level is maintained for at least 44h. The actual maximal level of inhibition reached appears to vary from repeat to repeat, possibly reflecting batch variation in the oligonucleotides. Cells treated with sense (LRP S2) oligonucleotides, or untreated cells display no reduction in LRP levels over time (Figure 3.4.4.3 (d) and (e)).

When treatment of the OAW42SR cells with LRP A1 and A2 was compared to treatment with the full range of control oligonucleotides, no differences in LRP expression were discernible (Figure 3.4.4.4). This is despite the fact that changes in LRP expression were clearly visible by RT-PCR for the treatment with LRP A2 over time (Figure 3.4.4.3). This indicates the intrinsic variability of the RT-PCR procedure.

4.3.4 Northern blot analysis of LRP mRNA expression in antisense treated OAW42SR cells

Northern blots, using probes for LRP and GAPDH, confirmed the ability of both LRP A1 and A2 to inhibit LRP expression at the mRNA level. Figure 3.4.7 (c) illustrates the three fold reduction in LRP mRNA seen after treatment with LRP A1, and the almost 2-fold drop after treatment with LRP A2, as compared to untreated cells. Sense

and lipofectin only treated cells, exhibited similar LRP expression levels as the untreated controls, while nonsense (NON) and scrambled (SCR) oligonucleotide treated cells, appeared to show an actual increase in LRP expression. Similar increases in target mRNA expression have been seen even after antisense treatment in some cell systems (Probst and Skutella, 1996).

4.3.5 Adriamycin toxicity assays on antisense treated OAW42SR cells

The above results have clearly demonstrated the potential of the anti-LRP antisense sequences to inhibit LRP expression at both the mRNA and protein level. Adriamycin toxicity assays were used to see if this reduction in LRP was correlated to a decrease in drug resistance. In order to eliminate any possible complication of toxicity assay results through the expression of Pgp and MRP, inhibitors of these proteins were employed in the toxicity assays. Indomethacin and cyclosporin A were used to inhibit MRP and Pgp respectively, and were used at the maximum non-toxic dose. This dose was calculated from toxicity assays using these compounds (data not shown). Toxicity assays were also performed in the absence of these inhibitors.

Table 3.4.5.1 illustrates that all of the antisense oligonucleotides caused a decrease in adriamycin IC_{50} in the OAW42SR cells, as compared to lipofectin only treated or untreated cells. Increasing the concentration of LRP A2 causing only a minor increase in sensitivity. This correlates with the minimal reduction in LRP protein expression seen when oligonucleotide concentration was increased from 1 to 5 μM (Table 3.4.3.1).

However, sense treated cells also displayed a significant, if somewhat smaller, decrease in adriamycin IC_{50} value as compared to the untreated controls. Mdr-1 antisense treated cells were shown to be more sensitive than the untreated cells. However, once again, the sense treated cells also displayed a significant reduction in IC_{50} value. All these effects were seen in either the presence or absence of indomethacin and cyclosporin A. This appeared to indicate that the effects seen were due to alterations in LRP alone.

It was not clear, at this point, whether the observed reductions in IC50 value after treatment with sense oligonucleotides were isolated phenomena, or whether all oligonucleotides would cause a similar effect. This would indicate that the antisense was acting non-specifically to induce reductions in adriamycin IC50 value.

When the action of LRP A1 and A2 was examined in parallel with the sense, nonsense and scrambled control oligonucleotides (Figure 3.4.5.2), it was found that there was no difference in IC_{50} value between antisense treated or control oligo treated cells. It has been clearly demonstrated that both LRP A1 and LRP A2 do reduce LRP expression levels, while the control oligos have no apparent effect. The absence of decreases in IC_{50} values for the antisense treated cells over control oligo treated cells, obviously points towards LRP having no role in drug resistance.

There were a number of considerations, however, before final judgement on LRP's role in drug resistance in antisense treated cells could be made. The toxicity assays, described above, were performed in 96-well plates. The cells, which were used for analysis of mRNA and protein expression, were grown in 25 and 75 cm^2 flasks. Flasks and 96-well plates cultivate different cellular environments. For instance, the IC50 value for a given drug would be different for cells grown in flasks and cells grown in 96-well plates. In addition, a number of the oligonucleotides displayed toxic effects on cells grown in 96-well plates, while no effects were generally visible in cells grown in flasks (results not shown). This may be due only to the difference in culture times between cells grown for mRNA/protein analysis (2/3 days) and toxicity assays (8 days), but this was not proven. It was decided, therefore, to perform a number of toxicity assays on cells grown in 25cm^2 flasks.

Another consideration was that during conventional drug toxicity assays, the cells are incubated with drug present for 7 days. Obviously, during the last few days of the assay, any antisense effect would have worn off, with the oligonucleotides being degraded. Therefore, the results seen may not truly reflect the effect of the antisense on protein expression. To overcome this problem, pulsed drug addition was used. After 72 h of antisense treatment, cells were pulsed with adriamycin for 2 hours. The cells were then incubated for a further 4 days in antisense- and drug-free media, to allow any toxic effect of the drug on cellular metabolism to be expressed. It has been

shown (Figure 3.4.6.1 and 3.4.6.2) that LRP protein expression is significantly reduced at 72h. It was postulated, therefore, that this method of pulsing cells with drug, when there was a significant reduction of LRP expression in antisense treated cells, should clearly indicate whether or not LRP played a role in drug resistance.

Table 3.4.5.3 shows that LRP A2 treated cells do show slightly reduced adriamycin IC50 values, as compared to sense treated and untreated cells. Nonsense treated cells, appear to show a significant increase in resistance to adriamycin. The fact that LRP A2 treated cells displayed not even a 2-fold reduction in resistance to adriamycin as compared to untreated cells, while LRP expression levels in antisense treated cells have been shown to be dramatically reduced, indicates a lack of any significant role for LRP in drug resistance. This is supported by the fact that adriamycin resistance was markedly elevated in nonsense treated cells, while no increase in protein expression was observed in these cells. However, the toxicity assays displayed a high degree of variation and the margins of error for these results are quite large. In spite of this, there appears to be no evidence from the above antisense experiments, to support a direct role for LRP in drug resistance.

4.4 LRP plays no major role in MDR in OAW42SR cell line

All of these results presented above contradict the theory that LRP is involved in drug resistance. The evidence that had been presented to date, postulating that LRP mediated a novel form of multidrug resistance, is mainly circumstantial. LRP was discovered through the selection of SW1573 lung carcinoma cell line to increasing concentrations of adriamycin (Scheper *et. al.*, 1993). These cells were found to be Pgp negative, but exhibited an energy dependent reduction of drug accumulation. They also displayed moderate cross-resistance to vincristine, gramicidin D and etoposide. A revertant cell line, which was cultured for 9 months without drug, exhibited a reduction in LRP expression back to parental levels, and a concomitant reversal of drug resistance.

LRP was found to be the major vault protein (MVP) (Scheffer *et al.*, 1995). The evolutionary conservation of the MVP sequence, and its abundant expression, implied its importance in cell function (Rome *et al.*, 1991). Structural similarities suggested that vaults comprise the central plug of the NPC and may mediate bidirectional transport of substrates between the nucleus and cytoplasm (Rome *et al.*, 1991; Chugani *et al.*, 1993).

Widespread overexpression of LRP was found in both drug-selected and drug-unselected drug resistant cancer cell lines of various origins (Scheper *et al.*, 1993; Verovski *et al.*, 1996; Moran *et al.*, 1997; see also Section 1.2.3). LRP is overexpressed in cell lines selected with a wide range of classical (i.e. anthracyclines, *Vinca* alkaloids and epipodophyllotoxins) and non-classical (i.e. antimetabolites, covalent DNA-binding drugs and non-covalent DNA-binding drugs) MDR drugs (Scheper *et al.*, 1993; Ikeda *et al.*, 1997; Komarov *et al.*, 1997).

LRP has been found to be a very useful prognostic indicator in the treatment of cancers. LRP is highly expressed in cancers that are refractory to chemotherapy, while chemosensitive tumors express LRP in only a minority of cases (Izquierdo *et al.*, 1996a). LRP is more likely to be expressed in malignant melanomas and lung cancers that have previously been exposed to chemotherapeutic drugs than untreated cancers (Schadendorf *et al.*, 1995; Dingemans *et al.*, 1996). This suggests an association between exposure to anticancer drugs and induction of LRP. Additionally, LRP is known to be a good indicator of poor response to chemotherapy in adult acute leukaemia (Klumper *et al.*, 1995). It has also been demonstrated that LRP overexpressing cell lines redistribute daunorubicin into a punctate cytoplasmic pattern. LRP negative parental cells localise daunorubicin in a diffuse nuclear and cytoplasmic pattern (Dietel *et al.*, 1990).

However, none of these findings provided direct evidence of LRP mediated drug resistance. There have also been reports that appear to contradict this theory. In the ovarian carcinoma cell line OAW42S, it was found that with increasing passages of the cells, the level of LRP expression increased significantly (Moran *et al.*, 1997). However, there was no concomitant increase in the cells resistance to chemotherapeutic drugs. The authors postulated that a non-functional form of LRP

might be present in these cells. However, it could equally be that LRP is normally co-expressed with some unknown resistance mechanism, that is absent in these OAW42S cells.

Transfection and expression of the full-length LRP cDNA construct into mouse 3T3 fibroblasts failed to confer a MDR phenotype (Scheffer *et al.*, 1995). It has also been reported by the same authors, that induction of LRP expression in drug selected cell lines caused a 15-fold increase in vault synthesis (Kickhoefer *et al.*, 1998). It is thought that LRP is the limiting factor in vault synthesis. If this is the case, the lack of even a minimal increase in drug resistance following massive overexpression of the LRP cDNA, indicates a minor role if any for LRP in drug resistance.

Since the discovery of LRP in 1993, there has been no evidence directly linking LRP to drug resistance. In no case has LRP expression been eliminated through the use of genetic manipulation, thus allowing clear assessment of its role in drug resistance. The work presented in this thesis provides this evidence. The clonal variations observed in the OAW42SR and OAW42S transfection clones masked somewhat the full effect of the ribozymes and antisense. Yet, it was this very variation that provided a wide base of clonal populations with varying levels of LRP expression which allow a thorough comparison of LRP expression with drug resistance. It was shown that, in the OAW42SR cell line at least, LRP played no direct role in mediating multidrug resistance.

It may be that in other cell lines LRP is involved in MDR, and until these types of experiments are repeated in different cell lines, it cannot be ruled out totally that LRP is involved in MDR. However, the evidence presented in this thesis for the OAW42SR cell line appears fairly conclusive. It may be that LRP is co-expressed with other resistance mechanisms. Kickhoefer *et al.*, (1998), postulated that LRP may interact with proteins or RNA that actively bind drugs. The fact that LRP was found to be downregulated to some extent in many cells (see Section 4.1.6), which exhibited low drug resistance, lends support to this theory.

5. SUMMARY AND CONLCUSIONS

5.1 Analysis of ribozyme and antisense RNA inhibition of LRP expression

Analysis of the anti-LRP ribozyme and antisense transfectant clones demonstrated that there was a high degree of variation in the levels of LRP expression and drug resistance between the clones. The main results were:

- There was up to a 10-fold and 20-fold decrease in adriamycin and vinblastine IC_{50} values respectively, for a number of ribozyme and antisense RNA transfected OAW42SR cells (Figures 3.1.2.5 and 3.1.2.6). However, some ribozyme and antisense RNA transfectants displayed no reduction in resistance. Additionally, while some control vector (pHβ) transfectants were unchanged in their resistance levels, others showed a marked reduction in resistance. This was the first indication of the high degree of clonal variation inherent within the heterogeneous OAW42SR population.
- The reductions in IC_{50} values for ribozyme and antisense RNA transfected OAW42S (Figures 3.1.2.7 and 3.1.2.8) cells were not as dramatic as for the OAW42SR clones. This may be due to the lower intrinsic resistance of the parental OAW42S cells. A number of the pHβ transfectants demonstrated a reduction in resistance to adriamycin and vincristine, similarly to the OAW42SR clones. However, as the OAW42S cell line is a homogeneous cloned population, it had been expected that the expression levels in the control clones should be less variable. There have been reports indicating that the expression level of a given gene can vary even within a cloned population, derived entirely from one cell (Hanchett *et al.*, 1994).
- LRP protein expression was shown to vary greatly between both the OAW42SR and OAW42S clones and their respective parental cells (Section 3.1.3). In a number of clones (e.g. SR-LRP-Rz 2 and SR-LRP-AS 1) LRP expression was virtually eliminated, while in others (e.g. SR-LRP-Rz 1 and 42S-LRP-AS 10) no change was evident. The differences in LRP expression seen for the different pHβ clones highlights the extent of the clonal variation.
- From this early analysis of LRP expression and drug resistance, there appeared to be little evidence correlating LRP with a direct role in multidrug resistance. Some clones (e.g. SR-LRP-Rz 2 and SR-LRP-AS 1) appeared to have low levels of LRP

and corresponding low resistance levels. In contrast, the clones SR-LRP-AS 6 and 42S-pHβ 1 displayed low LRP expression, but high drug IC_{50} values. Conversely, SR-pHβ 6 and 42S-LRP-Rz 4 exhibited high levels of LRP, yet low drug resistance. In summary, there was an equal number of OAW42SR clones that supported a correlation of LRP expression with drug resistance, as there was clones that failed to show any correlation. As regards the OAW42S clones, there were a greater number of clones that discounted a role for LRP in drug resistance than supported it.

No correlation between LRP and MDR could be concluded from the initial analysis of OAW42SR and OAW42S clones. Therefore, further, detailed analysis was performed on a selected number of clones.

- Only the clone SR-LRP-Rz 2 demonstrated a reduction in LRP mRNA, as measured by RT-PCR (Figure 3.2.1.1.1). Northern blot analysis (Section 3.2.2) also demonstrated a significant reduction in LRP mRNA in SR-LRP-Rz2, with smaller reductions being evident in SR-LRP-Rz 4 and SR-pHβ 1. All the other clones, along with the parental cells, appeared invariant in their levels of LRP expression.
- The levels of LRP mRNA expression generally correlated well with protein expression, as measured by immunocytochemistry (Section 3.2.3) and immunoprecipitation (Section 3.2.4). The only exception was SR-LRP-AS 1, which displayed only a minor reduction in LRP mRNA levels, with a virtual elimination at the protein level. This suggested that antisense RNA can effectively inhibit protein production without cleaving the precursor mRNA.
- No correlation was observed between LRP mRNA and protein levels in the OAW42S clones. However, mRNA levels were measured only by RT-PCR and, thus, were not quantitative.
- RT-PCR, immunocytochemistry and Western blot analysis demonstrated that there was little variation in the levels of mdr-1 and MRP expression between the clones and the parental cells (Sections 3.2.1, 3.2.5, and 3.2.6).
- Analysis of LRP expression in the OAW42SR clones suggests that the ribozyme and antisense constructs were functioning, but that the effects of clonal variation rendered analysis of the effect of LRP mRNA downregulation on drug resistance

difficult. RT-PCR analysis shows that the ribozyme and antisense constructs were being expressed in the respective clones. But as there was large variations in LRP expression in the pHβ clones, it cannot be assumed that the reductions in LRP expression seen in the other clones are due entirely to the expression of the ribozyme and antisense constructs.

- In spite of this uncertainty relating to ribozyme/antisense efficacy, the wide spectrum of LRP expression levels in the different clones provides a good basis for assessment of LRP's role in drug resistance.

Toxicity assays were performed with a wide range of classical and non-classical MDR drugs. This was done to indicate which drugs had a reduced efficacy in the transfected cells.

- It appears that in selected clones expressing the anti-LRP ribozyme and antisense constructs there was a reduction of resistance to anthracyclines and *Vinca* alkaloids, and possibly to a lesser extent epipodophyllotoxins (Section 3.2.7). The resistance of the clones to antimetabolites, covalent DNA-binding and non-covalent DNA-binding drugs was unaffected.
- No direct correlation could be made between the levels of LRP protein expression and levels of resistance to either adriamycin or vincristine. A number of different clones, with varying levels of LRP expression, expressed the same levels of resistance to the drugs tested.
- The similarity of the drug profile described above to that of Pgp, also raises doubts as to whether the observed reductions in drug resistance are mediated by LRP as opposed to mdr-1. Even though there were only small alterations in mdr-1 expression, altered activity of P-glycoprotein by altered phosphorylation is a possibility. Nevertheless, the results for VP16 may not be entirely concomitant with a P-170-mediated mechanism.

The high degree of clonal variation observed in this work prevented a clear examination of the efficacy of ribozymes and antisense RNA in modulating LRP expression. However, it did provide a number of clones with varying levels of LRP reduction, whether it was inherent or induced. These clones provided a clear basis with which to examine the effect of LRP expression on the drug resistance of the

cells. The above results clearly indicate that LRP is not involved in the multidrug resistance in the OAW42SR cells, or at least is not the rate-limiting step in drug resistance. The similarity of the drug profiles of the clones with that of P-glycoprotein overexpressing cells indicates that mdr-1 overexpression may be the predominant mechanism of resistance for these cells. Nevertheless, the SR-LRP-Rz 1 clone, which exhibited high levels of adriamycin, vincristine and VP-16 resistance, did not appear to be overexpressing mdr-1. However, there may have been some post-translational modifications in this clone, which induced the Pgp present to become active.

5.2 Analysis of uncloned ribozyme and antisense RNA transfected OAW42SRs

The transfection and selection of OAW42SR cells, without the cloning out of individual cells, was used as an attempt to overcome the problems of clonal variation. The transfected populations were analysed as a whole and therefore individual cell to cell variations should have been averaged out. The main findings were:

- The levels of LRP mRNA expression were found to be only minimally decreased in the ribozyme transfected cells (Section 3.3.2). This may indicate that the efficiency of ribozyme transfection was not sufficient throughout the population to result in a significant reduction in LRP expression. Individual cells may have exhibited high expression of the ribozyme and total elimination of LRP, but the apparent high LRP expression in the majority of cells would have negated this effect.
- The antisense RNA transfected OAW42SR cells exhibited an increase in LRP mRNA levels, as compared to the parental cells (Section 3.3.2). This may not necessarily indicate that the antisense construct was not functioning. It has been seen earlier that a cloned antisense RNA OAW42SR transfectant exhibited high LRP mRNA levels, but almost total elimination of protein levels. Cleavage of the target RNA is not necessary for inhibition of protein production.
- The control vector pHβ transfected cells exhibited a significant reduction in LRP mRNA expression, up to 2-fold in comparison to the parental cells (Section 3.3.2).

This once again highlights the problems associated with the transfection and selection of cells. Since the pHβ plasmid contains no constructs capable of cleaving LRP, or (as far as known) any other, mRNA, this reduction in LRP expression is a mystery. It reflects the reductions in LRP mRNA seen earlier for cloned pHβ transfectants.

- No changes in mdr-1 or MRP expression could be observed by RT-PCR analysis in any of the populations (Section 3.3.3).
- *In vitro* toxicity assays showed that the adriamycin and vincristine IC_{50} values for all the transfected populations were lower than the parental values (Section 3.3.1). The ribozyme and antisense RNA expressing populations exhibited a 2-fold reduction in resistance to both adriamycin and vincristine. The control pHβ vector transfected cells, however, exhibited almost a 3-fold reduction in IC_{50} values for both drugs.
- Repeated immunoprecipitation studies failed to produce useful results.
- There appears to be no correlation between the levels of LRP mRNA in the transfected cells and the levels of resistance to adriamycin and vincristine. The antisense RNA expressing cells show a much greater level of LRP mRNA expression than the ribozyme expressing cells, while both exhibit similar drug resistance levels. However, as seen earlier, LRP mRNA levels in the antisense transfected cells cannot be correlated with LRP protein expression.
- The levels of mRNA and protein expression were found to correlate for the ribozyme and control pHβ vector clones described earlier. Therefore, between these two populations a weak correlation may be drawn between LRP expression and drug resistance. However, there is some discrepancy between the magnitude of reduction in LRP mRNA and the magnitude of reduction in IC_{50} values. The pHβ-transfected population exhibits a 2-fold lower level of LRP mRNA expression than the ribozyme-transfected population, and yet exhibits only a 1.3-fold drop in adriamycin and vincristine IC_{50} values.

Once again, there is very little evidence to support the hypothesised role of LRP in MDR.

5.3 Analysis of antisense oligonucleotide mediated inhibition of LRP expression in OAW42SR cells

The use of antisense oligonucleotides to modulate gene expression is more widespread than the use of ribozyme and antisense expression vectors. Using antisense oligonucleotides avoids the problems associated with the stable transfection of cells and the selection of clones, which may exhibit inherent variation. The principal results were as follows:

- 6 antisense oligonucleotides were targeted to the 5' UTR, the initiation codon, the coding region and the 3'UTR of the LRP gene (Section 3.4.1). Sense, nonsense and scrambled oligonucleotide controls were used, along with lipofectin-only treated and un-treated cells.
- All of the antisense oligonucleotides tested, at a concentration of 1 μM, appeared to reduce LRP expression, as measured by immunocytochemistry (Section 3.4.3). LRP expression was unaffected by control sense oligonucleotides. No changes in LRP levels were observed in lipofectin-only treated or untreated cells.
- Increasing the concentration of one antisense oligonucleotide (LRP A2), targeted to the initiation codon of the LRP gene, from 1 to 5 μM had minimal effect on LRP expression levels. Therefore, all remaining experiments were carried out using oligonucleotide concentrations of 1 μM.
- Further immunocytochemical analysis of two of the antisense oligonucleotides (LRP A1 and A2), targeted to the 5' UTR and initiation codon, demonstrated their ability to reduce LRP expression, albeit with varying success (Section 3.4.3). While the control oligonucleotides (sense, nonsense and scrambled) continually had no effect on LRP expression, the variations in staining intensity for the two antisense oligos highlighted the need for a more accurate measure of LRP expression at the protein level.
- Repeated immunoprecipitation analysis of the antisense and control oligonucleotide treated cells, clearly demonstrated the ability of the LRP A1 and A2 antisense oligonucleotides to inhibit LRP expression (Section 3.4.6). The control oligonucleotide treated, lipofectin-only treated or untreated cells all maintained high levels of LRP expression. All samples for immunoprecipitation analysis were taken 72h post-initiation of treatment.

- RT-PCR analysis demonstrated that LRP mRNA expression in LRP A2 treated OAW42SR cells was greatly reduced by 4h of treatment, and this reduction was maintained for at least 48h post initiation of treatment (Section 3.4.4). Cells treated with a sense oligonucleotide, or untreated cells, demonstrated no changes in LRP mRNA expression over time.
- Northern blot analysis also demonstrated the ability of LRP A1 and A2 to significantly reduce LRP mRNA expression after 48h of treatment (Section 3.4.7). The sense, nonsense and scrambled oligonucleotide treated cells exhibited no reductions in LRP mRNA levels as compared to untreated cells.
- *In vitro* toxicity assays showed that all of antisense oligonucleotides tested reduced adriamycin IC_{50} values in OAW42SR cells (Section 3.4.5). However, the sense oligonucleotide treated OAW42SR cells also exhibited reductions in adriamycin IC_{50} as compared to untreated cells.
- Further toxicity assays revealed no difference in the ability of the antisense or control oligonucleotides to modulate adriamycin resistance (Section 3.4.5). It did appear, however, that all oligonucleotides exhibited a significant level of toxicity, which resulted in reduced adriamycin resistance as compared to lipofectin-only treated or untreated cells.
- Pulse toxicity assays in cell culture flasks were used as a means to more accurately reflect the condition of cells used for protein and mRNA analysis. These toxicity assays revealed a very marginal decrease in adriamycin IC_{50} value for LRP A2 treated cells as compared to sense oligonucleotide or untreated cells. It is felt however, that the margin of the reduction in adriamycin resistance did not reflect the large reductions in LRP mRNA and protein expression in cells treated with LRP A2.

The treatment of OAW42SR with anti-LRP antisense and control oligonucleotides appears, once again, to discount LRP's role in MDR. There was conclusive evidence of the ability of the antisense oligonucleotides to inhibit the expression of LRP at both the protein and mRNA level. There was some degree of variability in the efficacy of the oligonucleotides, and evidence of oligonucleotide toxicity, possibly due to their preparation and purification. However, the results failed to show any link between any LRP expression levels and resistance to adriamycin.

5.4 Conclusions

All the results presented in this thesis agree in one respect: the lack of substantial evidence to support a direct role for LRP in drug resistance in the OAW42SR or OAW42S cell line. The evidence cited in the literature, which supports a role for LRP in MDR, is purely circumstantial. There was equally, although somewhat less prolific, evidence which suggested that LRP may only be linked to drug resistance, and not directly involved. Reduced resistance is seen in clones that exhibit reduced LRP expression. However the levels of LRP expression and resistance do not correlate. It may be that LRP is co-expressed with some other resistance mechanism. The results for the ribozyme and antisense RNA transfections of OAW42SR cells indicates that variation in P-glycoprotein activity, possibly by post-translational modifications, may be solely responsible for the resistance mechanisms of these cells, although the results for VP-16 may not entirely support this.

However, an alternative explanation is that LRP may not be a relevant mechanism of drug resistance in the OAW42SR cell line, due to other resistance mechanisms being dominant. This has already been postulated for the OAW42S cell line. It has previously been observed that decreasing the levels of mdr-1 in a human non-small cell lung cancer cell line (SW1573) did not affect its resistance, as mdr-1 expression was secondary to MRP in mediating resistance in these cells (Eijdems *et al.*,1995a). Therefore, further ribozyme and antisense work needs to be carried out in an alternative LRP-expressing cell line, before LRP can be conclusively ruled out of drug resistance. In conclusion, it appears unlikely that LRP plays a significant role in the resistance mechanisms of OAW42SR cells.

5.5 Future Work

- Future work arising from this thesis should include transfection of alternative LRP overexpressing cell lines with the anti LRP-ribozyme and –antisense plasmids, along with the control pHβ vector. The cell lines chosen should be homogenous so as to minimise clonal variation. The transfectants should be analysed in a similar manner to that carried out in this thesis. This work should indicate whether the lack of a role in drug resistance for LRP is confined to the OAW42SR cell line, or if it is a widespread phenomenon.
- The *in vitro* cleavage activity of the ribozyme constructs should be assessed. This would confirm that the ribozyme construct can function properly, at least in an extra-cellular environment.
- Different ribozyme and antisense constructs should be employed to see if a more efficient target site on the LRP cDNA can be found. Other sites in the LRP sequence may be more accessible to cleavage, such as open sites at the end of stem loops. A more efficient ribozyme or antisense construct capable of total elimination of LRP expression would give a clearer picture of LRP's role in drug resistance. Constructing computer models of the LRP RNA secondary structure would facilitate the design of these ribozymes and antisense constructs.
- Using alternative backbone modifications in the antisense oligonucleotides may create less non-antisense specific toxic side effects that were evident with all the oligonucleotides. Phosphorothioate oligonucleotides are known to be somewhat toxic to cells. By substituting some or all of the phosphorothioate nucleotides for alternative less toxic analogues, this toxicity problem may be eliminated. The toxicity problem may also have been due to the preparation of the oligonucleotides, and could therefore possibly be eliminated by further purification
- The RT-PCR time-scale analysis of LRP mRNA downregulation using antisense oligonucleotides should be extended for a number of days. This would allow a determination of the longevity of the antisense mediated inhibition of LRP expression. The time-scale analysis could also be carried out at the protein level using immunoprecipitation.

- A dose-response curve assay with the antisense oligonucleotides should be carried out to further prove that the inhibitory effects seen are of a true antisense nature. Increasing the oligonucleotide concentration from 1 to 5 μM did not significantly increase the antisense effect. There is probably a saturation point of antisense concentration, above which no further inhibition is achieved. Therefore, decreasing concentrations of antisense should be employed in the dose-response curve.

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