Generation and Characterisation of Antibodies Specific for the Multidrug Resistance-Associated Protein, MRP.

A thesis submitted for the degree of Ph D

by

Lisa Connolly (B Sc)

November 1999

The research work described in this thesis was carried out under the supervision of

Prof Martin Clynes and Dr Elizabeth Moran

National Cell and Tissue Culture Centre,
School of Biological Sciences,
Dublin City University,
Glasnevin,
Dublin 9,
Ireland

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

Signed L Connolly ID No 94971277

Date 15 12 99

Abstract

Multidrug resistance associated protein 1 (MRP1) is a 190kD integral membrane glycoprotein which belongs to the ATP-binding cassette (ABC) superfamily of transport proteins The MRP1 specific MAbs, MRPr1, MRPm6 and QCRL-1 have facilitated both clinical and experimental investigations of this protein to date In this thesis we have used various types of MRP1-related immunogens in various species and utilising different immunisation protocols with the objective of producing additional MRP1 specific MAbs which might detect different epitopes Immunogens used included short synthetic peptides, fusion proteins and MRP1 over-expressing cells Species used for immunisation included New Zealand White rabbits, Balb\c mice and Wistar rats A number of immunisation routes, such as intraperitoneal, subcutaneous and the lymphatic system via footpad injections were used Variations in immunisation protocols included *in-vivo* and *in-vitro* immunisations

Resulting antibodies included three MRP1-specific polyclonal rabbit antibodies, a range of mouse monoclonal antibodies, and a number of apparently MRP1-specific rat monoclonal antibodies The mouse MAbs include one which detects a 190kD antigen present at equal or greater levels in sensitive cells compared to MRP1-overexpressing cells It may possibly detect a new form (possibly a conformational isoform which is less active or has different substrate specificity) of MRP1 One of the rat MAbs behaves in a manner similar to the well characterised MRPr1 MAb and detects both drug-selected and -transfected MRP1 by western blot analysis and stains drug selected and transfected MRP1 cells lines homogeneously by immunocytochemistry Another rat MAb detects a 190kD band in MRP1-transfected cells but not MRP1 drug-selected cells by western blot analysis and stains a sub-population of cells in both drug selected

and transfected MRP1 over-expressing cell lines None of these antibodies cross-reacted with the recently described MRP2 and MRP3 isoforms

Acknowledgements

I would like to sincerely thank Professor Martin Clynes for his tremendous encouragement and guidance during this thesis, especially for all the help during the last few months

I would especially like to thank my supervisor Dr Liz Moran for her unending help, advice and friendship I wouldn't have made it this far without your help, Liz

Thanks to all my colleagues in the NCTCC, especially Annemarie for her friendship and constant support (listening to my worries), Helena, Yizheng, Rob and Allan for their friendship, advice and humour Thanks to Liz, Helena, Annemarie and Rob for their help in the final version of this thesis Thanks to Rob for all his help with the computer problems also I couldn't forget Carol, Yvonne and Mairead for their help and kind words of encouragement

I wish to acknowledge the generosity of Mr George Schefer for his donation of antibodies and cell lines, the kind permission of Professor Marcel Kool to use the MRP3 cell line, the generosity of Professor Sarkadi for his donation of fusion proteins and Emese Sinko for further analysis of the polyclonal antibodies

A very special thanks to the boys in Beaumont, Nick, Derek and Martin for their input to a vital part of this thesis I would especially like to thank Derek and Martin for their help in the last few immunisations

Thanks to my friend Carmel for her friendship and sense of humour during the weekends we 'partied' the stress away

Finally, I would like to thank my parents and my sister for their continuous love and support, without which I would not have reached this far

This thesis is dedicated to Eddie, Rosie and Rachel The best father, mother and sister I could ever have.

TABLE OF CONTENTS

1.0. INTRODUCTION.

1 0. INTRODUCTION

1 1 Chemotherapy in cancer

Chemotherapy constitutes the principal therapeutic tool to treat unresectable or disseminated tumours resulting in a significant impact on survival in several malignancies such as Hodgkin's disease, testicular cancer and childhood acute leukaemias (De Vita, 1989) However, a major problem in the chemotherapeutic treatment of cancer is the occurrence of cellular resistance to cytotoxic drugs Chemotherapy against many types of cancers is often ineffective because tumour cells possess cellular mechanisms that enable them to resist the effects of these cytotoxic agents (Clynes, 1993, Coop, 1993) A number of different cellular mechanisms may be responsible for cytotoxic resistance and have been extensively studied in the laboratory using tumour cells made resistant by selection and growth in increasing concentrations of various cytotoxic drugs (Clynes, 1993)

1 1 1 Drug resistance mechanisms

1 1 2 Multidrug Resistance (MDR)

One particular mechanism to be revealed from these studies is that of multidrug resistance (MDR), that is, cells selected for resistance to one type of anti-cancer agent become simultaneously resistant to a range of structurally different agents These include anthracyclines, vinca alkaloids, taxanes, actinomycin D and the epipodophyllotoxins (Borst, 1991, Clynes *et al*, 1993) MDR affects patients with a wide variety of haematological cancers and solid tumours including those of the breast, ovarian, lung and lower gastrointestinal tract Tumours are often comprised of mixed populations of

malignant cells, some drug sensitive and others drug resistant In the progression of MDR, treatment with chemotherapeutic agents kills drug sensitive cells, leaving a higher proportion of drug resistant cells As the tumour begins to grow again, chemotherapy fails because the remaining cells of the tumour are drug resistant These cells can become resistant by actively lowering the intracellular drug concentration, indicating the presence of a transporter with the ability to efflux a range of different substrates The transporters responsible for this phenomenon have been identified as multidrug transporters To date two major human multidrug transporters have been identified, MDR1 (P-glycoprotein, P-170, P-gp) (Gottesman and Pastan, 1993) and MRP1 (multidrug resistance-associated protein 1, P-190 or MRP) (Cole *et al*, 1992) Both these membrane proteins are capable of transporting a range of drugs with different cellular targets and confer resistance by decreasing the intracellular concentration of drugs However, MDR1 and MRP1 are only distantly related sharing only 15% homology in their amino acid sequences (Cole *et al*, 1992)

1 1 3 Other mechanisms

Over-expression of other cellular proteins and mechanisms has also been observed, such as alterations in topoisomerase II levels (reviewed in Hoffman and Mattern, 1993), over-expression of the T antigen presenting protein (TAP) (Izquierdo *et al*, 1996a), alterations in the glutathionine system (reviewed in Moscow and Dixon, 1993) and over-expression of the lung resistance protein (LRP) (Scheper *et al*, 1993, Izquierdo *et al*, 1995)

1 1 4 The ATP-Binding Cassette (ABC) family

Membrane proteins belonging to the ATP binding cassette (ABC) family of transport proteins play a central role in the defence of cells against toxic compounds After exposure of mammalian tumour cells to one single cytotoxic drug, these cells can become resistant to a whole range of drugs by actively lowering the intracellular drug concentration Both MDR1 and MRP1 are integral membrane proteins belonging to the ABC (ATP-binding cassette) super family of transporter proteins The ABC superfamily are a structurally diverse group of protein transporters with over 50 members from eukaryote to prokaryote, but all sharing a common molecular architecture and containing at least one hydrophobic transmembrane region and a cytoplasmic nucleotide binding domain (NBD), (Higgins, 1992) These proteins are involved in the energy-dependent transport of a wide variation of chemical substrates across membranes including various cytotoxic drugs (Bellamy *et al*, 1996, Loe *et al*, 1996b) In eukaryote cells, ABC genes often encode four domains composed of two NBDs and two transmembrane regions, (Leveille-Webster and Arias, 1995) The ATP binding NBDs contain conserved residues (termed Walker A and B motifs) spaced by 90-120 amino acids (Walker C motif) (Walker *et al*, 1982) Eukaryotic ABC proteins require two nucleotide-binding regions and associated transmembrane regions in order to be functional In eukaryotes the two sites are usually found as two halves of a single polypeptide, while in prokaryotes ABC proteins have only one NBD and are therefore functional as dimers MRP1 requires both NBD's to actively extrude drug Mutation of lysines 773(NBD1) or 1333(NBD2) inactivates MRP1 transport possibly by destroying the structural integrity of these domains (Zhu *et al*, 1997)

1 1 5 P-glycoprotein.

The first major human multidrug transporter identified was P-glycoprotein (P-170, P-gp) encoded by the multidrug resistance protein 1 (MDR1) gene (Dano *et al*, 1973, Juliano and Ling, 1976, Gottesman and Pastan, 1993, Reviewed in Gottesman *et al*, 1996) P-170 is a 170 kD glycoprotein composed of 1280 amino acids P-170 is located in the plasma membrane and acts as an ATP-dependent efflux pump that actively transports chemotherapeutic agents (Gottesman and Pastan, 1993, Borst *et al*, 1991) P-170 substrates include several structurally and functionally unrelated agents, including the anthracychnes (daunorubicin and doxorubicin), the vinca alkaloids (vinblastine and vincristine), the epipophyllotoxins (teniposide and etoposide), the taxanes (paclitaxel and docetaxel) and actinomycin D P-170 is predominantly expressed in the apical membranes of organs with excretory functions (Georges *et al*, 1990, Van Der Valk *et al*, 1990) It is thought that P-170 may play a role in the elimination of exogenous toxins or their toxic metabolites from the body (Horio *et al*, 1989) P-170 is found in several normal human tissues (Sugawara *et al*, 1988) and is highly expressed in the adrenal cortex, kidney (proximal tubules), liver (bile caniculi), colon and jejunum (apical brush border cells) P-170 has also been found in untreated human cancers derived from normal tissues which express P-170, such as carcinomas of the colon, kidney, liver, adrenal gland and pancreas However, in other cancers P-170 has been more frequently detected at the time of relapse following initial chemotherapy (Bellamy *et al*, 1990) Studies have shown that P-170 expression in primary tumours has prognostic value in childhood sarcoma (Chan *et al*, 1990), neuroblastoma (Chan *et al*, 1991), osteosarcoma (Baldini *et al*, 1995) and acute myeloid leukaemia (Marie *et al*, 1995) In other major cancer types such as in melanoma, and cancers of the lung, breast and ovary, P-170 appears to play a minor role (Lai *et al*, 1989, Bellamy *et al*, 1990, Cole *et al*, 1992)

P-170 chemosensitisers (chemosensitisers are drugs or chemicals that enhance the therapeutic effects of chemotherapy drugs and therefore improve their effectiveness) include a variety of chemical classes such as calcium channel blockers (e g verapamil), calmodulin antagonists (e g trifluoperazine), vinca alkaloids (e g vindoline), steroidal agents (e g tamoxifen), immunosuppressive drugs (e g cyclosporin, SDZ PSC833) and antibiotics (e g erythromycin) (Hamada *et al*, 1987, Ford *et al*, 1995) There are however a number of human tumour cell lines that show decreased cellular accumulation and increased resistance to drugs associated with the MDR phenotype, but do not express P-170 (Center *et al*, 1993, Twentyman and Versantvoort, 1996)

1 2. The Multidrug resistance associated protein 1 (MRP1)·

The second major multidrug transporter termed MRP1 (multidrug resistance-associated protein 1, P-190 or MRP) (Reviewed in Cole and Deeley, 1998) was cloned and sequenced from a small-cell-lung cancer line which showed resistance to drugs associated with the MDR phenotype but did not express MDR1 (Cole *et al*, 1992) The MRP1 gene encodes a 190a membrane-bound glycoprotein of 1531 amino acids (Cole *et al*, 1992, Krishnamachary and Center, 1993)

MRP1 is an ATP-dependent drug efflux pump (Leier *et al*, 1994, Versantvoort *et al*, 1994) Reduced drug accumulation in cell lines expressing MRP1 have been reported (Barrand *et al*, 1993, Davey *et al*, 1995) Transfection experiments with different eukaryotic expression vectors containing full length complementary DNAs of the MRP1 gene have shown that MRP1 confers resistance to a broad range of natural product drugs, among which are anthracyclines, vinca alkaloids and epipodophyllotoxins (Cole *et al*, 1994, Grant *et al*, 1994, Kruh *et al*, 1994, Zaman *et al*, 1994) MRP1s function as

a drug extrusion pump has been established (Cole *et al*, 1994, Zaman *et al*, 1994), but the nature of the transported drugs and other chemicals is still controversial MRP1 appears to mediate the transport of a broad range of drugs across cellular membranes Like MDR1, many of the compounds transported by MRP1 are of natural origin However, MDR1 can directly transport unmodified, relatively large, hydrophobic, either uncharged or weakly basic molecules (Cole *et al*, 1992, Higgins, 1992, Gottesman and Pastan, 1993), whereas, MRP1 transports drugs in an unmodified form possibly conjugated to glutathione (Leier *et al*, 1994, Loe *et al*, 1996a, Zaman *et al*, 1996, Muller *et al*, 1994) or conjugated to acidic ligands such as glutathione, glucuronide, or sulphate On the one hand, it was concluded from the increased cellular drug efflux from MRP1-over-expressing or transfected tumour cell lines that MRP1 functions as a transporter of the typical 'MDR' anti-cancer agents (Versantvoort *et al*, 1992, Zaman *et al*, 1994) On the other hand, the observation that the efflux of anti-cancer drugs from MRP1-over-expressing cells was glutathione-dependent (Lutzky *et al*, 1989, Versantvoort *et al*, 1995) suggested that MRP1 is a transporter of anionic compounds and/or glutathione-conjugated drugs (Jedlitschky *et al*, 1994, Broxterman *et al*, 1995) It has been suggested that glutathione plays an essential role in MRP1 mediated drug resistance Glutathione conjugation of drugs or interaction of glutathione directly with MRP1 may be critical for drug transport (Loe *et al*, 1996b) Experiments using the glutathione (GSH) synthesis inhibitor, buthionine sulphoximine (BSO), show that intracellular GSH levels regulate drug transport in MRP1 tumour cell lines (Versantvoort *et al*, 1995) Important evidence for the identification of MRP1 as a transporter of multiple organic anions (MOAT) came from experiments showing ATP-dependent uptake of glutathione conjugates, such as leukotriene C_4, into inside out plasma membrane vesicles prepared form MRP1 over-expressing cells (Jedlitschky *et al*, 1994,

Muller *et al*, 1994) Transporters with these characteristics of MRP1 are known as GS-X pumps (Ishikawa, 1992), multispecific organic anion transporters (MOAT) (Jansen and Oude Elferink, 1993), or leukotriene C_4 (LTC_4) transporters (Keppler *et al*, 1992)

As yet the mode of action by which MRP1 confers a MDR phenotype on cells is not known However, the available data suggests that MRP1 acts both as a plasma membrane outward drug pump and as a pump for drug accumulation in intracytoplasmic vesicles (Cole *et al*, 1994, Zaman *et al*, 1994, Breuninger *et al*, 1995) By both mechanisms, cytoplasmic concentrations of free drug may be reduced to sublethal levels, and in that way MRP1 is able to promote cell survival

The amino acid sequence of MRP1 contains various sites known to be relevant for ATP binding and post-translational modification (Loe *et al*, 1996a) MRP1 has been detected immunologically as a 190 kD N-glycosylated phosphoprotein that binds ATP Various studies have shown that the unmodified MRP1 polypeptide has an apparent mass of 170 kD and is processed into a mature 190 kD form by addition of N-linked oligosaccharides It has been established that it takes approximately 90 minutes to produce the mature 190 kD MRP1 molecule from the 170 kD polypeptide, with the mature protein possessing a half life of 20 hours Human MRP1 contains twelve potential sites for N-linked glycosylation, but it is believed that only three of these sites are external to the plasma membrane and so ultimately glycosylated The effect of glycosylation on MRP1 activity is not fully known It has been demonstrated that tunicamycin-induced inhibition of glycosylation has little effect on the cellular drug accumulation characteristics of MRP1 expressing cells (Almquist *et al*, 1995)

1 2 1. Membrane Structure of MRP1

Due to the difficulty of crystallising large membrane proteins, no detailed 3-dimensional structure of any members of these transporters is currently available, and empirical prediction methods are used to obtain molecular models of their structure, especially to predict the locations and numbers of the membrane spanning helices In most cases these methods identify six short transmembrane domains (Gottesman and Pastan, 1993, Cole and Deeley, 1992, Higgins, 1992) The relevance of the prediction for the membrane topology of CFTR has been confirmed experimentally by insertional mutagenesis (Chang *et al*, 1994), thus reinforcing the 2 x 6 transmembrane helix model The same arrangement of transmembrane helices has been suggested in the case of P-glycoprotein (Gros *et al*, 1986, Chen *et al*, 1986), and a large body of experimental data strongly favours this model (Kast *et al*, 1997) On the other hand, Ling and co-workers (Zhang and Ling, 1991, Zhang *et al*, 1993), by suggesting an alternative 6- and 4- helix conformation, raised the possibility that P-glycoprotein may exist in two different topological forms in the cell membrane When the multidrug resistance associated protein 1 (MRP1) was cloned and sequenced, analysis of its primary amino acid sequence revealed that MRP1 is more closely related to CFTR than P-gp (Cole *et al*, 1992) Cole *et al*, (1992) predicted a unique transmembrane topology for MRP1, with eight N-terminal and four C-terminal transmembrane segments However, according to Bakos *et al*, (1996), the model predicted by Cole *et al*, (1992), is not consistent with their newly developed membrane topology of MRP1 They found that MRP1 has a characteristic, triple membrane bound domain structure (Figure 1 2 1 1) This model was examined by immunodetection with flow cytometry in intact and permeabilised cells, by limited proteolysis of isolated membranes with trypsin and chymotrypsin, and by immunoblotting of the proteolytic fragments with anti-peptide antibodies, reacting with

either the N-terminal or the C-terminal half of the protein By using glycosylated and unglycosylated forms of MRP1, its major sites of glycosylation could be determined They compared the experimental findings with a newly developed membrane topology of MRP1, based on the experimentally confirmed transmembrane topology of CFTR

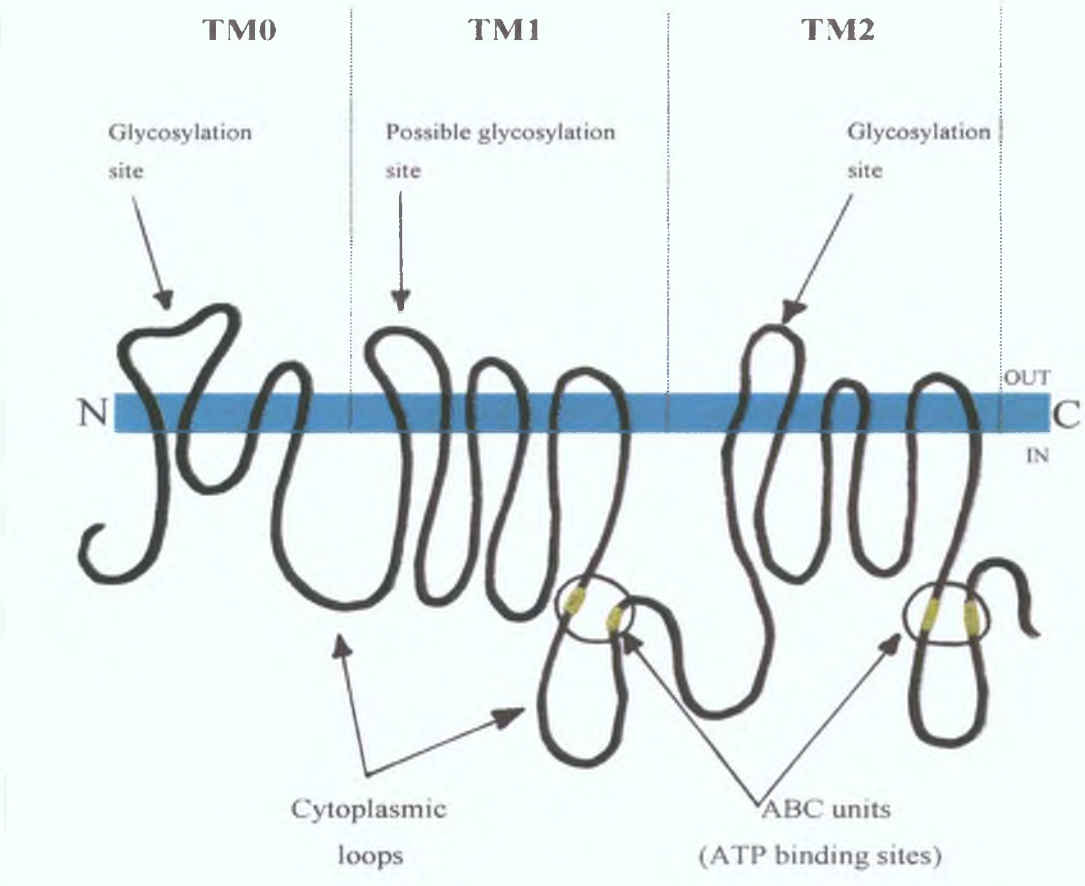


Figure 1.2.1.1. Membrane topology studies of the MRP1 protein (Bakos *et al.*, 1996) show three major membrane bound domains (TM0, TM1, and TM2) separated by two cytoplasmic loops. The first (TM0) and last (TM2) membrane bound domains are glycosylated when expressed in mammalian cells and a further possible glycosylation site is situated in (TM1). An ATP binding domain (ABC unit) follows TM1 and TM2.

1 2 2 MRP1 drug resistance profile

Cell lines over-expressing MRP1 are typically cross-resistant to anthracyclines, epipodophyllotoxins and vinca alkaloids (Loe *et al*, 1996a) Although the resistance profiles of drug-selected MRP1 or MDR1 over-expressing cell lines are similar, considerable differences exist particularly with regard to taxol resistance (Loe *et al*, 1996a) The explanation for a cell developing MRP1 rather than MDR1 mediated resistance during drug exposure remains unclear, but it is believed that overexpression of MRP1 may confer initial levels of resistance, while MDR1 overexpression develops as higher levels of resistance are required for survival A study by Brock *et al*, (1995), showed that in the small-cell lung cancer cell line, H69, MRP1 is over-expressed during selection in low concentrations of VP-16 Following further selection in higher concentrations of drug, MRP1 expression remained relatively constant, but MDR1 overexpression developed The expression of MRP1 protein and mRNA is not limited to drug-selected MRP1 over-expressing cells Berger *et al*, (1997a), reported that a significant number of drug sensitive non-small cell lung cancer cell lines expressed MRP1 mRNA and protein

1 2.3. Cellular location of MRP1

Although MRP1 was initially believed to be predominantly located in the endoplasmic reticulum of resistant cells (Krishnamachary and Center, 1993), significant levels are now known to be present in the plasma membrane (Flens *et al*, 1994, Muller *et al*, 1994, Zaman *et al*, 1994) and also in endocytic vesicles (Almquist *et al*, 1995) Immunohistochemical studies show that MRP1 in normal tissue is predominantly cytoplasmic whereas in malignant tissue it is predominantly plasma membrane located with some granular cytoplasmic staining observed (Flens *et al*, 1996) However, it has

been observed that resistant cell lines show predominantly plasma-membrane staining only (Flens *et al*, 1996, Zaman *et al*, 1994)

1 2 4 MRP1 expression in cell lines

The MRP1 transporter molecule has been shown to be present in non-MDR1 multidrug resistant cell lines obtained from a variety of tumour types These tumours include leukaemias, fibrosarcomas, small-cell and non-small cell lung carcinomas, breast, cervix, prostate and bladder carcinomas (Izquierdo *et al*, 1996b) There have also been reports of co-expression of MDR1 and MRP1 in a number of drug-selected cell lines (Brock *et al*, 1995) However, the high prevalence of MRP1 expression observed in a large number of cell lines which have not been subject to laboratory drug selection suggest that MDR mechanisms associated with this protein may be widespread in human malignancies Therefore, while acquired resistance in many human tumours may be attributed to MDR1, MRP1 may play an important role in the intrinsic resistance of certain human tumours

1 2.5 MRP1 expression in normal human tissue

MRP1 has been detected either at the protein or the mRNA level in normal human tissues including lung, stomach, colon, peripheral blood macrophages, thyroid, testis, nerve, bladder, adrenal, ovary, pancreas, gall bladder, duodenum, heart, muscle, placenta, brain, kidney, liver and spleen (Sugawara *et al*, 1997, Zaman *et al*, 1993, Cole *et al*, 1992, Kruh *et al*, 1995)

Distinct cell types which express MRP1 include epithelial cells, muscle cells and macrophages Negative MRP1 expression has been observed in many other cell types

including capillary epithelial cells (Flens *et al*, 1996)

Studies on a panel of normal human tissues carried out by Flens *et al*, 1996, revealed that the highest level of MRP1 protein (levels similar to or below that detected in the parental, drug sensitive lung cancer cell lines GLC4 and SW-1573/S1) are expressed in adrenal gland, lung, heart and skeletal muscle Lower amounts of MRP1 were detected in liver, spleen, kidney and erythrocyte membranes

1 2 6 MRP1 expression in malignant human tissues,

The widespread expression of MRP1 in normal human tissues is reflected in a broad range of adult solid tumours which over-express MRP1 (Kruh *et al*, 1995) This has been observed in breast carcinomas (Nooter *et al*, 1995, Filipits *et al*, 1996a), neuroblastomas (Bordow *et al*, 1994), anaplastic thyroid carcinomas (Sugawara *et al*, 1994), non-small-cell-lung carcinomas (Ota *et al*, 1995), colorectal carcinomas (Nooter *et al*, 1995), ovarian carcinomas (Nooter *et al*, 1995, Izquierdo *et al*, 1995) and relapsed acute leukaemias (Schneider *et al*, 1995) Generally, MRP1 positive tumours are derived from tissues that normally express MRP1, suggesting that MRP1 persists during malignant transformation Importantly, these tumours are also known to be intrinsically resistant to treatment with chemotherapeutic agents (Goldstein *et al*, 1992) suggesting that MRP1 may therefore contribute to this intrinsic resistance MRP1 may be an important factor in the clinical drug resistance of several tumour types, most notably non-small cell lung cancers (Ota *et al*, 1995, Nooter *et al*, 1996, Chuman *et al*, 1996, Giaccone *et al*, 1996), breast carcinomas (Nooter *et al*, 1997a, Nooter *et al*, 1997b, Ito *et al*, 1998) neuroblastoma (Norris *et al*, 1996), certain haematological malignancies (Kuss *et al*, 1996, Ross *et al*, 1996), and malignant melanoma (Berger *et al*, 1997b)

Immunohistochemical studies show that unlike the cytoplasmic staining observed in normal tissue, tumour tissue shows predominantly strong plasma-membrane staining with some granular cytoplasmic staining (Flens *et al*, 1996) Strong MRP1 staining, predominantly at the plasma-membrane, was observed in 20% of untreated solid tumours examined by Flens *et al*, (1996) This staining was mainly observed in squamous cell carcinomas such as the lung and oesophagus

MDR is a serious clinical problem encountered in the chemotherapy of lung cancer Human lung cancer is characterised by both high incidence and lethality, with non-small-cell lung cancer (NSCLC) accounting for at least 80% of the cases (Boring *et al*, 1993) The poor prognosis of this malignancy may be partially due to the pronounced resistance to chemotherapeutic drugs that is present intrinsically in most NSCLCs and is also acquired during treatment in many cases of initially drug sensitive small-cell lung cancer (SCLC) (Ihde, 1992) The response rate of NSCLC at initial chemotherapy is relatively low, suggesting the existence of intrinsic drug resistance On the other hand, while the primary response rate of SCLC is higher than NSCLC, SCLC usually relapses with the tumours displaying acquired MDR The drug resistance mechanisms in clinical lung cancer remain poorly understood However, human MDR1 may be linked to acquired MDR in SCLC and MRP1 is a potentially important candidate for intrinsic resistance in NSCLC

Differences in the expression of MRP1 in various histological types of lung cancer have also been reported Immunohistochemical studies have shown that in untreated lung cancer tumours, MRP1 is more prevalent in NSCLC than in SCLC (Wright *et al*, 1998) It has been observed that NSCLC tumours expressing moderate or high levels of MRP1

show significantly poorer prognoses than those with non or low level MRP1 expressing tumours (Ota *et al*, 1995) A clinical survey carried out by Dingemans *et al*, 1996, reported that all SCLC samples surveyed were MRP1 negative while a small number of NSCLC tumours were MRP1 positive suggesting that MRP1 may be a negative predictive marker in the response of NSCLC to chemotherapy

Recent work has focused on the role of MRP1 in breast cancer (Beck *et al*, 1998, Filipits *et al*, 1996, Ito *et al*, 1996, Lacave *et al*, 1998, Linn *et al*, 1997) A few studies have suggested that MRP1 may play a role in the prediction of response to chemotherapy in breast cancer (Mechetner *et al*, 1997, Nooter *et al*, 1997a, Nooter *et al*, 1997b) In a small immunohistochemical study of breast carcinomas, Larkin *et al*, (1999a), reported that in cases where intense MRP1 positivity was observed, the level of P-gp expression was low High MRP1 expression was observed in relapsed patient groups of primary breast carcinoma, suggesting that MRP1 is of prognostic significance in primary breast carcinomas and also a marker of a more malignant phenotype in breast cancer (Nooter *et al*, 1997a, Nooter *et al*, 1997b, Ito *et al*, 1998)

In neuroblastoma high levels of MRP1 gene expression are associated with reductions in both survival and event-free survival of patients (Norris *et al*, 1996) MRP1 expression also correlates with N-*myc* amplification, which is a well established prognostic indicator in this disease (Norris *et al*, 1997, Bordow *et al*, 1994) In a series of 40 neuroblastomas, Bader *et al*, (1999), analysed the relative mRNA levels of the MDR associated genes encoding MDR1, MRP1, LRP and TOPO II alpha and the N-*myc* gene by cDNA PCR The cellular proliferation marker, cyclin A, was included to examine proliferation activity They observed that tumours with N-*myc* gene amplification exhibit

significantly increased N-*myc* and MRP1 gene expression levels However, they also found a correlation of N-*myc* with TOPO II alpha and MRP1 with LRP Tumours with an allelic loss of the chromosomal 1p region showed lower MDR1 gene expression than tumours without loss They concluded that MDR in neuroblastomas might be caused by multiple resistance factors and that a higher proliferation rate of neuroblastoma cells possibly based on altered N-*myc* gene expression is associated with enhanced MRP1, Cyclin A and TOPO II alpha gene expression

Ikeda *et al*, (1999), observed an increased expression of MRP1 and LRP in chronic adult T-cell leukaemia (ATL) and suggested that the MRP1 and LRP genes in ATL are often activated by human T-cell leukemia-virus-1 (HTLV-1) infection and may confer a multidrug resistance phenotype on cells *in vivo*

The over-expression of MRP1 in *de novo* acute non-lymphocytic leukaemia (ANLL) is rare whereas both P-gp and LRP over-expression are common features at the onset of ANLL (Michieli *et al*, 1999) Increased MRP1 gene expression has been reported in relapsed acute leukaemias (Hart *et al*, 1994, Schneider *et al*, 1995) Filipits *et al*, (1997), surveyed MRP1 in *de novo* AML samples and concluded that unlike P-gp, MRP1 does not predict for outcome of induction chemotherapy or survival Recently the over-expression of the MRP1 gene has been implicated as another possible factor in the multidrug resistance phenotype of acute myeloid leukaemia (AML) Intermediate or high levels of MRP1 expression may be associated with shorter overall survival in some AML patients (Filipits *et al*, 1999) Brugger *et al*, (1999), carried out functional analysis studies of P-gp and MRP1 to study the role these two proteins mediate in the MDR of AML MRP1 and P-gp modulators were applied to blasts from AML patients

with relapses, followed by drug treatment studies The results concluded that chemotherapy responses might not be improved by targeting P-gp or MRP1 exclusively

The clinical relevance of the MRP1 gene in the chemoresistance of prostate carcinomas has recently been questioned In a study carried out by Schummer *et al*, (1999), MRP1 expression levels in human prostate tissue were determined by RT-PCR and capillary electrophoresis MRP1 expression was found in all 30 organ-confined prostate carcinoma samples, 9 adjacent normal tissue samples and 4 hormone unresponsive samples Normal tissue showed the same MRP1 mRNA level as the adriamycin-sensitive HL60 cells The adriamycin-resistant HL60-ADR cells were used as a positive control The expression of MRP1 was higher in organ-confined tumours than in hormone-unresponsive anaplastic tumours A higher tumour stage correlated with an increase of MRP1 expression, whereas G3 tumours displayed an MRP1 level of expression lower than that found in G2 tumours This study concluded that the small alterations in MRP1 expression do not seem to be involved in the chemoresistance of prostate carcinomas

Malignant melanoma is considered to be a chemotherapy-refractory tumour The cellular resistance mechanisms involved in melanoma chemoresistance have not yet been elucidated However, melanoma derived cell lines are often markedly chemoresistant suggesting the presence of intrinsic cellular resistance mechanisms MRP1 has been implicated in this intrinsic resistance in malignant melanoma (Berger *et al*, 1997b)

1.2.7. Physiological function of MRP1:

The physiological function of MRP1 remains unclear. However, the widespread expression of MRP1 in normal human tissues and haematopoietic cells suggests that MRP1 serves a function common to most cell types. Based on its prominent presence in many epithelia lining external surfaces, MRP1, like Pgp, may be associated with the transport of natural xenobiotics (Flens *et al.*, 1996). Studies on the physiological function of MRP1 by Wijnholds *et al.*, (1997), showed that MRP1 knockout mice which are homozygous for the mutant allele $MRP^{-/-}$ were viable and fertile but hypersensitive to etoposide. Evidence that cancer cells require glutathione for MRP1 drug transport has been demonstrated by the cellular depletion of glutathione (Versantvoort *et al.*, 1995; Zaman *et al.*, 1995).

Flens *et al.*, (1996), reported high levels of MRP1 in cells with endocrine functions such as those of the adrenal cortex, the islets of Langherans in the pancreas, in the placenta trophoblast and in testosterone producing cells of the testis and ovary, suggesting that like Pgp, MRP1 may play a role in hormone transport.

1.2.8. Other ABC transporters involved in MDR:

In addition to MDR1 and MRP1, evidence suggests that there may be other ABC transporters that are involved in MDR. In the case of natural product drugs, resistant cell lines that display a multi-drug resistance phenotype associated with a drug accumulation deficit, but do not over-express MDR1 or MRP1 have been described (Lee JS. *et al.*, 1997.). ABC transporters have also been linked to cisplatin resistance, and several lines of evidence suggest the possibility that pumps specific for organic ions may be involved:

(a) Decreased cisplatin accumulation is observed consistently in cisplatin-resistant cell lines (Gately *et al*, 1993)

(b) Cisplatin is conjugated to glutathione in the cell, and this anionic conjugate is toxic in an *in-vitro* biochemical assay (Ishikawa *et al*, 1993)

(c) Biochemical studies using membrane vesicle preparations have shown that cisplatin-resistant cell lines have enhanced expression of an ATP-dependent transporter of cisplatin-glutathione and other glutathione S-conjugates, such as the cystinyl leukotriene C_4 (Ishikawa *et al*, 1994)

Whereas MRP1 is an organic anion transporter, the reported drug resistance profile of MRP1-transfected cells does not extend to cisplatin (Breuninger *et al*, 1995, Cole *et al*, 1994), and to date only one cisplatin-resistant cell line has been reported to over-express MRP1 (Ishikawa *et al*, 1996) These observations suggest the possibility that organic anion transporters other than MRP1 may contribute to cisplatin resistance Consistent with this possibility, MRP2 (canalicular MRP / cMRP / cMOAT), an MRP1-related transporter that functions as a major organic anion transporter in liver, has been reported to be overexpressed in cisplatin-resistant cell lines (Taniguchi *et al*, 1996, Kool *et al*, 1997) A more direct link between MRP2 and cytotoxic drug resistance is suggested by a recent report in which transfection of an MRP2 antisense construct into a liver cancer cell line resulted in sensitisation to cisplatin, daunorubicin and other cytotoxic agents (Koike *et al*, 1997)

1 3 MRP1 Homologues

Recently it has been reported that besides MRP1 and MRP2, there are at least four more MRP homologues expressed in human tissues, called MRP3, MRP4, MRP5 and MRP6 (Kool *et al*, 1997, Kool *et al*, 1999a, Kool *et al*, 1999b) Other groups have also sequenced and reported these recently discovered MRP homologues (Belinsky *et al*, 1998, Lee *et al*, 1998) Therefore, various names for each homologue have arisen The names of each homologue can be matched by comparison of their cDNA sequence (see Table 1 3 1)

Table 1.3.1.

MRP Isoform (as described by Kool *et al* 1997)	**Other names** (as described by Belinsky *et al* 1998, Lee *et al*, 1998)
MRP1	MRP
MRP2	Canalicular MOAT cMOAT cMRP
MRP3	MOAT-D
MRP4	MOAT-B
MRP5	MOAT-C
MRP6	-

The newly identified MRP homologues are all located on chromosomes distinct from those containing the MRP1 and MRP2 genes confirming that they are new genes and not splice variants of MRP1 or MRP2 (see Table 1 3 2)

Table 1.3 2

MRP Isoform	Gene location
MRP1	16p13 1
MRP2	10q21 3
MRP3	17q21 3
MRP4	13q32
MRP5	3q27
MRP6	16q13 11

The cDNA sequences of all six human MRP family members, MRP1 (Cole *et al*, 1992), MRP2 (Taniguchi *et al*, 1996), MRP3 (Kool *et al*, 1999a, Kuichi *et al*, 1998, Uchiumi *et al*, 1998, Belinsky *et al*, 1998), MRP4 (Lee *et al*, 1998), MRP5 (Belinsky *et al*, 1998) and MRP6 (Kool *et al*, 1999b) are known Each member is more homologous to MRP1 than to any other ABC-transporter, justifying the classification of these genes into one subfamily of ABC-transporter genes called MRP1-6 by Kool *et al*, (1997) Based on the complete sequences two subgroups can be recognised One group with MRP1, MRP2, MRP3 and MRP6 sharing 45 - 58% amino acid identity, is characterised by the presence of an NH_2-terminal membrane-bound extension of about 280 amino acids,

which adds 5 putative transmembrane segments to an MDR1-like core (Tusnady *et al*, 1997) This extra domain is also present in the yeast MRP1 homologue YCF1 and the SUR proteins SUR1 and SUR2, but not in the other MRP homologues, MRP4 and MRP5 MRP4 and MRP5 have less homology to MRP1 (34 - 39% amino acid identity) and their structure is more similar to CFTR and MDR1 The function of the NH_2-terminal extension is unknown but recent data suggest that this domain is not required for catalytic function or routing to the plasma membrane (Bakos *et al*, 1998)

1.3 1 MRP2 (canalicular MRP / cMRP / cMOAT).

The human MRP2 cDNA sequence (Taniguchi *et al*, 1996) codes for a protein of 1545 amino acids MRP2 has been established as another GS-X pump which can confer drug resistance in cell lines but has yet to be proven to be involved in clinical multidrug resistance MRP2 has been shown to transport vinblastine and overexpression of MRP2 has been found in many cell lines resistant to cisplatin While MRP1 is expressed ubiquitously in the basolateral plasma membrane (Zaman *et al*, 1994), MRP2 is mainly expressed in the canalicular membranes of hepatocytes (Paulusma *et al*, 1997) and in other apical domains of polarized cells such as the epithelial cells of the proximal tubules of the kidney (Schaub *et al*, 1997) Like MRP1, MRP2 uses ATP to transport bilirubin glucuronides Bilirubin is secreted from the liver into bile mainly as glucuronosyl and bisglucuronsyl conjugates Studies with mutant rats (TR/GY or EHBR), which lack the MRP2 protein in the canalicular membrane of hepatocytes, have shown that the substrate specificity of MRP2 is very similar to that of MRP1 (reviewed in Oude Elferink *et al*, 1995, Keppler *et al*, 1997) However, Jedlitschky *et al*, (1997), demonstrated that substrate specificity for bilirubin glucuronides is more strongly favoured by MRP2 MRP2 also contributes to transport of anti-cancer drugs and metals The mutant rats

showed a reduced biliary clearance of methotrexate (Masuda *et al*, 1997), of the topoisomerase inhibitor CPT-11 and its metabolites (Sugiyama *et al*, 1998) Cells transduced with an MRP2 cDNA construct transport the cytostatic drug vinblastine (Evers *et al*, 1998) Moreover, overexpression of the MRP2 gene has been found in several cisplatin resistant cell lines (Taniguchi *et al*, 1996, Kool *et al*, 1997), and transfection of an MRP2-antisense construct into liver cells was reported to confer an increased sensitivity to cytotoxic drugs (Koike *et al*, 1997) All these observations strongly suggest that MRP2 may confer multidrug resistance in these cells, but whether it does so in cancer patients remains to be established However, defects in MRP2 are known to cause Dubin-Johnson syndrome (DJS), an autosomal recessive disorder in humans causing hyperbilirubinemia (an increase in the urinary excretion of coproporphyrin isomer I), deposition of a melanin-like pigment in hepatocytes, and prolonged retention of sulfobromophthalein, but otherwise normal liver function (Kartenbeck *et al*, 1996) Therefore, MRP2 mediates heptobilliary excretion of numerous organic anions and may function as a cellular transporter of cisplatin but as of yet the involvement of MRP2 in clinical multidrug resistance is unknown

1 3 2 MRP3 (MOAT-D).

Of all the MRP family members, MRP3 has the highest sequence homology to MRP1 with a 58% amino acid identity between these two proteins and 47% amino acid identity to MRP2 MRP3 is located on chromosome 17q21 3 and the mature protein yields a 170-190 kD doublet band in MRP3-transfected cells by Western blot analysis (Kool *et al*, 1999a) Like MRP1 and MRP2, MRP3 has also been established as a GS-X pump and is able to confer resistance to the anti-cancer drugs methotrexate, etoposide and teniposide (Kool *et al*, 1999a) Like MRP1, it also exports drug towards the basolateral

side of polarised cells (Kool *et al*, 1999a) However, MRP3 has a different tissue distribution from MRP1 In a previous report, Kool *et al*, (1997), showed that MRP3 mRNA is mainly expressed in the liver, colon, intestine, and adrenal gland, and to a lower extent in several other tissues In agreement with Kool *et al*, (1997), high levels of MRP3 mRNA in human liver have also been reported by others (Kiuchi *et al*, 1998, Uchiumi *et al*, 1998, Belinsky *et al*, 1998, Fromm *et al*, 1999) MRP3 mRNA expression has also been reported in tissues such as the prostate, testis and various regions of the brain (Fromm *et al*, 1999) However, results with new MRP3 antibodies (Kool *et al*, 1999a), show that there is little MRP3 protein in normal human liver These results may be explained by a massive induction of MRP3 expression in diseased liver which may have been possibly used in the mRNA studies while normal liver was used in the protein studies This hypothesis is further supported by Hirohashi *et al*, (1998), who reported that Mrp3 mRNA levels are low or undetectable in normal rat liver but that the level is increased in rats made cholestatic by bile duct ligation Another study carried out by Konig *et al*, (1999), reported MRP3 mRNA expression in human liver They also observed a particularly strong expression of the MRP3 protein in the basolateral hepatocyte membrane of two patients with Dubin-Johnson syndrome who are deficient in MRP2 They concluded that the basolateral MRP1 isoform, MRP3, may be upregulated when the canalicular secretion of anionic conjugates is impaired However, more studies are needed to assess what determines liver MRP3 levels in humans The physiological role of MRP3 has been linked to biliary excretion Kool *et al*, 1999a, have suggested that MRP3 may be the transporter responsible for secretion of bile salts into the blood

mRNA levels of both MRP1 and MRP3 have been reported to be higher in NSCLC cell lines than in SCLC cell lines (Young *et al*, 1999) The levels of both MRP1 and MRP3

mRNA correlated with resistance of 23 unselected lung cancer cell lines to the chemotherapeutic drugs tested, including a strong correlation of MRP3 protein over-expression with doxorubicin resistance This difference of MRP3 expression in the two major subclasses of lung cancer was also reflected in tumour samples Therefore, both MRP1 and MRP3 may contribute to the multifactorial multidrug resistance phenotype of lung cancer cells, particularly that of NSCLC

1.3 3 MRP4 (MOAT-B)

The MRP4 cDNA sequence (Lee *et al*, 1998) predicts a structure more similar to CFTR and MDR1 than MRP1 or MRP2 because of the lack of an NH_2-terminal membrane-bound extension of about 280 amino acids However, comparison of the MRP4 protein sequence with other transporters revealed that it is most closely related to MRP1, MRP2 and the yeast organic anion transporter YCF1 In addition, the MRP4 tissue distribution is distinct from MRP1 and MRP2 In contrast to MRP1, which is widely expressed in tissues including liver, and MRP2, the expression of which is largely restricted to liver, the MRP4 transcript is widely expressed, with particularly high levels in prostate, but is barely detectable in liver (Lee *et al*, 1998) A low level of MRP4 mRNA in the pancreas was also reported (Kool *et al*, 1997) These data indicate that MRP4 is a ubiquitously expressed transporter that is closely related to MRP1 and MRP2 and raises the possibility that it may be an organic anion pump relevant to cellular detoxification

1 3 4 MRP5 (MOAT-C)

The MRP5 cDNA sequence (Belinsky *et al*, 1998) like MRP4, predicts a structure more similar to CFTR and MDR1 than MRP1 or MRP2 because of the lack of an NH_2-terminal membrane-bound extension of about 280 amino acids However, comparison of the MRP5 protein sequence of 1437 amino acids with other transporters revealed that it is most closely related to MRP1, MRP2 and MRP4 sharing about 36% amino acid identity

MRP5 mRNA is expressed at highest levels in skeletal muscle, kidney, testis, heart and brain It is also expressed in most other tissues but is barely detectable in the lung and liver (Belinsky *et al*, 1998)

1 3 5 MRP6

MRP6 is located on chromosome 16, band 16q13 11, immediately next to MRP1 MRP6 is predicted to encode a protein of 1503 amino acids with a predicted molecular weight of 165 kD and shares a 45% identity to human MRP1 Another recently identified MRP-like gene, named Anthracycline Resistance Associated protein (ARA) (Longhurst *et al*, 1996) is almost 100% identical with the 3' end of MRP6 and has been identified in epirubicin selected leukaemia cells ARA, a 49 5 kD unglycosylated protein is, therefore, possibly a splice variant of MRP6

Whether the complete MRP6 gene is also transcribed in tissues and cell lines has recently been investigated (Kool M *et al*, 1999b) MRP6 mRNA has been reported to be highly expressed in liver and kidney and to a lower or very low extent in a few other tissues No evidence of an independent expression of the ARA gene in tissue was found

Analysis of a large panel of resistant cell lines for the over-expression of MRP6 did not reveal a correlation between expression and multidrug resistance Over-expression of the ARA gene was only found in those cell lines with high over-expression and high amplification of the MRP1 gene These results suggest that MRP6 does not play a role in the resistance of the resistant cells analysed and ARA is only co-amplified with MRP1 because of its location immediately next to it on the same chromosome

1 3 6 Summary of MRP1 and its isoforms

MRP1 and its isoforms are expressed in various human tissues (see Table 1 3 6 1) MRP1 is widely expressed in tissues including liver MRP2 is expressed mainly in the liver MRP3 is widely expressed in tissues but not in normal liver at the protein level MRP4 is widely expressed with particularly high levels in prostate, but barely detectable in liver MRP5 is expressed in skeletal muscle, kidney, testis, heart and brain Lower levels are detected in most tissues but MRP5 mRNA is barely detectable in the lung and liver MRP6 is highly expressed in liver and kidney and to a lower or very low extent in a few other tissues (similar to MRP1)

MRP1 and MRP2 have been characterised as organic anion transporters, giving rise to MDR and possibly to cisplatin resistance Not much is known about the substrate specificity of the putative new transporters However, MRP3 has only recently been characterised as another organic anion transporter which is able to confer resistance against a range of drugs similar to that of MRP1 and MRP2 (Kool *et al*, 1999a) MRP3, MRP5 and MRP6 but not MRP4 were found to be over-expressed in some drug resistant cell lines, but no correlation was found thus far between their expression and the resistance of these cells (Kool *et al*, 1997, Kool *et al*, 1999a, Kool *et al*, 1999b)

Over-expression of the MRP6 gene in MDR cells is invariably associated with the amplification of the adjacent MRP1 gene and MRP6 probably does not contribute to resistance Although several members of the MRP family can be up-regulated simultaneously in MDR or cisplatin resistant cell lines, it is not yet known whether MRP4-6 can transport drugs

The functions of MRP4 and MRP5 remain to be elucidated Analysis by Kool *et al*, 1997, of a panel of cell lines did not reveal any correlation between MRP4 or MRP5 mRNA expression and MDR or cisplatin resistance The absence of the N-terminal membrane spanning domain and their weaker amino acid similarities to MRP1 and MRP2 suggest that their functions and substrate specificities may be different from that of MRP1, MRP2 and MRP3

Table 1.3.6.1. Protein or mRNA expression of MRP1 and its homologues in normal human tissues

ISOFORM	GENERAL TISSUE EXPRESSION	POSITIVE TISSUES	NEGATIVE TISSUES
MRP1	Widely expressed (protein)	Lung Heart Liver	Expressed in most tissues at a very low level
MRP2	Expressed mainly in the liver (Protein)	Liver	Most other tissues
MRP3	Widely expressed (Protein)	Pancreas Adrenal gland Cholestatic liver	Normal liver
MRP4	Widely expressed (mRNA)	Prostate	Liver
MRP5	Low levels in most tissues (mRNA)	Skeletal muscle Kidney Testis Heart Brain	Lung Liver
MRP6	Low extent most tissues (mRNA)	Liver Kidney	Low in most tissues

1 4 Monoclonal antibodies in research and diagnostics

The discovery of antibodies at the end of the 19th century provided the means to search for cancer specific antigens and opened the way for extensive studies of antibodies as potential diagnostic indicators and immunotherapies for cancer Over the past century, investigators injected human cancer cells into innumerable horses, sheep, rabbits, mice and rats, closely analysing the polyclonal antibodies the animals produced in response If the immune systems of the animals reacted to the foreign tumour cells by producing antibodies that did not react with normal cells, this finding would signal the presence of antigens that could subsequently be identified and pressed into service as targets for antibody based techniques Many workers tried this approach and claimed to identify cancer-specific antigens Unfortunately, none of these claims held up to careful scrutiny

The search for cancer antigens became easier in 1975, thanks to a discovery made by Cesar Milstein and Georges J F Kohler of the University of Cambridge This model was the first mouse model of monoclonal antibody (MAb) secreting hybridomas These researchers demonstrated that antibody-producing cells could be made to survive indefinitely if they were fused with cancer cells This method had a profound effect on cancer immunology for several reasons First, it provided a powerful new method to search for cancer antigens and second, workers could at last produce defined antibodies in sufficient amounts to put antibody-based therapies to the test The ability of antibodies to recognise fine distinctions between molecules is what has made them extremely useful in the search and study of cancer antigens

1.4.1. Monoclonal antibodies specific for MRP1 and its homologues

A number of MRP1 monoclonal antibodies have been produced and are available commercially (see Table 1 4 1 1) All the commercial MRP1 MAbs react with internal epitopes on various regions of the MRP1 protein Recently, new MAbs that detect a number of the newly discovered MRP1 isoforms, MRP2, MRP3 and MRP5 have been produced (Scheffer, G L Unpublished work) and are summarised in Table 1 4 1 2) To date, antibodies which detect the MRP4 and MRP6 isoforms have not been produced

Table 1.4.1.1.

Commercially available MRP1 MAbs

ANTIBODY	HOST SPECIES	EPITOPE (Human MRP1)	SPECIES SPECIFICITY
MRPr1 (Flens *et al* 1994)	RAT	a a 238 – 247 Epitope conserved in murine mrp except for one amino acid Human – GSDLWSLNKE Murine – SSDLWSLNKE	Human and murine MRP1 specific
QCRL-1 (Hipfner *et al* 1994)	MOUSE	a a 918 – 924 Epitope not conserved in murine mrp Human – SSYSGDI Murine – SSHSGDT	Human MRP1 specific
MRPm6 (Flens *et al*, 1994)	MOUSE	a a 1511 – 1520 Epitope not conserved in murine mrp Human – PSDLLQQRGL Murine – PSELLQQRGI	Human MRP1 specific

Table 1 4.1.2.

MRP1 isoform MAbs

ANTIBODY NAME	ISOFORM DETECTED	HOST SPECIES
MRPr1	MRP1	RAT
MRPm6	MRP1	MOUSE
M2I-4	MRP2	MOUSE
M2III-6	MRP2	MOUSE
M3II-21	MRP3	MOUSE
M3II-9	MRP3	MOUSE
M5I-1	MRP5	RAT
M5II-54	MRP5	RAT

1 4 2 Monoclonal antibody production techniques

The techniques conventionally used for the production of MAbs are based on variations of the original report by Kohler and Milstein (1975) This technology has proven to be capable of producing monoclonal antibodies of a single pre-determined specificity to a wide variety of antigens which can potentially be produced in unlimited quantities

The general scheme for the production of MAbs by hybridoma technology is shown in Figure 1 4 2 1 Animals, usually mice are immunised by injection of the antigen to which a MAb is required When an immune response has been raised, B cells are harvested from the rodent, usually from the spleen, and these are fused with immortal myeloma cells by fusion of the cell membranes with polyethylene glycol (PEG) Fusion results in the production of immortalised hybrid cells or hybridomas The remainder of the process is then one of isolation and propagation of hybridoma cells which have retained the ability of the B cell to produce antibody and the good growth characteristics of the myeloma cell The process of MAb production can therefore be considered in three parts immunisation, fusion and selection of the required hybridoma Many protocols for the immunisation and generation of hybridomas are available (Harlow and Lane, 1988)

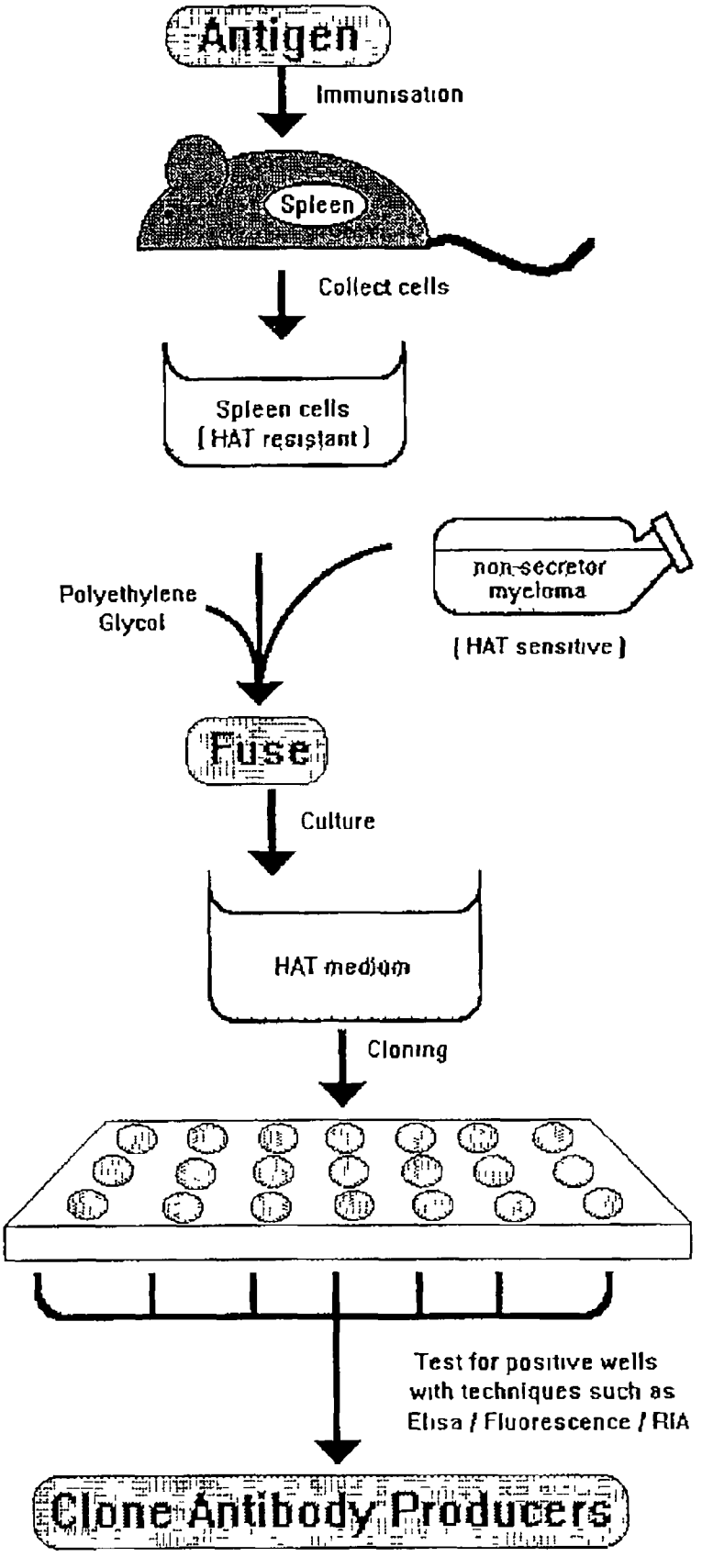


Figure 1 4.2.1 General scheme for MAb production

1.4.3. Immunogens:

MAbs can be produced against a wide variety of antigens including proteins, nucleic acids, carbohydrates and haptens (Harlow and Lane, 1988; Claassen *et al.*, 1993). The production of MAbs specific for proteins can be performed by immunising with a variety of sources and forms of antigen. Live cells may be used to generate MAbs specific for surface antigens. Fusion proteins produced by recombinant DNA technology make excellent antigens and provide a large quantity of antigen which can be purified. Short synthetic peptides can be used for site specific MAbs when a proteins coding sequence is available.

However, all immunisations using peptide antigens run the risk of producing antibodies that recognise the linear peptide but do not recognise the native protein which may have a complex conformation, thus blocking the MAb epitope (Claassen *et al.*, 1993). Therefore, it is important to consider the conformation of the molecule which the MAb is desired to bind to. For example, will a protein be in its native conformation or denatured? MAbs of exquisite specificity can be generated which recognise a desired isomer or molecular form of a particular antigen.

There are two important exceptions to the range of immunogens which can be used (Harlow and Lane, 1988). Antibodies will not normally be raised to 'self' antigens, and small antigens (less than approximately 1000 Daltons in molecular weight) will not raise a response unless conjugated to a high molecular weight carrier protein. For this purpose carrier proteins such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) are used, which are very immunogenic in rodents, easily available and may be conjugated to small molecules.

Adjuvants produce an emulsion which is injected into the animal and provides a long-lasting local 'depot' of antigen which is slowly released and thus continually boosts the response (Harlow and Lane, 1988) In addition, other components are sometimes included in the adjuvant mixture to activate the immune system non-specifically For example, complete Freund's adjuvant contains killed mycobacteria for activation as well as oil and an emulsifying agent Complete Freund's adjuvant is used for the first immunisation and subsequent booster immunisations are carried out in incomplete Freund's adjuvant (without the mycobacteria component) to maximise response to the antigen and avoid excessive inflammatory responses to mycobacteria

1 4 4 Design of Synthetic Peptides for Immunisation.

The use of synthetic peptides for MAb production requires careful thought in their choice and design Searches for the full-length amino acid sequences of a protein can be carried out in the NCBI SWISS-PROT data bank (www3 ncbi nlm nih gov) After considering the criteria for choosing peptide sequences for immunisation, chosen peptide sequences can then be entered into the BLITZ data bank by email (BLITZ@ebi ac uk) and aligned to compare sequence homology with other protein sequences Usually sequences sharing five or more amino acids in sequence with any other mammalian protein are disregarded The main criteria for peptide choice are listed below

CRITERIA FOR CHOOSING THE PEPTIDES

- Choose peptides with a preponderance of polar amino acids and proline residues (these sequences are more likely to be exposed on the surface of the native protein)
- Choose sequences as near as possible to termini (these sequences are also more likely to be exposed)

- Choose sequences with a higher "mobility" of the amino acid residues (stretches of amino acids with higher flexibility) as determined by NMR and X-ray structure when available Higher temperature sensitivity in crystallography and NMR distinguishes regions of a protein that are more mobile from regions that are more static It has been suggested that regions of higher mobility present a higher chance of forming a structure that is similar to the linear peptide (Moore, 1980)
- Avoid hydrophobic regions
- Avoid transmembrane segments (only possible when information on 3D structure is available)
- Avoid sequences including valines
- Look at sequences used for successful polyclonals
- Avoid sequences sharing five or more amino acids in sequence with any mammalian protein

To optimise the chance of a MAb being able to gain access to its epitope, it is advantageous to choose sequences which are more likely to be situated on the exposed surface of the native protein Peptide sequences with a preponderance of polar amino acids and proline residues are more likely to be exposed on the surface of the native protein In contrast, hydrophobic regions are more likely to be encased in the inner core of a protein, making them less likely to be accessible to MAbs Sequences close to either terminus of a protein are also more likely to be exposed on the surface of a protein Obviously transmembrane sequences should be avoided, as the membrane would block access of the MAb to its epitope (Harlow and Lane, 1988) Crystallography and NMR distinguishes regions of the protein that are more likely to be exposed on the surface of a

protein and present a higher chance of forming a structure that is similar to the linear peptide (Moore, 1980) Successful polyclonals may be used as a precursor to testing peptide sequences They may indicate accessible epitopes and pinpoint peptide sequences which will work optimally for their intended use It is our experience that peptides including valines are insoluble and therefore unsuitable for use as a soluble immunogen

1 4 5 Species·

There are a number of species which are useful for hybridoma production such as rats, mice, chickens, hamsters and rabbits (Harlow and Lane, 1988) Mice are usually the first choice for immunisation because they are small, easy to handle and are suitable for fusion with the most commonly available SP2 murine myeloma cells There are in fact suitable myeloma cells for each species available now However, cross species fusions with murine SP2 cells and rat lymphocytes have been carried out successfully by our laboratory and others (Flens *et al*, 1994) Choosing a species which does not express any proteins sharing a high amino acid homology with the immunogen is advised because antibodies will not normally be raised to 'self' antigens Various strains of the same species can react differently to the same antigen, resulting in successful immunisation in only some of the strains Also, individual species of animals may elicit various immune responses to immunogens For example the rat antibody repertoire is different from that of the mouse in a number of ways Most importantly some antigens from origins other than mouse or rat species can induce much stronger responses in rats than in mice and the reciprocal may be true for other antigens However, obtaining rat-rat hybridomas still seems to be considered difficult and several experienced investigators have had difficulty in keeping rat-rat hybrids alive for more than 2 to 3 weeks possibly due to an unusually

high number of natural killer cells in the spleens of rats from some colonies Therefore, alternative immunisation *via* the lymphatic system and collection of B cells from lymph nodes may be more suitable in rat models

1 4 6 Route of immunisation·

There are many routes of injection which can be employed to immunise an animal However, each route has advantages and disadvantages which must be taken into consideration when designing an immunisation protocol (Harlow and Lane, 1988) For example, different routes present varying immunogenicity and the structure of the antigen can be changed dependent on the environment where antigen is presented (Claasen *et al*, 1993) The most common route of injection for mouse and rat is intraperitoneally, followed by collection of B cells from the spleen for fusion The most common route of injection for rabbit is subcutaneously followed by collection of polyclonal serum from bleeds Other routes include subcutaneous, intravenous, intramuscular and intradermal The lymphatic system can also be employed by administering rear footpad injections followed by collection of B cells from the popliteal node (see Materials and Methods, section 2 2 8 2)

1 4.7 Immunisation

The immunisation protocol, used to raise an immune response in the animal, is of key importance (Harlow and Lane, 1988) Immunogens of different molecular types can be used and they do not need to be pure materials, as a hybridoma secreting a MAb of the required specificity may be selected out later Different molecules vary greatly in their immunogenicity and thus require different immunisation protocols for an optimal response Factors such as the form of the antigen, number and route of immunisations,

carrier, adjuvant and the species and the strain of animal used need to be considered To ensure that an immune response has been elicited against the antigen before harvesting B cells, blood samples are obtained from the immunised animal and tested for the presence of specific antibodies If positive, final boosts of soluble antigen are given intravenously to increase the number of specific immune B cells present in the spleen prior to harvest The type of antibody produced is dependent on the number of immunisations If IgM is required, only one immunisation is carried out, as only a primary response is required, whereas IgG antibodies require multiple injections (usually three or four) at intervals of approximately 3-4 weeks to allow an effective secondary response

1 4 8 Fusion.

Prior to fusion, blood samples should be obtained from the immunised animal, tested to ensure the presence of specific antibodies, followed by a final boost The spleen is the most common source of immune B cells, although cells can also be harvested from lymph nodes Fusion of immune B cells with immortal myeloma cells is usually accomplished with polyethylene glycol (PEG) to induce membrane fusion After fusion, a mixture of hybridoma cells, B cells and myeloma cells are present in the culture medium B cells fused to myeloma cells form hybridoma cells which have a selective growth advantage over B cells and myeloma cells B cells fail to grow in culture because they are not immortal and die after a few days Myeloma cells used for fusion are deficient in the hypoxanthinephosphoribosyltranseferase (HPRT) enzyme and consequently are not able to use the HRPT - based salvage pathway for RNA synthesis Selection is then achieved by using hypoxanthine, aminopterin and thymidine (HAT) medium Aminopterin is an effective inhibitor of RNA and DNA synthesis and thus will block the growth of the myeloma cells However, hybrid cells which have the HRPT enzyme from the B cells

will be able to use the added hypoxanthine and thymidine to produce RNA via the salvage pathway and survive

1 4 9 Screening

After selection in HAT medium, hybrid cells are grown and tested to determine which colonies of cells produce antibodies of interest Not all of the spleen cells which form hybrids are B cells, and thus there will be some hybrids which do not produce antibody at all, and also many cells will be present which produce antibodies which are not of interest An important step is therefore the screening of clones of cells to identify those which produce the MAbs required A large number of clones may need to be screened quickly to allow selection of those required for further culture A suitable screening assay should therefore be rapid, able to screen many samples and capable of giving a result with the small amount of antibody produced by early clones of cells There are many types of suitable assays to measure antigen-binding properties including ELISA (enzyme linked immunoadsorbent assay), dot blots, Western blots, immunohistochemistry and immunofluorescence If possible, the assay which uses the antibody in the closest way to that required for the intended end use of the antibody is the most suitable, as this will ensure that the optimal properties of the antibody are selected

1 4 10 Maintenance of stable hybridoma cell lines

Once suitable hybridomas have been identified, these must be cloned several times to ensure a stable, homogeneous colony of cells and to ensure that the antibody is indeed monoclonal To do this requires the growth of a colony from a single cell This is achieved by subculturing the cells by limiting dilution so that each well of the culture

plate contains an average of less than one cell Alternatively, cloning can be achieved by plating out cells in semisolid agar of by single cell manipulation techniques Individual clones of cells can then be screened for antibody production as before Once cloned, the hybridoma cell is ready to be used for the production of large amounts of antibody, but it is also important to ensure that samples of cells are preserved by freezing in liquid nitrogen at each stage so that valuable hybridomas are not lost

1.5 Aims of this thesis

In order to elucidate further the role of multidrug transporters in multidrug resistance specific antibodies can be used to determine the frequency and pattern of expression of a particular protein in different tumour types MAbs specific for various regions of a protein can determine variations in topology Each individual protein may contain various functional epitopes MAbs which bind to a functional epitope of a protein can be used to determine the substrate specificity of a protein External epitope MAbs are desirable because of their suitability for use on viable unfixed cells and their potential clinical applications for treating drug resistance

Several well-performing MAbs have been described that detect both internal and external epitopes of the MDR1 transporter molecule A limited number of MAbs have also been described for MRP1 However, all MRP1 MAbs described to date detect internal epitopes and require permeabilisation of the cells in order for the antibody to bind to their epitopes It is possible that like P-gp, MRP1 may exist in a number of topological forms This can only be confirmed by use of a range of MRP1 antibodies While this thesis was in progress five homologues of MRP1 were discovered MAbs detecting internal epitopes of the MRP homologues MRP2 MRP3 and MRP5 have also been described (Scheffer, G L , Unpublished work) MAbs detecting MRP4 and MRP6 have not been reported

It was the aim of this thesis to produce new MRP1 antibodies which do not cross-react with other members of the MRP family and can be included in the further study of MRP-1 These MAbs could then be used as tools to unravel the role of MRP1 in multidrug resistance and furthermore may possibly have clinical applications

1.6. Summary of cell lines used in this thesis.

Table 1.6.1. Cell lines used in this thesis.

CELL LINE	ORIGIN	USE	REFERENCE
COR-L23S Parental	Human large cell lung.	MRP1 negative control.	Twentyman *et al.*, 1986.
COR-L23R Drug-selected (Adriamycin)	Human large cell lung.	MRP1 positive control.	Twentyman *et al.*, 1986
HL60S Parental	Human leukaemia, promyelocyte.	MRP1 negative control.	Marsh *et al.*, 1986.
HL60ADR Drug-selected (Adriamycin)	Human leukaemia, promyelocyte.	MRP1 positive control.	Marsh *et al.*, 1986.
2008 Parental	Human ovarian.	MRP1, MRP2 and MRP3 negative control.	Naredi *et al.*, 1994.
2008 MRP1 Transfected (MRP1)	Human ovarian.	MRP1 positive control.	Hooijberg *et al.*, 1999.
2008 MRP2 Transfected (MRP2)	Human ovarian.	MRP2 positive control.	Hooijberg *et al.*, 1999.
2008 MRP3 Transfected (MRP3)	Human ovarian.	MRP3 positive control.	Kool *et al.*, 1999a.
SF9 (Spodoptera frugiperda)	Insect cells.	Negative control for glycosylated MRP1.	Germann *et al.*, 1990.

Table 1 6 1 (Continued) Cell lines used in this thesis

CELL LINE	ORIGIN	USE	REFERENCE
SF9 MRP1 Transfected (MRP1)	Insect cells	Positive control for glycosylated MRP1	Bakos *et al*, 1996
DLKP Parental	Human non-small-cell lung carcinoma	Cell line survey	Law *et al*, 1992
DLKPA Drug-selected (Adriamycin)	Human non -small-cell lung carcinoma	Cell line survey	Clynes *et al*, 1992
A549 Parental	Human adenocarcinoma, lung	Cell line survey	Berger *et al*, 1997a
RPMI 2650 Parental	Cultured from pleural effusion of a patient with metastatic anaplastic squamous cell carcinoma of the nasal septum (human)	Cell line survey	Moore and Sandberg, 1964
RPMI 2650 Drug-selected (Taxol)	Cultured from pleural effusion of a patient with metastatic anaplastic squamous cell carcinoma of the nasal septum (human)	Cell line survey	Yizheng Liang, PhD Thesis, 1999
RPMI 2650 Drug-selected (Melphalan)	Cultured from pleural effusion of a patient with metastatic anaplastic squamous cell carcinoma of the nasal septum (human)	Cell line survey	Yizheng Liang, PhD Thesis, 1999

2.0. MATERIALS AND METHODS

Addresses of suppliers are provided in Appendix 2

2 1 1 Water

Ultrapure water was used in the preparation of all media and solutions This water was purified to a standard of 12-18 MΩ/cm resistance by a reverse osmosis system (Millipore Milli-RO 10 Plus, Elgastat UHP) A conductivity meter in the system continuously monitored the quality of the UHP

2.1 2 Glassware

Most solutions pertaining to cell culture and maintenance were prepared and stored m sterile glass bottles All glassware including these bottles, used for cell culture work were soaked in a 2% (v/v) solution of RBS (Chemical Products) fort at least one hour after which, they were scrubbed and rinsed several times in tap water The glassware was washed in an industrial dishwasher, using Neodisher detergent (an organic, phosphate-based acid detergent) rinsed twice with UHP The materials were finally sterilised by autoclaving as described in section 2 1 3

2 1 3. Sterilisation

Water, glassware and all thermostable solutions were sterilised by autoclaving at 121^{0}C for 20 minutes at 15 p s i Thermolabile solutions were filtered through a 0 22μm sterile, low protein-binding filter (Millipore, Millex-GV SLGV025BS) Large volumes, (up to 10 litres) of thermolabile solutions were filter sterilised through a micro-culture bell filter (Gelman, 12158)

2 1 4. Media preparation

The basal media used during routine cell culture were prepared according to the formulations shown in table 2 1 4 10X media were added to sterile ultrapure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7 45-7 55 using sterile 1 5M NaOH and 1 5M HCl The medium was then filtered through a sterile 0 22µm bell filter (Gelman G1423S) and stored in 500ml bottles at 4^0C up to the expiry date recorded on each individual 10X medium container (up to three months from production date) Prior to use, 100ml aliquots of basal media were supplemented with 2mM L-glutamine (Gibco 043-0503) and 5-15% foetal calf serum This was then used as routine culture medium This was stored for up to 2 weeks at 4^0C after which time fresh culture medium was prepared Hybridoma and SP/2/O-Ag14 myeloma cells were grown in a basic basal medium of commercially available DMEM, DMEM with Glutamax I (, Glutamax is L-Amyl-L-Glutamine, high glucose concentration - 4 5mg, Gibco 61965-026) supplemented with 10% heat inactivated FCS (Myoclone, Gibco 10082-147) This was further supplemented for hybridoma growth and hybridoma cloning with 1% Penicillin streptomycin and 5% Briclone (Bioresearch Ireland) Briclone (Bioresearch Ireland) is a conditioned medium collected from a human cell line, for use in post fusion stages of hybridoma production and cloning, replacing the function of feeder cells Sterility checks were routinely carried out on all media, media supplements and reagents used in cell culture as described in section 2 2 6

Table 2 1 4 Preparation of basal media

	DMEM (Gibco 042-0250M)	**Hams-F12** (Gibco04201430M)
10X Medium	500 ml	500 ml
Ultrapure H_2O	4300 ml	4700 ml
1M HEPES (Sigma H9136)	100 ml	100 ml
7 5% $NaHCO_3$ (BDH 30151)	45 ml	45 ml

2 2 Cell culture procedures

All cell culture work was carried out in a class II down-flow re-circulating laminar air-flow cabinet (Nuaire Biological Cabinet) All experiments involving toxic compounds were conducted in a cytoguard laminar air-flow cabinet (Gelman Sciences, CG series) Strict aseptic technique was adhered to at all times The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items brought into the cabinet Each cell line was assigned specific media and waste bottles At any time, only one cell line was used m the laminar air-flow cabinet, and upon completion of work with any given cell line the laminar air-flow cabinet was allowed to clear for at least 15 minutes This was to eliminate any possibilities of cross contamination between the various cell lines The cabinet itself was cleaned weekly with industrial disinfectants (Virkon, Antec International or TEGO, TH Goldschmidt) as were all incubators used in the culture of cell lines and hybridomas These detergents were alternated every month

2 2 1 Cell lines

Details pertaining to the cell lines used for the experiments detailed in this thesis are provided in Table 2 2 1 Cell lines were maintained in 25 cm^2 (Costar 3035), 75 cm^2 (Costar 3075) or 175 cm^2 (Nunclon, NUNC) tissue culture flasks at 37^0C and fed every 2-3 days Cell lines were cultured through 7-10 passages before they were discarded and new cultures grown from frozen stocks Confluent hybridoma cell lines were grown for at least 7 days with no change of growth media when antibody was being harvested

Table 2.2.1. Source description and media requirements of cell lines used in experiments described in this thesis.

CELL LINE	CELL TYPE	BASAL MEDIUM	SOURCE
COR-L23S	Human large cell lung.	RPMI 1640	Dr. Peter Twentyman
COR-L23R	Human large cell lung. Drug-selected (Adriamycin)	RPMI 1640	Dr. Peter Twentyman
HL60S	Human leukaemia, promyelocyte.	RPMI 1640	Dr. Melvin Center
HL60ADR	Human leukaemia, promyelocyte. Drug-selected(Adriamycin)	RPMI 1640	Dr. Melvin Center
2008	Human Ovarian carcinoma.	DMEM	Mr George Scheffer
2008 MRP1	Human Ovarian carcinoma. Transfected (MRP1)	DMEM	Mr George Scheffer
2008 MRP2	Human Ovarian carcinoma. Transfected (MRP2).	DMEM	Mr George Scheffer
2008 MRP3	Human Ovarian carcinoma. Transfected (MRP3).	DMEM	Mr George Scheffer
SF9 (Spodoptera frugiperda)	Insect cells.	Not grown in our laboratory.	Prof. Balazs Sarkadi
SF9 MRP1	Insect cells transfected with MRP1.	Not grown in our laboratory.	Prof. Balazs Sarkadi
DLKP	Human non-small-cell lung carcinoma.	ATC	NCTCC
DLKPA	Human Non-small-cell lung carcinoma. Drug-selected (Adriamycin)	ATC	NCTCC
A549	Human Adenocarcinoma, lung.	ATC	ATCC
RPMI 2650	Human Nasal Carcinoma.	MEM	ATCC
RPMI 2650 Taxol	Human Nasal Carcinoma. Drug-selected (Taxol)	MEM	Yizheng Liang, NCTCC
RPMI 2650 Melphalan	Human Nasal Carcinoma. Drug-selected (Melphalan)	MEM	Yizheng Liang, NCTCC
SP2/O-Ag14	Myeloma (murine)	DMEM	ECACC

ECACC : European Collection of Animal Cell Cultures, Salisbury, Wiltshire SP4 OJG, UK.
ATCC : American Type Culture collection, Rockville, MD, USA.
NCTCC : National Cell and Tissue Culture Centre, Dublin City University, Glasnevin, Dublin 9, Ireland.
MEM was supplemented with 1%(V/V) Non-essential amino acids (NEAA) (Gibco 043-01140)
ATC basal media consists of a 1:1 mixture of DMEM and Hams F12.
RPMI 1640 media supplied as a 1X stock (Gibco, 52400-025).
DMEM media supplied as a 1X stock (Gibco, 61965-026).

2.2.2 Subculturing cell lines

During routine subculturing or harvesting of adherent cell lines, cells were removed from their flasks by enzymatic detachment Waste medium was removed from the cells which were then rinsed with pre-warmed trypsin-versene/EDTA (TV) solution (0 25% trypsin (Gibco 043-05090), 0 01% EDTA (Sigma) solution in PBS (Oxoid BR14A) This ensured that any naturally occurring trypsin inhibitor in residual serum was deactivated Fresh TV was then placed in the flask and incubated until the cells were seen to have detached (2-10 minutes) The TV solution was deactivated by the addition of pre-warmed basal medium containing serum The entire solution was then transferred to a 30ml sterile universal tube (Sterilin 128a) and centrifuged at 1,000 rpm for 5 minutes The resulting pellet was then re-suspended in pre-warmed growth medium, cells counted and tissue culture flasks re-seeded at the required density

Hybridoma and SP/2 cells are loosely adherent Cells were passaged by tapping the flask lightly and/or gentle pipetting with a 10ml sterile pipette (Elkay, Ireland) Cell suspensions were pooled and centrifuged at 1,000 rpm for 5 minutes The cell pellet was then re-suspended in culture medium and a cell count performed (section 2 6 3) and the cells re-suspended at the desired density Cells were grown in 5% CO_2

2 2 3 Cell counting

Cells were trypsinised, pelleted and re-suspended in media as described in section 2 2 2 Cell counting and viability were carried out by using trypan blue (Gibco, 15250) dye exclusion technique An aliquot of Trypan blue was added to a single cell suspension at a ratio of 1 2 After 3 minutes incubation at room temperature, a sample of the mixture was applied to the chamber of a haemocytometer over which a glass cover slip had been placed Cells in the 4 outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the dilution factor taken into account and the final number multiplied by 10^4 to determine the number of cells per ml Non-viable cells were those which stained blue while viable cells excluded the trypan blue dye and remained unstained

2 2 4. Cell freezing

Cells of various passage numbers were frozen and cryo-preserved to serve as master stocks Vials could then be thawed and cultured for study

Cells to be frozen were harvested in the log phase of growth and counted as described in Section 2 2 3 The pellets were re-suspended in foetal calf serum (pre-cooled to 4^0C) and an equal volume of freezing medium (DMSO (Sigma D-5879)/serum 1 9 (v/v)) was added drop-wise to the cell suspension to give a final concentration of at least $5x10^6$ cells per ml 1 5ml of the cell suspension was quickly placed in a cryovial (Greiner 122278) which was placed in the vapour phase of liquid nitrogen container for 2 5-3 5 hr After this, the cryovials were stored in liquid nitrogen until required

2 2 5 Cell thawing

The cryopreserved cells were removed from the liquid nitrogen and thawed at 37^0C Immediately prior to removal of a cryovial from the liquid nitrogen, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells (thus reducing the exposure time to DMSO, which is toxic at room temperature) Following the addition of the thawed cell suspension to the growth medium, the suspension was centrifuged at 1,000 rpm for 3 minutes, after which the pellet was re-suspended in fresh growth medium A viability count was carried out (Section 2 2 3) and the thawed cells were placed in tissue culture flasks and allowed to attach over night, The following day the cultures were re-fed with growth medium to remove any residual DMSO

2 2 6 Monitoring of sterility of cell culture solutions

Sterility testing was performed for all cell culture media and cell culture related solutions Samples of prepared basal media were inoculated onto Columbia blood agar plates (Oxoid CM331), Saburaud dextrose (Oxoid CM217), and Thioglycollate broths (Oxoid CM173) and incubated for 2 days to 3 weeks at 37^0C and 25^0C Complete cell

media were sterility tested at least four days prior to use, using Columbia blood agar plates and incubation of 5ml media samples at 37^0C

2 2 7 *Mycoplasma* analysis of cell lines

Mycoplasma exammations were carried out routinely (every 3 months) on all cell lines used in this study

2 2 7 1 Indirect staining procedure for *Mycoplasma* analysis

Mycoplasma negative NRK cells (Normal Rat Kidney fibroblasts) were used as indicator cells for this analysis The cells were cultured with supernatant (harvested from test cell lines after 2-3 days confluency) from test cell lines for 4 to 5 days, fixed and stained with Hoescht fluorescent stain which binds specifically to DNA, thus it would stain the nucleus of the cell and any *Mycoplasma* infection, which can be seen as small fluorescent bodies in the cytoplasm of the NRK cells Positive controls consisted of NRK cells infected with *Mycoplasma* enriched supernatants

2 7 2 2 Direct staining procedure for *Mycoplasma* analysis

The direct procedure for *Mycoplasma* involved inoculating test samples onto an enriched *Mycoplasma* culture broth to optimise the growth of any contaminants and incubated at 37^0C for 48h Samples of this broth were then streaked onto plates of *Mycoplasma* agar base and incubated for 3 weeks at 37^0C and 5% CO_2 Plates were checked microscopically every 7 days for the growth of small oval shaped colonies which was indicative of *Mycoplasma* infection

2 3 Polyclonal and monoclonal antibody production

All experiments on animals were carried out under license and in compliance with the rules of The Cruelty to Animals Act, 1876, E C Directive 86/609/EC

2 3 1 Immunogens

A range of immunogens were employed in three different animal species (rabbit, mouse and rat) for this thesis to produce both polyclonal and monoclonal antibodies specific to the MRP1 protein These included (1) whole cell lysates of the COR-L23R MRP1 over-expressmg cell line, (2) cell membranes isolated by differential centrifugation from the COR-L23R MRP1 over-expressmg cell line, (3) synthetic peptides and (4) fusion proteins

2 3.1 1 (1) Whole cell lysates (Used for immunisation of mice)

The MRP1 over-expressing COR-L23R cells which is an adherent cell line was trypsinsed as described in section 2 2 2 , counted as described in section 2 2 3 to give a final concentration of 2 x 10^6 cells and washed three times in PBS by centrifugation @ 1000rpm for 10 minutes The cell pellet was re-suspended in 250µl PBS containing protease inhibitors (Protease inhibitor cocktail tablets, prepared according to the manufacturers instructions Boehringer Mannheim 1697498) Cells were then lysed by sonication and completion of cell lysis was confirmed by viewing under an inverted microscope Equal volumes (i e 250µl) of Freund's Complete Adjuvant (Sigma F-5881) were added immediately prior to primary immunisation and Freund's Incomplete adjuvant (Sigma F-5506) added for booster injections Mice received one intraperitoneal injection of 500µl each

2.3.1.2. (2) Cell membranes (Used for immunisation of mice and rats.)

The COR-L23R cell membrane isolation, outlined below, was carried out according to a modification of the method Gerlach *et al.*, 1987.

- At least $2x10^7$ cells were trypsinised (or thawed directly from liquid nitrogen) and washed 3 times with PBS by centrifugation at 2000rpm.
- NB. Cells are kept on ice from this stage on.
- The cells were then suspended in approximately 5ml of lysis buffer (PBS / Protease Inhibitors (Boehringer Mannheim 1697498) - added just before use and stored on ice). The re-suspended cells were then sonicated until cells are lysed - this is determined by viewing under the microscope after each sonication.
- The lysed cells were centrifuged at 7,800rpm in an ultracentrifuge (Beckman XL80) for 10 minutes at 4^0C.
- The supernatant was collected and centrifuged at 38,000rpm in an ultracentrifuge (Beckman XL80) for 1hour at 4^0C.
- The pellet obtained was re-suspended in 500µl - 1000µl lysis buffer.
- An aliquot of 50µl is taken for total protein analysis and the remaining supernatant was divided into aliquots (equivalent to 100ug protein) which were lyophilised overnight in a freeze-drier (VirTis Consol 4.5).
- The lyophilised samples are stored at -20^0C (closed tightly and sealed with parafilm) until required.

Mice received one intraperitoneal injection (500µl per mouse) of 100ug of freeze-dried membrane protein reconstituted in 250µl PBS and mixed with equal volumes of appropriate adjuvant. As in previous section, rats received injections (100µl per footpad) into both rear footpads of 100ug freeze dried membrane protein reconstituted to 50µl in PBS and mixed with equal volumes of adjuvant.

2.3.1.3. (3) Synthetic Peptides (Used for immunisation of rabbits, mice and rats.)

The entire amino acid sequence of the encoded MRP1 protein was obtained from EMBL'S Swiss-Prot (EMBL:http://www.embl-heidelberg.de/) protein sequence database databank using the NETSERV program. The three short synthetic peptides (their location within the complete protein sequence for MRP1 is described in Section 3.5.2. of the Results) used in immunisations were chosen after alignment searches of the above databank using the Mail-Fasta program (Pearson and Lipman 1982). The three peptides were synthesised by Biosyn Ltd, 10 Malone Road, Belfast, BT9 5BN, Northern Ireland and Immune Systems Ltd., PO Box 120, Paignton, TQ4 7XD, UK according to the deduced MRP1 sequence (Cole *et al.*, 1992). Three sequences having the following sequences were prepared :

Peptide 1 CALFYSMAKDAGLV (amino acids 1520 – 1531) conjugated to KLH
Peptide 2 PLEGSDLWSLNK (amino acids 235 – 246) conjugated to BSA
Peptide 3 KEDTSEQVVPVLVKN (amino acids 246 – 260) conjugated to KLH

For immunisation of rabbits, 1mg of Peptide 1, Peptide 2 or Peptide 3 was reconstituted in 1ml PBS and mixed thoroughly with equal volumes of Freund's Complete Adjuvant (Sigma F-5881) for the primary immunisation and Freund's Incomplete Adjuvant (Sigma F-5506) for all subsequent booster injections. Rabbits received subcutaneous injections on 10 sites along their back equivalent to100ug protein/site.

For immunisation of mice, synthetic peptides were reconstituted at 100ug protein in 250µl PBS. Equal volumes of Freund's Complete Adjuvant (Sigma F-5881) were added for primary immunisations and Freund's Incomplete adjuvant (Sigma F-5506) for booster injections. Mice received one intraperitoneal injection of 500µl each (100ug peptide/injection).

For immunisation of rats, 100ug of each of the two synthetic peptides used (Peptide 2 and Peptide 3) were reconstituted together in 100 µl PBS and mixed with equal volumes of Freund's Complete Adjuvant (Sigma F-5881) for the primary immunisation and

Freund's Incomplete adjuvant (Sigma F-5506) for booster injections Rats received 100µl volumes in both rear footpads (50ug mixed peptides/injection)

2 3 1 4 (4) Fusion Proteins (Used for immunisation of mice)

DNA technology provides the ability to isolate, characterise and sequence genes From these gene sequences (i e the nucleotide sequence) it is possible to predict the amino acid sequence of the encoded gene This information makes it possible to express large quantities of the protein of interest in cell expression vector systems These proteins are usually expressed fused to a large protein such as Maltose Binding Protein (MBP) for their purification and are termed ''Fusion Proteins'' Shorter synthetic peptides may be synthesised using the solid-phase techniques introduced by Merrifield (1963) These short synthetic peptides are usually coupled to a carrier protein such as bovine serum albumin (BSA) (Sigma) or keyhole limpet hemacyanin (KLH) (Sigma) The two Fusion Proteins used in immunisations (described in Section 3 5 2 of the Results) were a kind gift from Dr Balazs Sarkadi, The Institute of Enzymology, Biological Research Center, Hungarian Academy of sciences, H-1113 Budapest, Hungary Both Fusion proteins (location described in Section 3 5 2 of the Results) used in immunisations were produced according to the deduced MRP1 sequence (Cole *et al*, 1992) and their amino acid numbers are listed below

Fusion Protein 1 (amino acids 1294 - 1531) fused to Maltose Binding Protein (MBP)
Fusion Protein 2 (ammo acids 647 - 912) fused to Maltose Binding Protein (MBP)

Fusion proteins were reconstituted at 100ug protein in 250µl PBS Equal volumes of Freund's Complete Adjuvant (Sigma F-5881) were added for primary immunisations and Freund's Incomplete adjuvant (Sigma F-5506) for booster injections Mice were the only species used for immunisation with fusion proteins Each mouse received one intraperitoneal injection of 500µl each (100ug peptide/injection)

2 3 2. *In-vivo* Immunisation procedure

2 3 2 1 **Rabbits** (female New Zealand White rabbits)

Prior to immunisations and test bleeds, the rabbits (6 months old) were given 0 5ml (equivalent to 0 015mg) Tengesic (Buprenorphorine) as an analgesic Before the primary immunisation a control pre-bleed of 1-2ml was taken from a vein visible under the skin surface of the earlobe after shaving the area Directly after this the rabbits were injected at least 3 times at 6 week intervals with 100ug peptide in 200µl injection volume per site (10 sites on back (1mg of Peptide 1, Peptide 2 or Peptide 3 reconstituted in 1ml PBS and mixed thoroughly with Freund's Complete Adjuvant (Sigma F-5881) for the primary immunisation and Freund's Incomplete adjuvant (Sigma F-5506) for all subsequent immunisation boosts)) After each booster, a blood sample (5-6ml) was taken as before and tested for specific antibody presence by Western blot analysis A period of 9-11 days were left after boosters for the test bleeds on immunised rabbits to allow peaking of the immune response 10 days following the final booster injection, the animals were administered Pentobarbitone Sodium (Sagatal) (60mg/ml/kilo bodyweight) until they were completely anaesthetised The animals were then exsanguinated and finally euthanased using Pentobarbitone sodium (Sagatal) (120mg/ml/kilo bodyweight) at the end of the procedure

2 3 2.2 **Mice** (male Balb/c mice)

Mice (6-8 weeks old) received a primary booster of 100ug peptide, 100ug fusion protein or 100ug COR-L23R cell membranes or $2x10^6$ lysed COR-L23R cells in Freund's Complete Adjuvant (Sigma F-5881), followed by at least 3-4 booster injections of the same quantity of immunogen in Freund's Incomplete adjuvant (Sigma F-5506) at three weekly intervals, injections administered intraperitoneally For the synthetic peptides and fusion proteins, the same regime applied with each mouse receiving 100ug peptide reconstituted into 250µl PBS and mixed with equal volumes of appropriate adjuvant In all cases, three days prior to a fusion being performed, a final booster was given

2 3 2 3 Rats (female Wistar rats)

Rats (10-12 weeks old) received at least 4 rear footpad injections (100µl per footpad) of either COR-L23R cell membranes or a combination of both Peptide 2 and Peptide 3 at three weekly intervals 200ug of freeze-dried cell membrane protein was reconstituted to 100µl in PBS and mixed with equal volumes of Freund's Complete Adjuvant (Sigma F-5881) for the primary immunisation and Freund's Incomplete adjuvant (Sigma F-5506) for booster injections 50ug of each of the two synthetic peptides used (Peptide 2 and Peptide 3) were reconstituted together in 100µl PBS and mixed with equal volumes of Freund's Complete Adjuvant (Sigma F-5881) for the primary immunisation and Freund's Incomplete adjuvant (Sigma F-5506) for booster injections In all cases, three days prior to performing the fusion, a final booster was given

2 3 3 Fusion Procedure

2 3 3 1 Mouse

The fusion procedure used for the production of mouse monoclonal antibodies was a modification of the protocol outlined by Kohler and Milstein (1975) Prior to the removal of the spleen from the sacrificed mouse, SP/2/O-Ag14 myeloma cells (Shulman et al 1978) were prepared for cell fusion by harvesting from 75 cm^2 flasks and centrifuging at 1,000 rpm for 5 minutes in HEPES-free serum free medium This step was repeated twice A cell count was then performed (Section 2 2 3) and the cells kept at 37^0C A Balb/C mouse was then sacrificed by cervical dislocation The animal was swabbed with 70% IMS and the spleen removed in a laminar flow cabinet with sterile dissection instruments Single cells were obtained by forcing the spleen through a sterile Falcon cell strainer (Becton Dickinson 2360) using the plunger from a sterile 10ml syringe into serum free DMEM (the DMEM referred in this section is DMEM with Glutamax I (high glucose concentration - 4 5mg Gibco 61965-026) containing pyridoxine and without sodium pyruvate or HEPES This cell suspension was placed in a 50ml centrifuge tube and the volume adjusted to 20ml Large clumps of cells were

allowed to pellet by standing at room temperature for 2-3 minutes The supernatant was then transferred to a fresh centrifuge tube and centrifuged at 1,000 rpm for 5 minutes A cell count was performed as before (Section 2 2 3) Splenocyte and SP/2 myeloma cells were mixed in a 50ml universal tube at a ratio of 10 1 (a minimum of $1x10^7$ SP/2 are required for this procedure), centrifuged at 1,000 rpm for 5 minutes and re-suspended in serum free medium This step was repeated twice Following the final washing step, 1ml of PEG (polyethylene glycol, Boehringer Mannheim, 783641 pre-warmed to 37^0C) was added to the cell pellet with a Pasteur pipette using a gentle swirling and aspirating action for 30 seconds After 30 seconds the aspiration was discontmued 75 seconds after the start, 0 5ml of plating medium (DMEM with Glutamax I, 10% heat inactivated FCS, 5% Briclone (Bioresearch Ireland) and 1% HAT (Hypoxanthine, Aminopterin, Thymidine) (Boehringer Mannheim 644579) was added slowly down the side of the 50ml centrifuge tube while continuing to swirl gently 8ml of plating medium was added over the next 5 minutes (3ml at 1 minute intervals followed by the addition of 5ml) Following this step, the cell suspension was centrifuged at 500 rpm for 5 minutes The supernatant was removed and the cells were re-suspended in 10ml of plating medium and incubated at room temperature for 15 minutes Prior to performing the fusion, 0 5ml of plating medium was dispensed into each well of 8x48 well plates (Costar, 3548) and the plates were equilibrated in the CO_2 incubator @ 37^0C Following the 15 minute incubation at room temperature one drop of fused cells was added to each well and the plates were incubated for 10-12 days at 37^0C, 5% CO_2

2 3 3 2 Rat

The fusion procedure for the production of rat monoclonal antibodies was identical to the procedure used for mouse monoclonal antibody production except that immune activated B cells were collected from the popliteal node in the lymphatic system as seen in Diagram 2 3 2 This single node is found in a cavity, the popliteal fossa, amidst the leg muscles at the back of the knee The rat is placed on its ventral surface and the hind limbs are stretched out A small longitudinal incision is made in the skin at the back of the leg behind the knee Superficial connective tissue is cleared, and by careful observation the area where the semitendinous, the biceps femoris and the triceps sural muscles cross is located This area is often denoted by a small white fatty area into

which the vein running along the lower border of the triceps sural muscle disappears Forceps are pushed into this area which delineates the popliteal fossa The popliteal lymph node lies within the fatty tissue but is distinguished from it by being a small round discrete creamy-yellow coloured organ The node can be removed with forceps The popliteal node was removed aseptically and squeezed through a sterile falcon cell strainer (Becton Dickinson 2360) to gain a single cell suspension These cells were then treated in an identical manner to that of the mouse spleen cells (Section 2 3 3 1) for the remainder of the fusion

A trial run using a number of rats was carried out, in order to become familiar with the location of this popliteal node prior to performing immunisations and fusions Lymphazurin* 1% (Isosulfan Blue), which is a contrast agent used for the delineation of lymphatic vesicles, was injected into the footpads of rats The chemical name for Isosulfan blue is N-[4-[[4-(diethylamino)phenyl](2,5-disulfophenyl)methylene]-2,5-cyclohexadien-1-ylidene]-N-ethylethanammium hydroxide, inner salt, sodium salt Following subcutaneous administration, isosulfan blue is selectively picked up by the lymphatic vesicles Within 10 minutes of injecting the dye, a distinct blue colour could be traced along the vesicles of the lymphatic system under the skin Upon dissecting the rat and following the dyed lymphatic vesicles, the popliteal node is distinguished at the point of final concentration of all lymphatic vesicles in that area within the fatty tissue

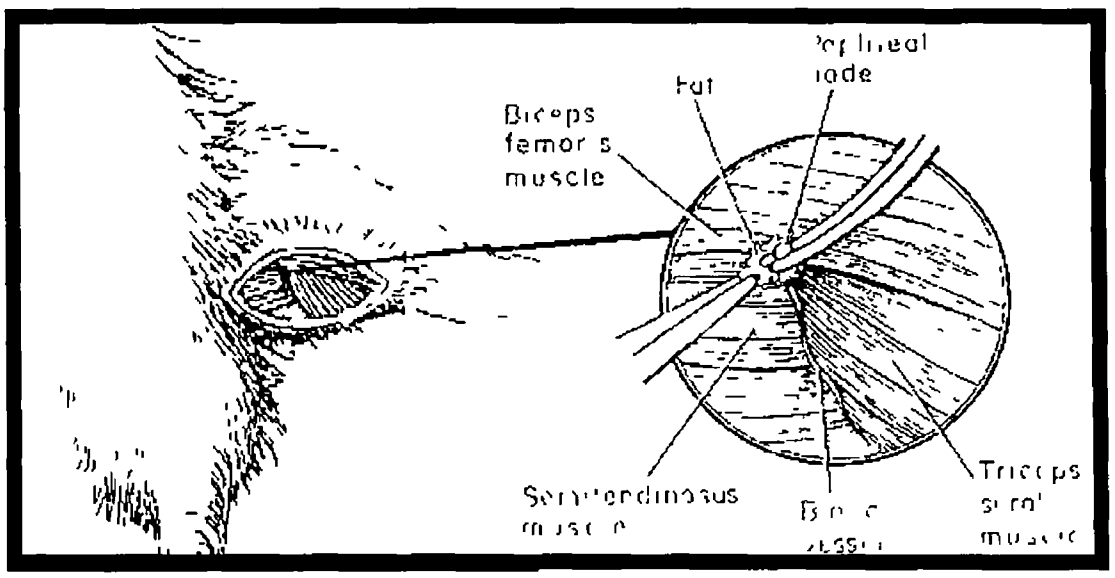


Diagram 2 3.2 Removal of the popliteal lymph node

2.3.4. *In-vitro* immunisation

In-vitro immunisations for murine monoclonal antibody production were carried out using a kit (Cell Prime) supplied by Immune Systems, Bristol, UK. The spleen was removed from a BALB/c mouse, and the spleen cells were immunised *in-vitro* with 60ug of conjugated peptide following the instructions provided by the *in-vitro* immunisation kit manufacturer. Three days after *in-vitro* immunisation, spleen cells were fused to SP/2 as described in section 2.3.3.1. Alternatively, a combination of *in-vivo* and *in-vitro* immunisations were carried out by immunising the mouse *in-vivo* prior to removal of the spleen for *in-vitro* immunisation.

2.3.5. Screening of hybridomas

Hybridomas were screened by Peptide ELISA, Western blotting against COR-L23R whole cell lysates or live cell immunofluoresence. Hybridomas were allowed to form large colonies and grow for at least 10 days undisturbed before supernatant was removed to screen for specific antibody production.

For the peptide ELISA, plates were coated with peptide (1ug per well in 100µl carbonate buffer (BUFFER 1: 1.68g $NaHCO_3$ dissolved in 100ml distilled H_2O; BUFFER 2: 2.12g Na_2CO_3 dissolved in 100ml distilled H_2O. 74ml of BUFFER 1 was mixed with 26ml of BUFFER 2. The pH was 9.4-9.7. Stored at 4^0C.) and incubated at 4^0C overnight. The plates were washed once in PBS and then incubated with blocking buffer (1% (w/v) BSA, 0.1% (w/v) Sodium Azide, PBS) for 1.5 hours at room temperature. The plates were washed once in PBS again. 100µl of hybridoma supernatant was then added to the peptide coated plates and incubated at 37^0C for 1 hr. This solution was then discarded and the plates washed 3 times with wash buffer (0.1% (v/v) Tween 20 (Merck) in PBS). 100µl of secondary antibody, alkaline -phosphatase-linked rabbit anti-mouse immunoglobulins, I_gG, I_gM (Dako, diluted 1/10,000 in PBS 0.1% (v/v) Tween 20) was added to each well and incubated at 37^0C for 1 hr. The secondary antibody was removed and the plates washed 3 times with wash buffer as before. Plates were then incubated with the substrate solution (1 mg/ml p-nitrophenyl

phosphate (PNPP, Sigma 104-0) in 0 1M glycine, 0 001M $MgCl_2$, 0 001 M $ZnCl_2$, pH 10 4) at 37^{0}C for 0 5-1 hr or until a yellow colour appeared in the wells The reaction was stopped by the addition of 1M NaOH which also enhanced colour Absorbencies were read on a Titerex ELISA plate reader at 405nm Positive reactivity was determined by comparing supernatant containing wells with those which had been incubated with PBS instead of supernatant

2.3.6 Subculture of Hybridomas

Positive clones were further sub-cultured to 6 well plates (Costar) and gradually transferred (two feeds in HAT medium followed by gradual change into HT medium - half HAT and half HT medium, HT medium) into medium containing HT (Hypoxanthine, Thymidine) (Boehringer Mannheim, 623091) Eventually (within two weeks) hybridoma clones were weaned off HT (by decreasing HT gradually in feeds every three days) and fed with DMEM medium supplemented with 5% Briclone (Bioresearch Ireland) and 10% heat inactivated FCS

2 3 7 Single cell cloning by limiting dilution

Using a multi-channel pipette (eppendorf) 100µl of DMEM growth medium was pipetted into each well of a sterile 96 well tissue culture treated plate 100µl of cell suspension from rapidly growing hybridomas at a concentration of $1x10^4$ cells was added to the top left hand well and mixed by pipetting 1 in 2 doubling dilutions were performed down the left hand row of the plate (8 wells, 7 dilution steps) and mixed by pipetting, ensuring to change the pipette tip each time 1 in 2 dilutions were also performed across the plate using a multi-channel pipette Plates were then incubated for 7-10 days at 37^{0}C, 5 CO_2 Wells with a single colony were chosen Hybridomas were cultured in 25 cm^2 flasks and the procedure repeated The selected clones were screened by ELISA, Western blotting and immunocytochemistry and frozen stocks made of positive clones

2 3 8 Isotype analysis

The mouse MAbs were isotyped using an isotyping kit specific for mouse MAbs, The Isostrip Mouse Monoclonal Antibody Isotyping Kit (Boehringer Mannheim, 1493027)

The rat MAbs were istoyped using an ELISA based rat MAb isotyping kit, MAb-based Rat Ig Isotyping Kit (PharMingen, 04117K)

2 4 Western blot analysis

2 4 1 Determination of Protein concentration

Protein concentration was determined by the BCA method (Smith *et al*, 1985) using a kit obtained from Pierce (Pierce, 23225) Protein samples (cell lysates or cell membranes) were diluted to a final volume of 100µl in PBS BSA protein standards of known concentration were also prepared from a 2mg/ml stock solution in PBS in borosicilliate test tubes Negative controls consisted of PBS 100µl of the BCA reagent (prepared according to the manufacturers instructions) was added to each protein sample, mixed and incubated at 60^0C for 30 minutes Following a brief cooling period, samples were transferred to plastic cuvettes (Elkay) and the absorbencies read on spectrophotometer (Titertek, Multiscan Plus) at 562 nm A standard curve of absorbance vs protein concentration of the BSA standards was constructed The protein concentration of the test samples was then estimated

2 4 2 Sample preparation for gel electrophoresis

At least 2 x 10^7 cells were collected by trypsinisation as outlined in section 2 2 2 However, instead of re-suspending the cell pellet in medium, the pellet was re-suspended and washed three times in PBS by centrifugation at 1000rpm for 10 minutes

The washed cell pellet was re-suspended in 25µl PBS Protein inhibitors were also added to the cell suspension (protease inhibitor cocktail tablets, Boehringer Mannheim 1697498) Cells were lysed by sonication Complete lysis was confirmed by viewing under an inverted microscope Protein concentration was determined by the BCA method (Section 2 4 1) Protein samples were prepared for electrophoresis by adding equal volumes of 2X loading buffer (5 0ml 1 25M-Tris/HCL pH 6 8, 2 0g SDS, 5 0ml mercaptoethanol (Sigma M6250), 11 6ml glycerol and 10mg bromophenol blue (Sigma B8026) reconstituted to a final volume of 50ml in H_2O) Protein samples in 2X loading buffer were only heat treated (boiled for 3 minutes at 100^0C) if necessary for antigen detection Detection of the antigen by MRPr1, or any other antibody mentioned in this thesis which worked in Western blotting techniques, did not require heat treatment Samples were stored at -20^0C until required for a maximum of one month

2 4 3 Gel electrophoresis

Proteins for Western blotting were separated by SDS-polyacrylamide gel electrophoresis (Laemmli *et al*, 1970) Gels were prepared as outlined in table 2 4 3 and poured into clean 10cm x 8cm gel casting cassettes (Mighty Small II, Hoefer) which consisted of one glass plate and one aluminium plate separated by 0 75cm^2 plastic spacers The resolving gel was poured first and allowed to set A layer of saturated isobuthanol was gently layered over the resolving gel to prevent drying out When the resolving gel was set the layer of isobuthanol was washed off with several changes of distilled H_2O The stacking gel was then poured and a comb was fitted allowing the formation of wells for sample loading Once the gels had set (at room temperature) they were wrapped in tinfoil and stored at 4^0C if not used immediately 10-15 µg of protein was applied to each well of the polyacrylamide gel Empty wells were loaded with 1X loading buffer to ensure even running of the gel Pre-stained molecular weight markers (Sigma, SDS-6H) were also loaded onto the gel for the determination of the molecular weight of unknown protein samples Gels were run at 250 volts and 45 milliamps (Atto power pack, Atto Corp , Japan) for 1-1 5 hr (values were halved if only one gel was being run) When the bromophenol dye front had reached the end of the gel,

electrophoresis was stopped, the gel removed and equilibrated in transfer buffer (0 25 M Tris - 1 92M Glycine pH 8 4, 10X (Sigma T-4904)) for 15 minutes

Table 2 4 3 Preparation of electrophoresis gels

Stock solutions	Resolving gel (7.5% acrylamide)	Stacking gel (5% acrylamide)
40% acrylamide (Sigma)	2 7 ml	0 6 ml
2% Bis-acrylamide (Sigma)	1 9 ml	0 42 ml
dH_20	8 3 ml	3 67 ml
8X Tris-HCl pH 8.8	1 88 ml	-------
16X Tris-HCl pH 6 8	------	0 3 ml
10% SDS	150 µl	50 µl
10% Ammonium persulphate	60 µl	17 µl
TEMED	10 µl	5 µl

2 4 4 Western blotting

Between 8 to 10 sheets of Whatman filter paper were soaked in transfer buffer (0 25 M Tris - 1 92M Glycine, 10X Sigma T-4904, diluted 1 in 10 in dH_2O) and placed on the anode of a semi-dry blotting apparatus (Biorad Transblot SD) taking care to remove all bubbles by rolling a glass pasteur pipette across the sheets PVDF membrane (Boehringer Mannheim, 972206), pre-soaked according to the manufacturers instructions) was placed over the filter paper and the gel layered on top of this A further 8-10 sheets of pre-soaked filter paper were paced over the gel and any air bubbles removed The cathode was carefully laid on top of the stack and the proteins transferred from gel to PVDF membrane at 15 volts and 34 milliamps for 25 minutes

2 4 5 Development of Western blots by enhanced chemiluminescence (ECL)

ECL Western blotting detection is a highly sensitive, rapid, quantifiable, non-isotopic method for detecting proteins immobilised on membranes The primary antibody is located with the Horseradish-Peroxidase (HRP)-labelled species specific secondary antibody The HRP-labelled molecule catalyses the oxidation of luminol, resulting in the emission of light - chemiluminescence Enhanced chemiluminescence (ECL) is the sustained emission of light provided by the inclusion of chemical enhancers in the HRP catalysed oxidation of luminol, and gives approximately 1000 fold more light that the oxidation of luminol alone

Following Western blotting, the filter paper was removed and the membrane blocked for non-specific binding by incubating for 2 hours on a rocking platform with non-fat milk (Marvel, Cadbury) After the blocking step was complete, the blot was rinsed once in TBS, cut into strips (if required) and incubated with the primary antibody optimally diluted in TBS/Tween overnight at 4^0C on a rocking platform Mouse IgG diluted 1/5,000 in TBS/Tween served as a negative control The following day blots were washed 3 times within 30 minutes with TBS 0 5% (v/v) Tween 20 Blots were then incubated with a rabbit anti-mouse HRP-conjugated secondary antibody (Dako, P0447) diluted 1/4,000 in TBS/Tween for 1 hour at room temperature on a rocking platform The blots were washed as before, laid out on a glass plate covered in parafilm and incubated with ECL reagents (Amersham RPN 2109) for 1 minute at room temperature Or if used to enhance low signals, Super Signal Ultra (Pierce) was mixed according to the instructions and applied to the blots for 5 minutes at room temperature Following this the solution was tapped off, the blot laid between two sheets of cling film and exposed to LUMI film, chemiluminescence film (Boehringer Mannheim 166657) for various time periods and processed using standard x-ray developing procedures Dried film was aligned with pre-stained molecular markers for molecular weight determination

2.4.6. Immunoprecipitation

Immunoprecipitation of specific proteins was carried out using a modification of a protocol described in a study carried out by Masterson A, PhD Thesis 1998. This protocol utilised a new cellular labelling and immunoprecipitation kit (Boehringer Mannheim 1647652) with modifications. The principle of this procedure is based on non-radioactive labelling of proteins or glycoproteins on intact cells (or in cell lysates) with D-biotinoyl-ε-aminocarporic acid-N-hydroxysuccinimide ester (biotin-7 NSH) which covalently binds free amino groups (mainly lysine residues). These labelled proteins can then be immunoprecipitated and detected using a biotin specific antibody. However, in this study the protein was directly labelled with the primary antibody, immunoprecipitated and then detected by Western blot analysis using the same antibody to re-probe the blot.

Unless stated otherwise, all reagents were supplied in the immunoprecipitation kit. Briefly, the modified procedure was carried out as follows; whole cell lysates were prepared from cell pellets containing a maximum of $5x10^7$ cells/ml. Cell lysates (kept on ice for the rest of the procedure) were prepared in lysis buffer and sonicated (as outlined in Section 2.4.2.), followed by a 20 minute incubation period in lysis buffer (to solubilise membrane protein. Cell lysates were then diluted 1:1 with dilution buffer and pre-cleared by incubating with 2 changes of Protein A or G-linked Agarose beads (depending on Ab isotype and species) for four hours each clearance. Following the final incubation step, the primary antibody was optimally diluted in the cell lysates (15 µg/ml, mouse immunoglobulins at a concentration of 15 µg/ml served a negative control) and incubated at 4^0 C over night on a rocking platform. The following day 50µl of protein A/G-linked agarose beads (Boehringer Mannheim) were added to the cell lysates and incubated for a minimum of 4 hours at 4^0C on a rocking platform to precipitate the antibody-antigen complex. Pelleted beads were then washed with two changes of wash buffer 1, two changes of wash buffer 2 or 2 changes of a low salt buffer (100mM NaCl) and one change of wash buffer 3. Following the removal of any remaining supernatant, pellets were re-suspended in 60µl of loading buffer and heat treated at 100^0C for 3 minutes to disassociate the beads from the complex. After

centrifugation at 1000rpm for 5 minutes, the supernatant was collected and analysed by Western blotting, section 2.4.4. and blot development using ECL section 2.4.5.

2.5. Immunocytochemical analysis

2.5.1. Coating slides with poly-L-lysine

Washed microscope slides were coated with poly-L-Lysine hydrobromide (Sigma P1274) following the procedure of Huang *et al.*, (1983). Slides were stored at room temperature for up to 1 month.

2.5.2 Preparation of cytospins

Cells from actively growing cultures were trypsinised (Section 2.2.2.), washed 3 times in 10 minutes in PBS and diluted to a final concentration of $1x10^6$ cells/ml in PBS. 100µl of the cell suspension was then applied to modified eppendorf component of a cytofuge and spun onto Poly-L-lysine coated glass slides at 400 rpm for 4 minutes Cytospins, consisting of 2 "sections" were allowed to air dry over night. Slides were allowed to air dry and stored wrapped in tin foil at -20^0 C until required.

2.5.3. Immunofluorescence analysis of live cells

When immunofluorescence is carried out on live cells only cell surface components are detected (Schachner *et al.*, 1982). All reagents were kept at 4^0C. Cell suspensions at $1x10^6$ cells/ml were prepared in PBS. 100µl of cell suspensions was placed in an eppendorf tube mixed and incubated at 4^0C for 30 minutes. Negative controls consisted of cell suspension incubated with mouse immunoglobulins (Vector Laboratories, I-2000), diluted 1/10,000 in PBS. Cells were washed 3 times over 12 minutes in PBS by

centrifuging at 2,000rpm in a microfuge. Cell pellets were re-suspended in 100 µl of anti-mouse IgG FITC-labelled secondary antibody, diluted 1/50 in PBS, (Boehringer Mannheim 814385) and incubated in the dark for 30 minutes at 4^0C. Cell suspensions were washed as described and following the final washing step the supernatant was removed. Cells were then aspirated in 1 drop of Vectashield Mounting Medium (Vector Labs Ltd.UK H1000). 20µl of the suspension was placed on a microscope slide, covered with a coverslip and the edges sealed with clear nail polish. Slides were viewed using a Nikon phase contrast microscope fitted with an FITC filter.

2.5.4. Immunofluorescence analysis of fixed cells

Indirect immunofluorescence on fixed cells was carried out on cytospin preparations (Section 2.9.2.). Cytospins were fixed in ice cold acetone (BDH) and allowed to air dry at room temperature. The test antibody was applied to one cytospin section on the cytospin while mouse IgG diluted 1/10,000 in PBS was applied to the other section. The cytospins were incubated for 30 minutes at room temperature in a humidified chamber. Slides were then washed in PBS 0.1% (v/v) Tween 20 3 times over 10 minutes 50µl of an anti-mouse FITC-labelled secondary antibody diluted 1/50 in PBS was applied to each section and incubated at room temperature for 30 minutes Slides were washed as before. Slides were mounted with 1 drop of Vecta Shield (Vector Laboratories) mounting medium applied to the centre of the slide, and the coverslip and sealed with clear nail varnish.

2.5.5. Immunocytochemical analysis using the StrepAB complex/HRP method

All immunocytochemical studies on cytospins or formalin fixed paraffin-embedded archival material were performed following the method of Hsu *et al.*, (1981) using an avidin-biotin (ABC) horseradish peroxidase (HRP) conjugated kit (Dako K337). The use of diaminobenzidine (DAB) (liquid DAB solution prepared according to instructions; Dako K3465) as a substrate for the peroxidase enzyme produced an insoluble brown precipitate which was indicative of primary antibody activity.

Frozen cytospin preparations were allowed to equilibrate to room temperature (approximately 10-15 minutes) Slides were then fixed in ice cold acetone for 4 to 10 minutes, depending on the antibody (all antibodies worked when fixed for 10 minutes), and allowed to air dry A wax pen (Dako S2002) was used to encircle each "section" to contain the various solutions involved Cytospins were then incubated with 0 3% (v/v) hydrogen peroxide (BDH) in methanol (BDH) to quench endogenous peroxidase activity followed by a brief rinse in distilled H_2O and incubation in 1xTBS for 5 minutes Following this cytospins were incubated in 20% (v/v) normal rabbit serum (Dako X0902), in 1xTBS for 20 minutes at room temperature to block any non-specific binding of the secondary antibody (a rabbit host) The excess serum was tapped off and the optimally diluted primary antibody was applied to one of the cytospm sections while control mouse immunoglobulins (Vector Laboratories) served as a negative control Slides were placed in a humidified atmosphere and incubated at room temperature for 2 hour or at 4^0C overnight Following primary antibody incubation, cytospins were washed 3 times over 15 minutes in 1xTBS, 0 1% (v/v) Tween 20 (TBS/Tween) and then incubated with an appropriate biotinylated secondary antibody (anti-rat (Dako E0468) or anti-mouse (Dako E0354)) diluted 1/300 in TBS/Tween, for 30 minutes at room temperature Following this cytospins were washed as before in TBS/Tween The Strep AB complex (Dako K337 prepared according to manufacturers instructions) was applied to the cytospin and incubated at room temperature for 30 minutes Following the washing steps, liquid DAB (Dako K3565 prepared according to the manufacturers instructions) was applied to each "section" of the cytospin for 7-10 minutes at room temperature Cytospins were rinsed in tap water, terminating the reaction, counterstained in Harris' haematoxylin (Sigma Diagnostics HHS-16) (15 seconds), differentiated in 1% (v/v) hydrochloric acid in 70% IMS (15 seconds) and blued in Scotts tap water (Clintech, Clacton-on-Sea, Essex, England)(10 seconds) rinsing in water between each step Alternatively cells were counterstained with methyl green (Vector Laboratories) Cytospins were then dehydrated in graded alcohols (2 x 3 minutes in 70%, 90% and 100% (v/v) IMS in dH_2O and cleared in xylene (BDH, 2 changes of 5 minutes each) Cytospins were mounted in DPX mounting medium (BDH) and allowed to dry overnight Cytospins were viewed under a light microscope

3.0 RESULTS.

3 1 CHARACTERISATION OF MRP ISOFORM MAbs AND MRP ISOFORM TRANSFECTED CELL LINES

Various MAbs which detect specific isoforms of the MRP family were a kind gift from Mr George Scheffer of Professor Rik Scheper's Laboratory, Department of Pathology and Oncology, Free University Hospital, 1081 HV Amsterdam (see Table 3 1 1) The MRPr1 antibody included is the well characterised MRP1 specific rat MAb (Flens *et al* , 1996) The ovarian 2008 parental cell lines, which were transfected with the MRP1, MRP2 or MRP3 cDNAs, were also a kind gift from Mr George Scheffer (see Table 3 1 2) Permission to use the MRP3 transfected 2008 cell line was kindly provided by Marcel Kool, Academic Medical Center, Laboratory of Neurozintuigen, Room K2-212, Meibergdreef 9, 1105 AZ Amsterdam Both the MRP isoform MAbs and MRP isoform transfected cells were characterised by Western blot and immunocytochemical analysis (see following results) These MAbs and cell lines were further used to characterise the specificity of the MRP1 antibodies produced in this thesis

Table 3.1.1. **MRP Isoform MAbs**

ANTIBODY NAME	ISOFORM DETECTED	HOST SPECIES
MRPr1	MRP1	RAT
MRPm6	MRP1	MOUSE
M2I-4	MRP2	MOUSE
M2III-6	MRP2	MOUSE
M3II-21	MRP3	MOUSE
M3II-9	MRP3	MOUSE
M5I-1	MRP5	RAT
M5II-54	MRP5	RAT

Table 3.1 2. **MRP Isoform transfected cell lines**

CELL LINE	TRANSFECTED WITH
2008 PARENTAL	-
2008 M1	MRP1
2008 M2	MRP2
2008 M3	MRP3

3 2 CHARACTERISATION OF THE MRP1 SPECIFIC MAb, MRPr1

The expression of MRP1 in various cell lines was examined by Western blotting and immunocytochemical techniques Known MRP1 over-expressing drug-selected cell lines and their parental MRP1 negative cell lines (see Table 3 2 1) were compared with the various MRP isoform transfected cell lines (previously described in section 3 1) MRP1 cDNA-transfected SF9 insect cell membranes, which express the unglycosylated form of MRP1, were also examined (see Table 3 2 2) These membranes were a kind gift from Dr Balazs Sarkadi, The Institute of Enzymology, Biological Research Center, Hungarian Academy of sciences, H-1113 Budapest, Hungary After characterisation these cell lines and membranes were then used for controls during antibody screening and characterisation of the chosen antibodies

Table 3.2.1

Drug-selected MRP1 over-expressing cell lines and their corresponding parental cell lines

PARENTAL CELL LINE (VERY LOW MRP1 EXPRESSION)	DRUG SELECTED CELL LINE (MRP1 OVER-EXPRESSION)
HL60S (Leukemia cell line)	HL60ADR
COR-L23S (lung carcinoma cell line)	COR-L23R

Table 3.2.2

MRP1 cDNA transfected insect cells

CELL LINE	TRANSFECTED WITH
Sf9 insect cells	MRP1 cDNA

Western Blot analysis of MRP1 expression with the rat monoclonal MRPr1

Western Blot analysis of MRP1 expression with the rat monoclonal MRPr1 showed that MRP1 is over-expressed as a 190kD protein in the drug selected HL60ADR and COR-L23R cell lines No MRP1 expression was observed in the parental HL60S cell line and a very faint expression was observed in the parental COR-L23S cell line (see Figures 3 2 1 and 3 2 2) The unglycosylated form of MRP1 was observed as a 150kD band in the MRP1 transfected SF9 insect membranes (see Figure 3 2 3) Strong MRP1 over-expression at 190kD was apparent in the MRP1 transfected ovarian cell line, 2008 MRP1 A very low or faint 190kD band of expression was seen in the parental, MRP2 and MRP3 transfected 2008 cell lines (see Figure 3 2 4) The faint expression of MRP1 in some sensitive and un-transfected lines is probably due to an intrinsic basal level of MRP1 present prior to drug selection or transfection

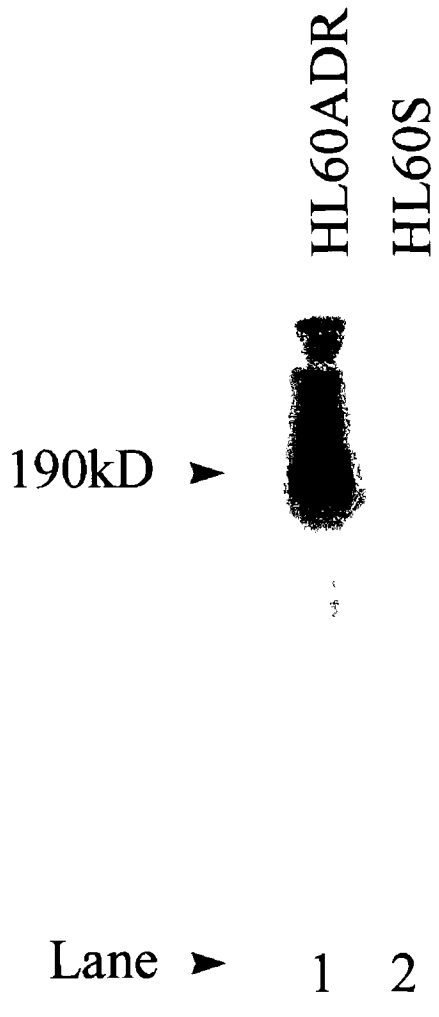


Figure 3.2.1. Detection of MRP1 with the rat monoclonal antibody, MRPr1 MRP1 is detected at 190kD in the drug-resistant HL60ADR cell line MRP1 is not detected in the drug-sensitive HL60S cell line

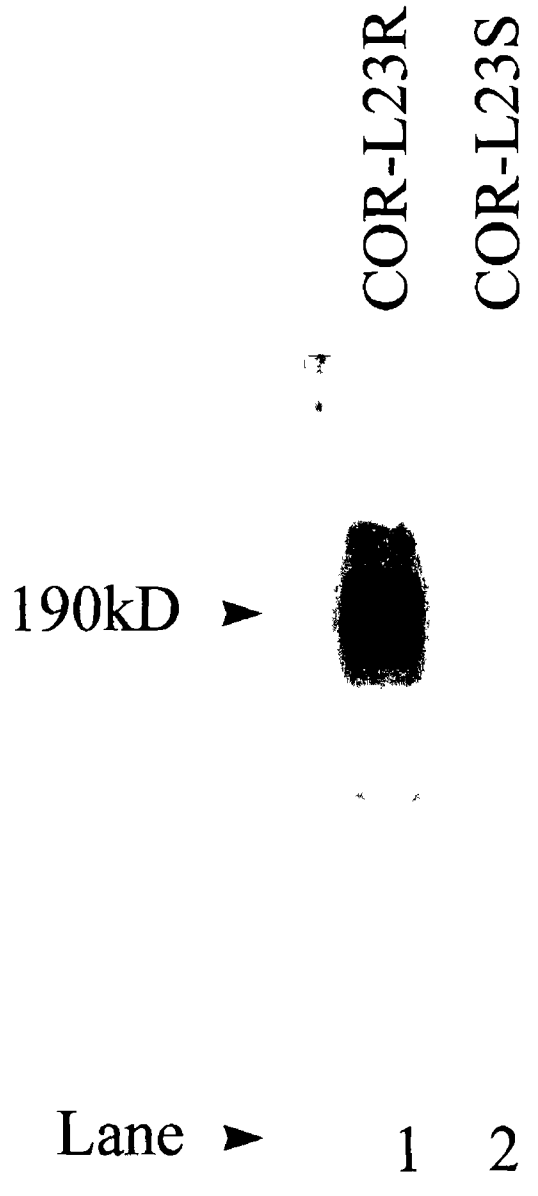


Figure 3.2.2 Detection of MRP1 with the rat monoclonal antibody, MRPr1 MRP1 is detected at 190kD in the drug-resistant COR-L23R cell line MRP1 is very weakly detected in the drug-sensitive COR-L23S cell line

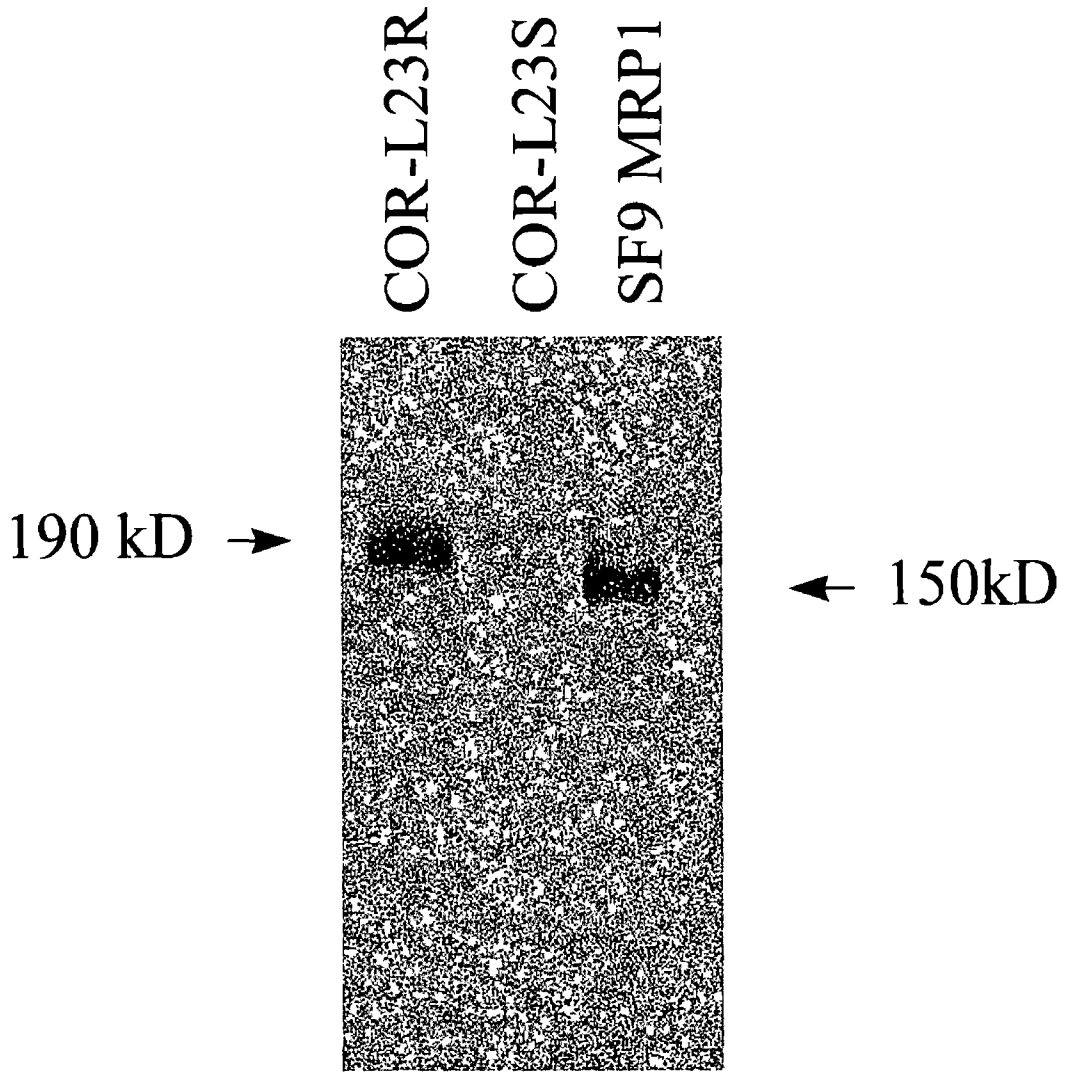
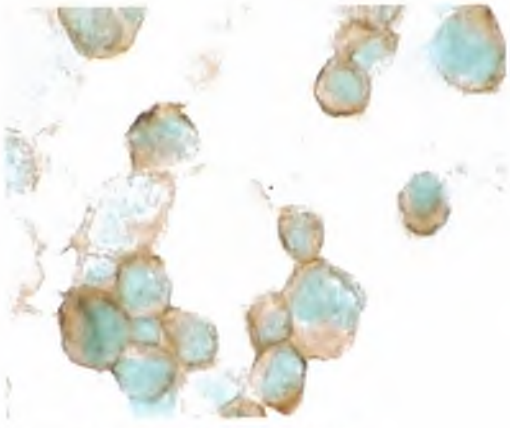


Figure 3.2.3. Detection of MRP1 with the rat monoclonal antibody, MRPr1 MRP1 is detected at 190kD in the COR-L23R cell line, faintly detected in the COR-L23S cell line and detected at 150kD in the MRP1-transfected SF9 insect membranes

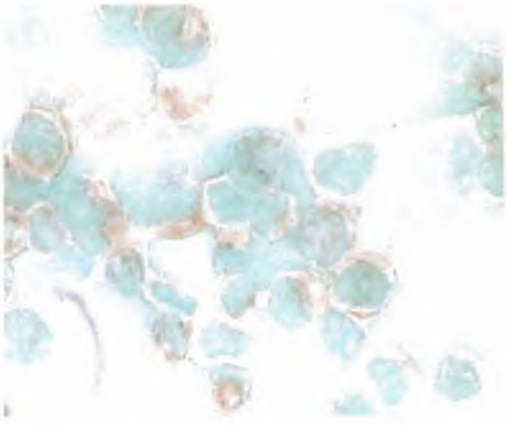
Figure 3.2.4. Detection of MRP1 with the rat MAb, MRPr1. MRP1 is detected at 190kD in the drug-resistant COR-L23R cell line and the MRP1-transfected 2008 MRP1 cell line. MRP1 is very weakly detected at 190kD in the drug-sensitive COR-L23S cell line, the parental 2008 cell line, the 2008 MRP2 and 2008 MRP3 -transfected cell lines.

Immunocytochemical analysis of MRP1 expression in various cell lines with the rat monoclonal MRPr1

Immunocytochemical analysis of MRP1 expression with the rat monoclonal MRPr1 showed that MRP1 is over-expressed in the plasma membrane of the drug-selected HL60ADR and COR-L23R cell lines Very low basal levels of MRP1 expression were observed in the parental HL60S and COR-L23S cell lines (see Figures 3 2 5 and 3 2 6) Immunocytochemical analysis of the 2008 parental cell line and MRP2 and MRP3 isoform transfectants with the rat monoclonal antibody MRPr1, showed intense staining of the plasma membrane of the 2008 MRP1 isoform transfected cell line MRPr1 detects a basal level of staining in the 2008 parental and 2008 MRP2 and MRP3 isoform transfectants (see Figure 3 2 7)



HL60ADR cells with MRPr1 MAb

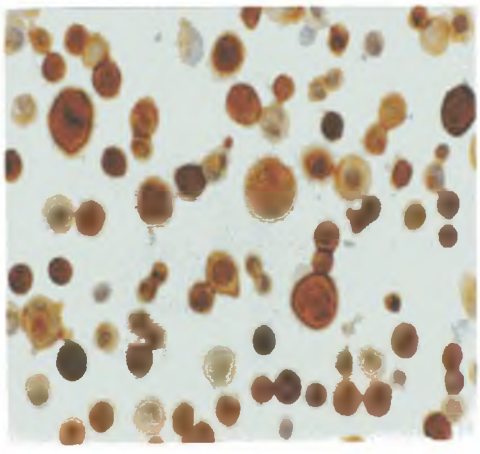


HL60S cells with MRPr1 MAb

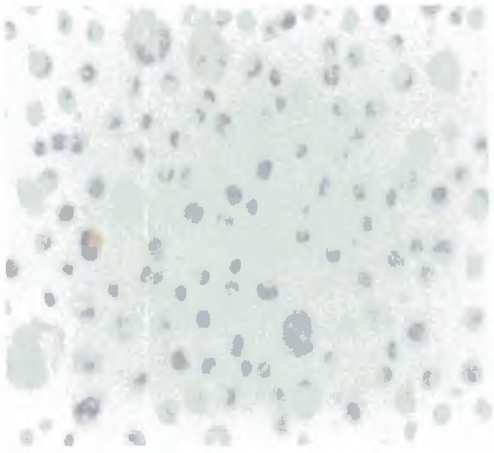
HL60ADR cells with negative control

HL60S cells with negative control

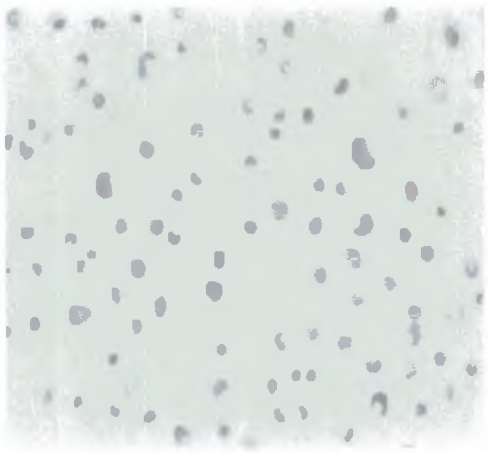
Figure 3.2.5. Immunocytochemical analysis of HL60ADR and HL60S cells with the rat monoclonal MRPr1 and negative control. Plasma membranous staining is observed in the HL60ADR cells (100x magnification).



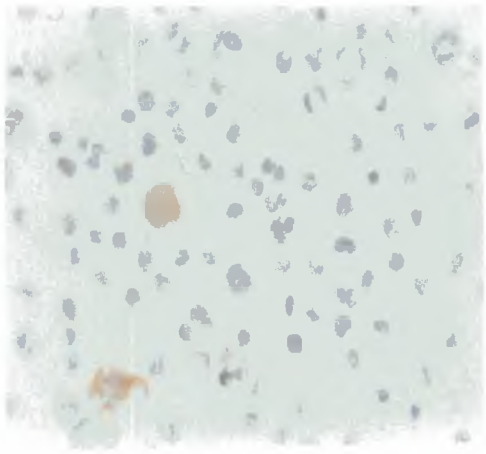
COR-L23R cells with MRPr1 MAb



COR-L23S cells with MRPr1 MAb



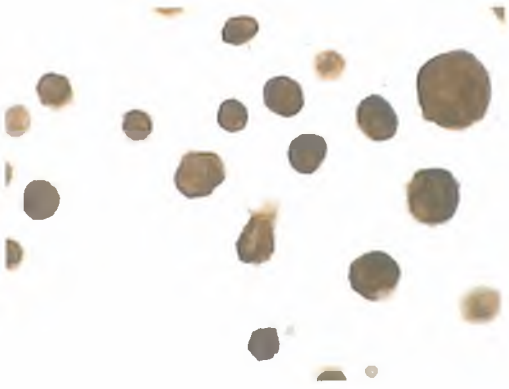
COR-L23R cells with negative control



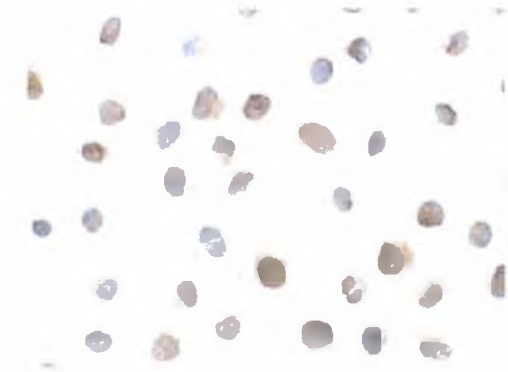
COR-L23S cells with negative control

Figure 3.2.6. Immunocytochemical analysis of COR-L23R and COR-L23S cells with the rat monoclonal antibody MRPr1 and negative control. MRPr1 shows intense plasma membranous staining in the COR-L23R cell line (100x magnification).

1. 2008 Parental.



2. 2008 MRP1



3. 2008 MRP2

4. 2008 MRP3

Figure 3.2.7. Immunocytochemical analysis of the 2008 parental cell line and MRP isoform transfectants with the rat monoclonal antibody MRPr1. MRPr1 shows intense plasma-membranous staining in the 2008 MRP1 isoform transfected cell line. MRPr1 detects a basal level of staining in the 2008 parental and 2008 MRP2 and MRP3 isoform transfectants.

Heat treatment studies on the epitope recognised by the MRPr1 antibody by western blot analysis

Heat treatment studies in the MRP1 over-expressing drug selected COR-L23R cell line and the MRP1-transfected cell line 2008 MRP1 prior to electrophoresis (Refer to section 2 4 2 for usual sample preparation prior to electrophoresis), showed that heat treatment for 3 minutes @ 100^0C destroys the MRP1 epitope recognised by the MRPr1 antibody in the drug selected COR-23R cell line but not the MRP1-transfected 2008 MRP1 cell line (see Figure 3 2 8 and 3 2 9) This result may be due to a conformational difference between the drug-selected and the transfected MRP1 protein

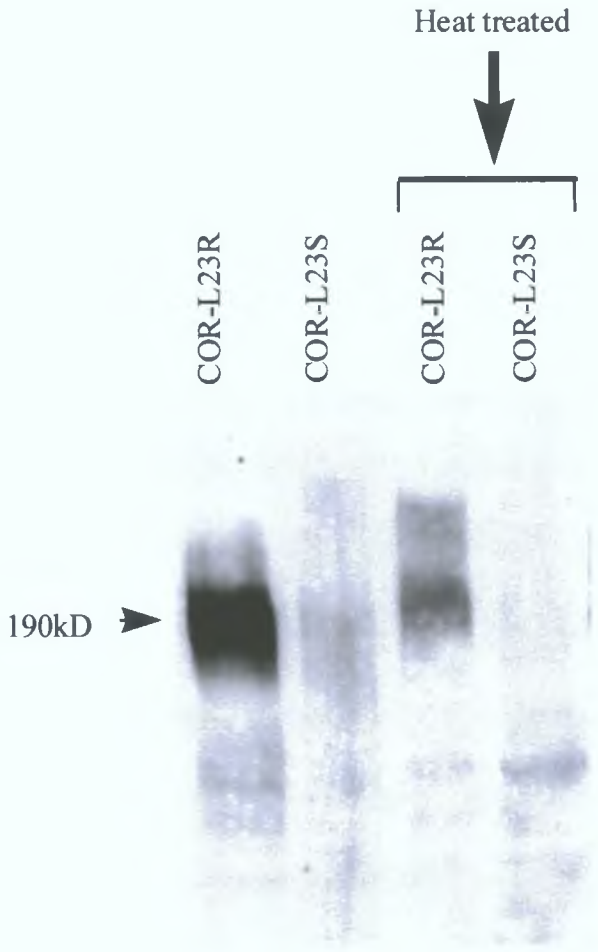


Figure 3.2.8. Western blot analysis of COR-L23R and COR-L23S whole cell lysates with MRPr1. Samples loaded onto lanes 3 and 4 were heat treated @100^{0}C for 3 minutes prior to loading. A major reduction in the 190kD band is observed in the heat treated COR-L23R whole cell lysates suggesting that the epitope recognised by MRPr1 on the MRP1 protein induced by drug selection is heat sensitive.

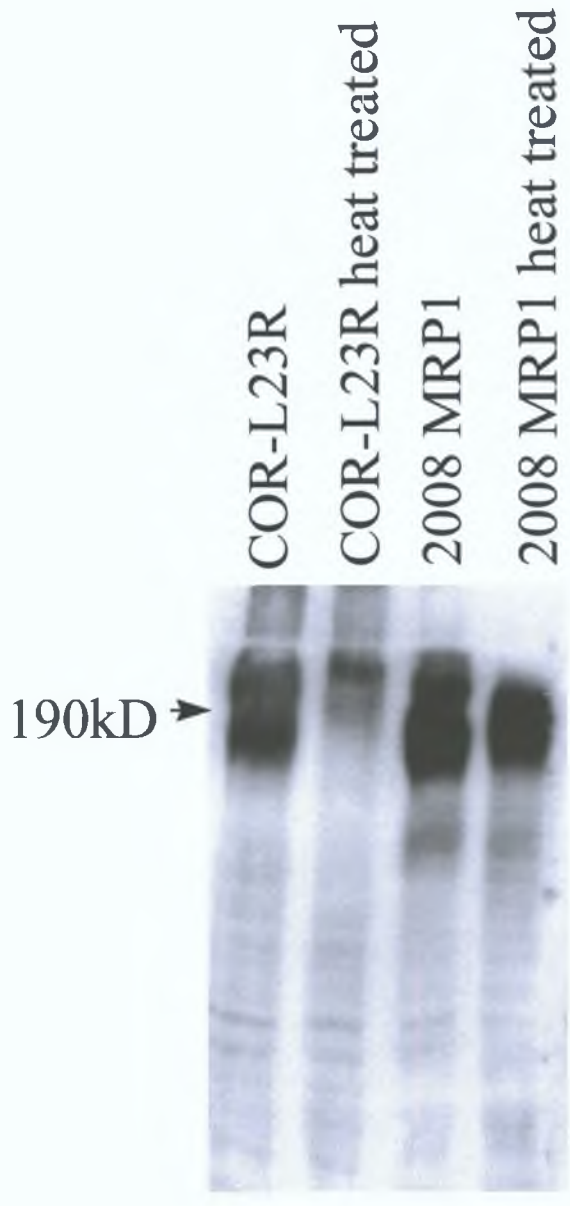


Figure 3.2.9. Heat treatment studies in the MRP1 over-expressing drug selected COR-L23R cell line and the MRP1 transfected cell line 2008 MRP1, by Western blot analysis with the rat MAb, MRPr1. Heat treatment at 100^0C for 3 minutes destroys the epitope recognised by the MRPr1 antibody in the intrinsically expressed MRP1 protein but not the MRP1 transfected 2008 MRP1 cell line. This result may be due to a conformational difference of the intrinsic and the transfected protein.

3 3 CHARACTERISATION OF THE MRP2 SPECIFIC MAbs, M2I-4 and M2III-6

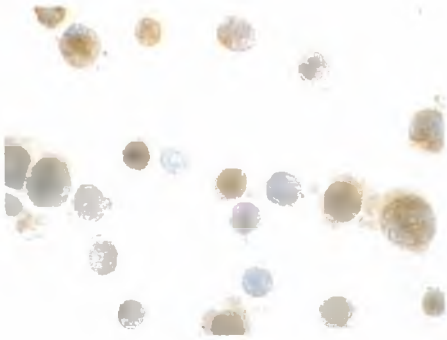
Immunocytochemical analysis

A number of cell lines, including the 2008 MRP2-transfected cell line were examined by immunocytochemical analysis with the MRP2 specific antibodies M2I-4 and M2III-6 The mouse monoclonal antibody M2I-4 weakly stained all the cell lines examined (see Figure 3 3 1) However, immunocytochemical analysis of the 2008 parental cell line and MRP isoform transfectants with the mouse monoclonal antibody M2III-6 showed that MAb M2III-6 strongly stained the 2008 MRP2 cell line only (see Figure 3 3 2) These results show that MRP2 protein is expressed in the 2008 MRP2 transfected cells While the M2I-4 is unsuitable for immunocytochemical analysis, the M2III-6 MAb is highly specific for the MRP2 protein expressed in the 2008 MRP2 transfected cells and does not cross-react with the MRP1 or MRP3 2008 transfectants or the COR-L23R MRP1 over-expressing cell line

1. COR-L23R

2. COR-L23S

3. 2008 Parental

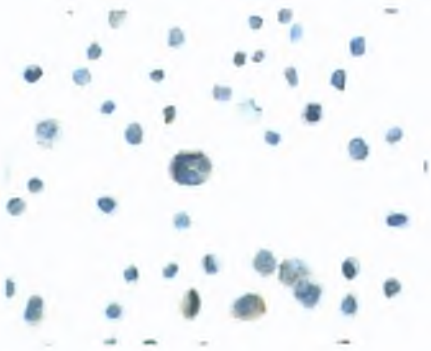


4. 2008 MRP1

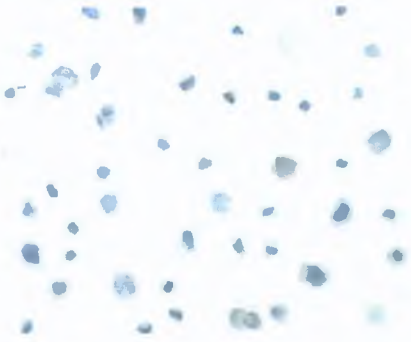
5. 2008 MRP2

6. 2008 MRP3

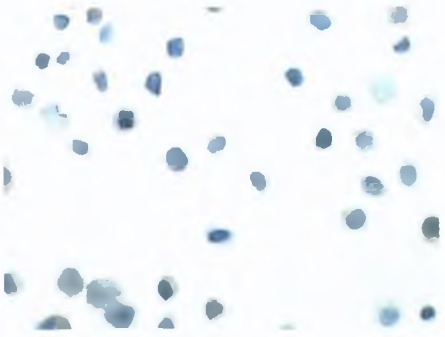
Figure 3.3.1. Immunocytochemical analysis of the 2008 parental cell line and MRP isoform transfectants with the mouse monoclonal antibody M2I-4. MAb M2I-4 stained all cell lines weakly.



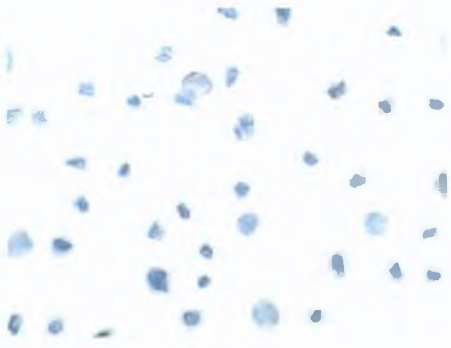
1. COR-L23R



2. COR-L23S



3. 2008 Parental



4. 2008 MRP1

5. 2008 MRP2

6. 2008 MRP3

Figure 3.3.2. Immunocytochemical analysis of the 2008 parental cell line and MRP isoform transfectants with the mouse monoclonal antibody M2III-6. MAb M2III-6 strongly stained the 2008 MRP2 cell line.

Western blot analysis

A number of cell lines, including the 2008 MRP2-transfected cell line were examined by Western blot analysis with the MRP2 specific antibody M2III-6 The M2III-6 MAb was used because of the specific results achieved with this MAb in the MRP2 immunocytochemical study (Figure 3 3 2) Analysis of the 2008 parental cell line and MRP isoform transfectants with the M2III-6 MAb showed that it strongly reacted with a 190kD band in the 2008 MRP2 cell line (see Figure 3 3 3) These results show that MRP2 protein is expressed in the 2008 MRP2 transfected cells and that MRP2 expression can be detected with the M2III-6 MAb by Western blot analysis The M2III-6 MAb is highly specific for the MRP2 protein and does not cross-react with the MRP1 or MRP3 2008 transfectants or the COR-L23R MRP1 over-expressing cell lme

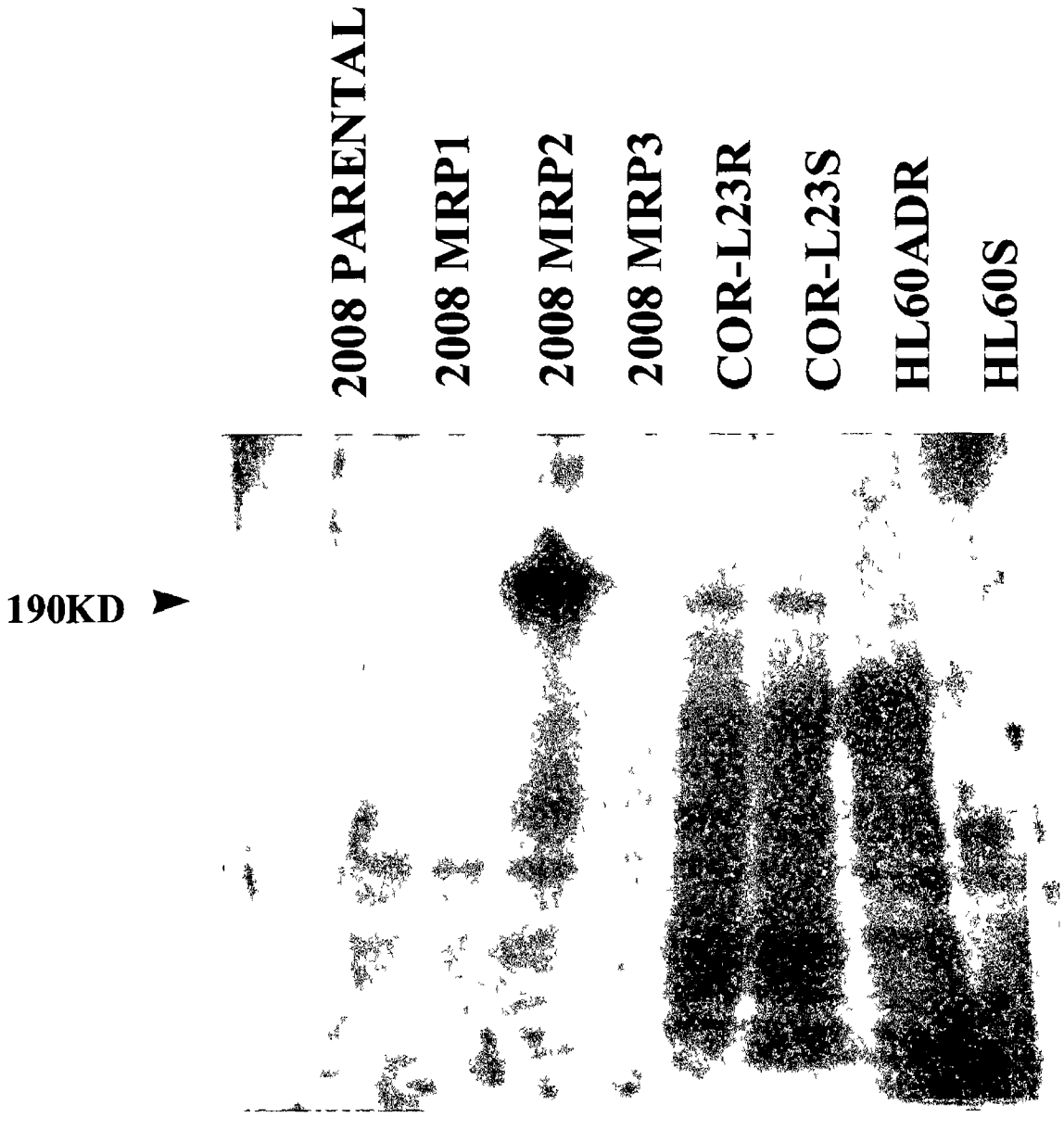
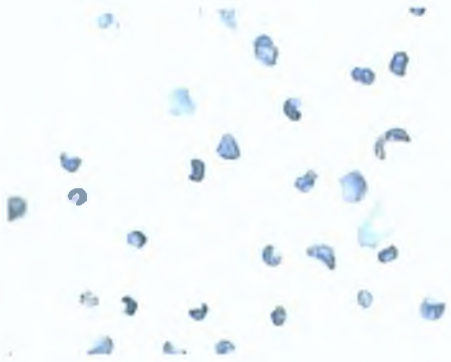


Figure 3.3.3. Detection of MRP2 by Western blot analysis with the MAb, M2III-6 MRP2 is detected at 190kD in the MRP2-transfected cell line, 2008 MRP2 MRP2 is not expressed in the 2008 parental, MRP1-transfectant, MRP3-transfectant or the COR-L23R, COR-L23S, HL60ADR and HL60S cell lines

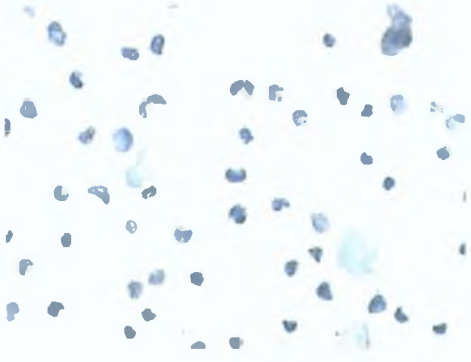
3 4 CHARACTERISATION OF THE MRP3 SPECIFIC MAbs, M311-21 and M3II-9

Immunocytochemical analysis

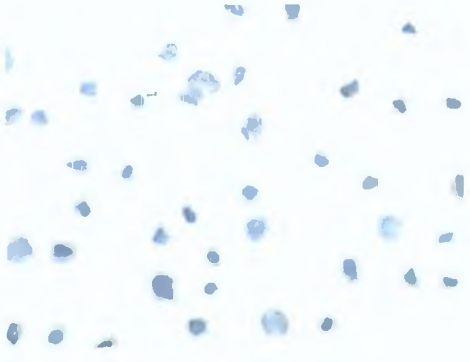
A number of cell lines, including the 2008 MRP3 transfected cell line were examined by immunocytochemical analysis with the MRP3 specific M311-21 and M3II-9 The mouse monoclonal antibody M311-21 showed granular plasma-membranous staining in a sub-population of the 2008 MRP3 cell line (see Figure 3 4 1) The mouse monoclonal antibody M311-9 also showed granular plasma-membranous staining in a sub-population of the 2008 MRP3 cell line (see Figure 3 4 2) These results show that MRP3 protein is expressed in a subpopulation of the 2008 MRP3 transfected cells The MRP3 protein was not found to be expressed in any of the other cell lines exammed Both of these MRP3 MAbs were found to be suitable for immunocytochemical analysis and did not cross-react with the MRP1 or MRP3 2008 transfectants or the COR-L23R MRP1 over-expressing cell line, indicating that they are MRP3 specific



1. COR-L23R

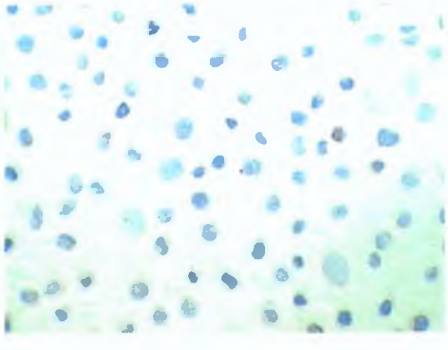


2. COR-L23S

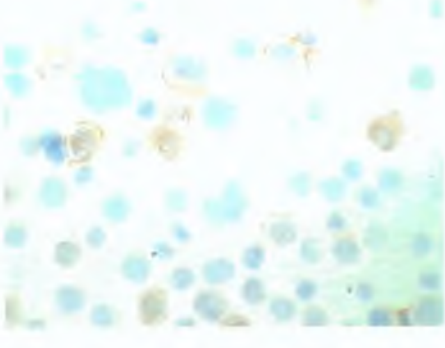


3. 2008 Parental

4. 2008 MRP1

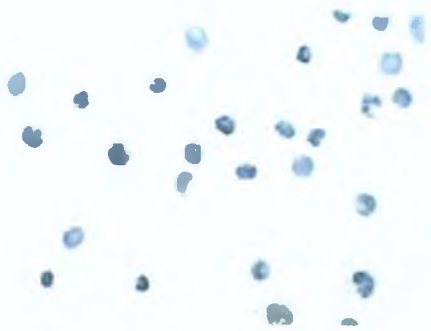


5. 2008 MRP2

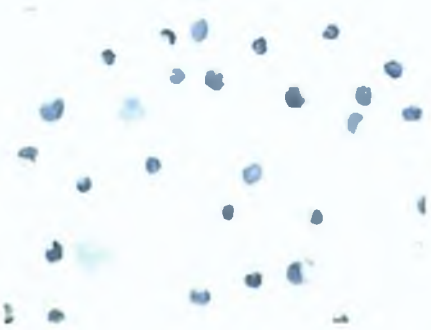


6. 2008 MRP3

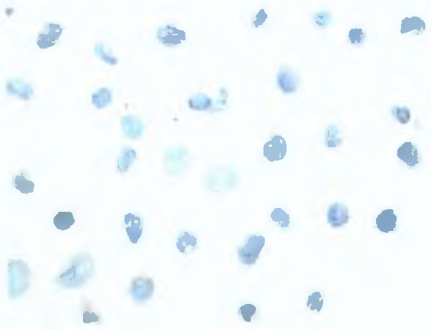
Figure 3.4.1. Immunocytochemical analysis of the 2008 parental cell line and MRP isoform transfectants with the monoclonal antibody M3II-21. MAb M3II-21 showed granular plasma-membranous staining in a sub-population of the 2008 MRP3 cell line.



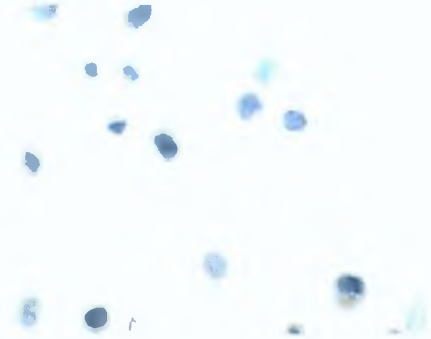
1. COR-L23R



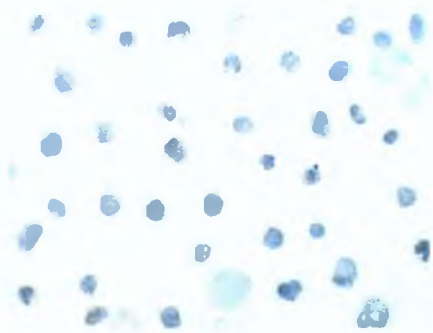
2. COR-L23S



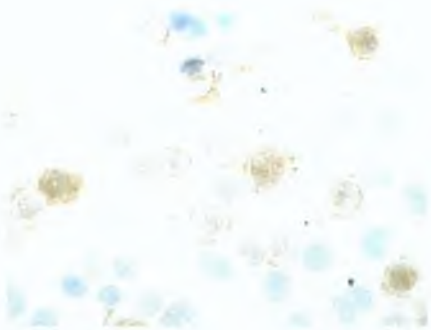
3. 2008 Parental



4. 2008 MRP1



5. 2008 MRP2



6. 2008 MRP3

Figure 3.4.2. Immunocytochemical analysis of the 2008 parental cell line and MRP isoform transfectants with the monoclonal antibody M3II-9. MAb M3II-9 showed granular plasma-membranous staining in a sub-population of the 2008 MRP3 cell line.

Western blot analysis

A number of cell lines, including the 2008 MRP3 transfected cell line were examined by Western blot analysis with the MRP3 specific antibody M3II-21 The M3II-21 MAb was used because of its characterisation in publications (Kool *et al*, 1999a) Analysis of the 2008 parental cell line and MRP isoform transfectants with the M3II-21 MAb showed that it strongly reacted with a 190kD band in the 2008 MRP3 cell line (see Figure 3 4 3) These results show that MRP3 protein is expressed in the 2008 MRP3 transfected cells and that MRP3 expression can be detected with the M3II-21 MAb by Western blot analysis The M3II-21 MAb is highly specific for the MRP3 protein and does not cross-react with the MRP1 or MRP2 2008 transfectants or the COR-L23R MRP1 over-expressing cell line

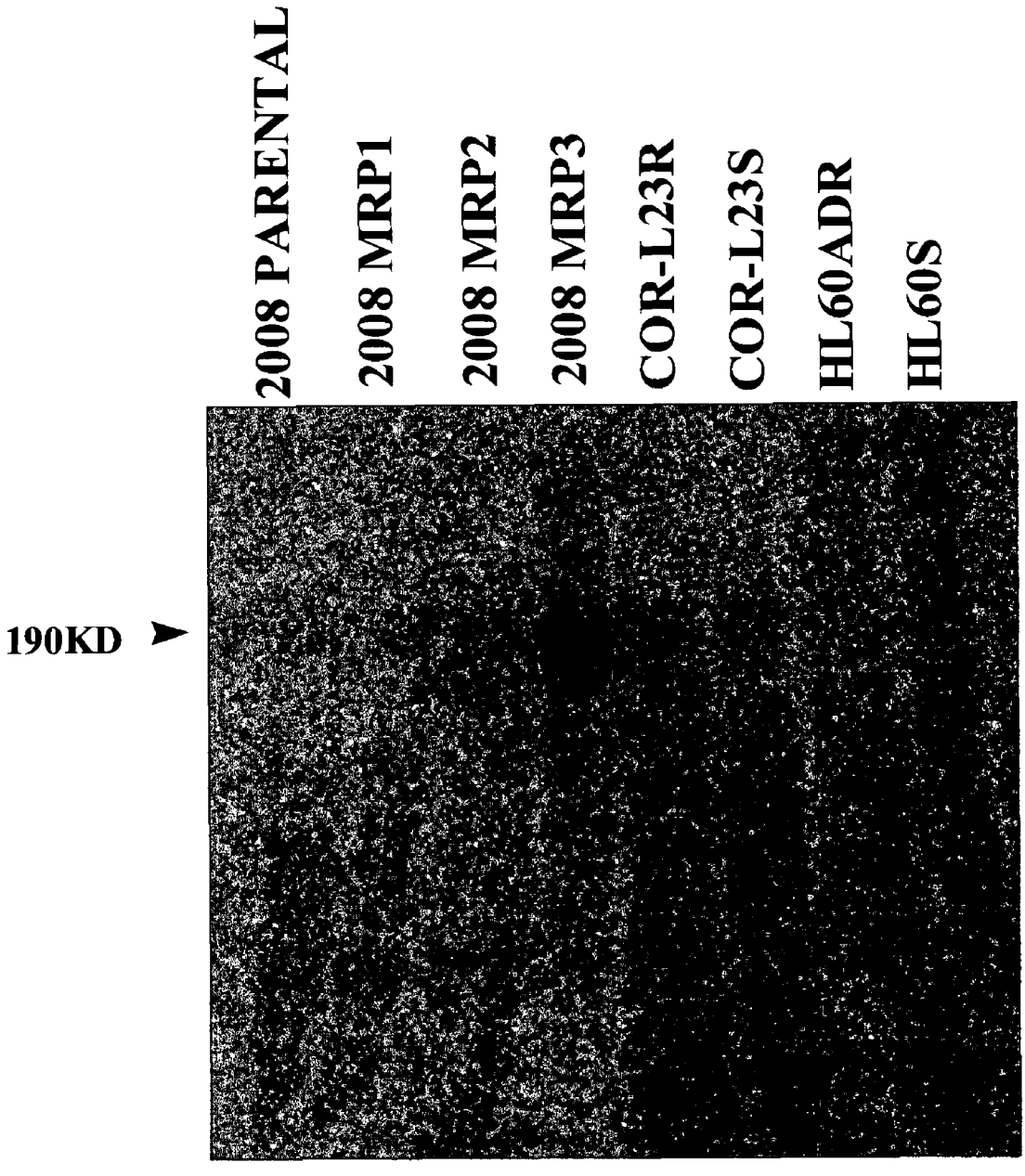


Figure 3.4.3. Detection of MRP3 by Western blot analysis with the MAb, M3II-21 MRP3 is detected at 190KD in the MRP3 transfected cell line, 2008 MRP3 MRP3 is not detected in the 2008 parental, MRP1-transfectant, MRP2-transfectant or the COR-L23R, COR-L23S, HL60ADR and HL60S cell lines

3 5 IMMUNOGENS

3 5 1 Native protein

Native protein can be a valuable source for the production of antibodies which are required to recognise the native protein The most obvious source of native protein is the protein expressing cell itself The COR-L23R MRP1 over-expressing cells are a rich source of the native MRP1 protein Whole cell lysate preparations or cell membranes partially purified by centrifugation techniques may be used as immunogens The COR-L23R cells were used as sources for whole cell lysates and cell membranes for immunisation

3 5 2 Synthetic peptides and Fusion proteins

Short synthetic peptides or Fusion proteins expressed in vector systems can be produced for use as immunogens These proteins are linear and may not possess the original conformation of the native protein However, a multitude of techniques can help to predict linear regions on the native protein Therefore, these proteins are another valuable source of immunogen in certain cases The 3 short peptides used in this thesis were chosen from the predicted model proposed by Bakos *et al*, 1996 (see Figure 1 2 1 1) The locations of each of these 3 peptides on the MRP1 protein are shown in Diagram 3 5 1 Peptide 1 (amino acids 1520 - 1531) is situated intracellularly in the C-terminus Peptide 2 (amino acids 235-246) and peptide 3 (amino acids 246-260) are both situated in the cytoplasmic loop separating transmembrane domain 0 (TM0) and transmembrane domain 1 (TM1) in the N-terminus

The locations of the two Fusion Proteins used in this thesis are shown in Diagram 3 5 2 Fusion Protein 1 (amino acids 1294-1531) is situated intracellularly in the C-terminus of MRP1 Fusion Protein 2 (amino acids 647-912) is situated intracellularly in the N-terminus of MRP1

MRPr1, the MRP1-specific rat MAb was produced by immunising with a Fusion Protein (Flens *et al*, 1996) Diagram 3 5 3 shows the location of the Fusion Protein used in the of production MRPr1 and the mapped epitope it recognises The fusion protein incorporates ammo acids 192-360 of human MRP1 This region is mainly situated in the large cytoplasmic loop separating transmembrane domain 0 (TM0) and transmembrane domain 1 (TM1) in the N-terminus (see Diagram 3 5 4 , Region 1) The epitope recognised by MRPr1 has been mapped to amino acids 238 to 247 This epitope is similar to the region encompassed by one of the short synthetic peptides, Peptide 2, used in this thesis as an immunogen (see Diagram 3 5 1)

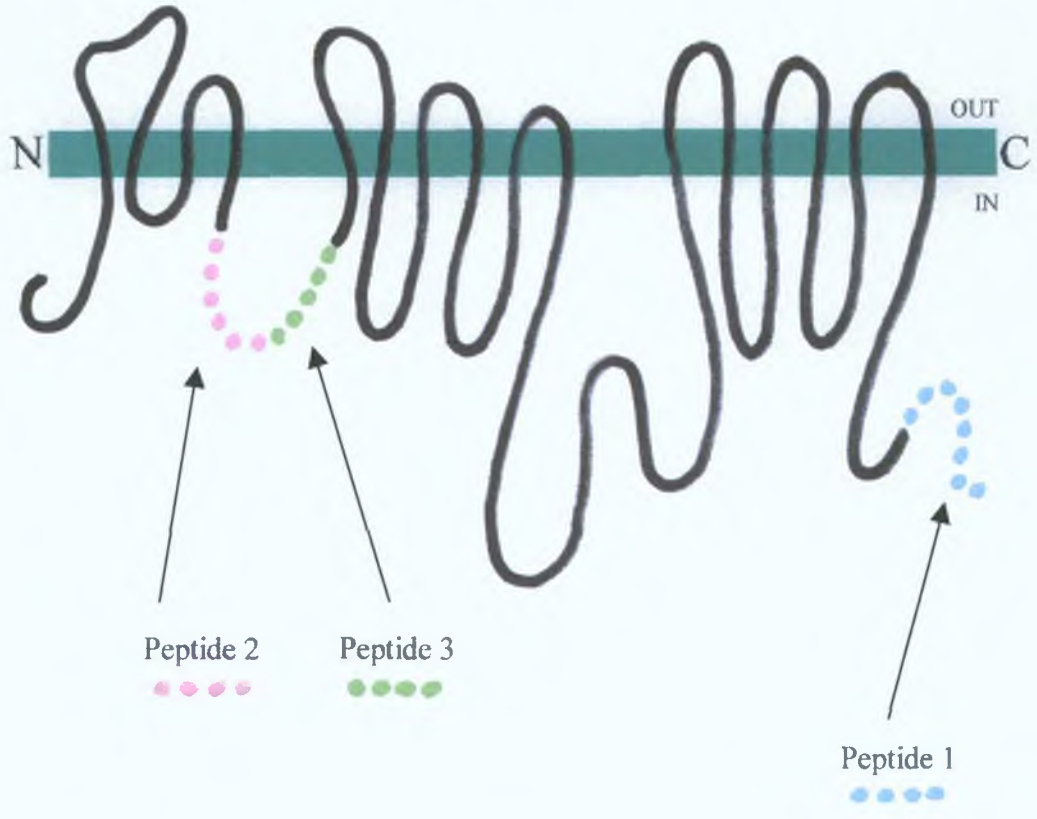


Diagram 3.5.1. Presumed location of immunisation Peptides 1, 2 and 3 based on the model of Bakos *et al.*, 1996. Peptide 1 (amino acids 1520 - 1531) is situated intracellularly in the C-terminus. Peptide 2 (amino acids 235-246) and peptide 3 (amino acids 246-260) are both situated in the cytoplasmic loop separating transmembrane domain 0 (TM0) and transmembrane domain 1 (TM1) in the N-terminus.

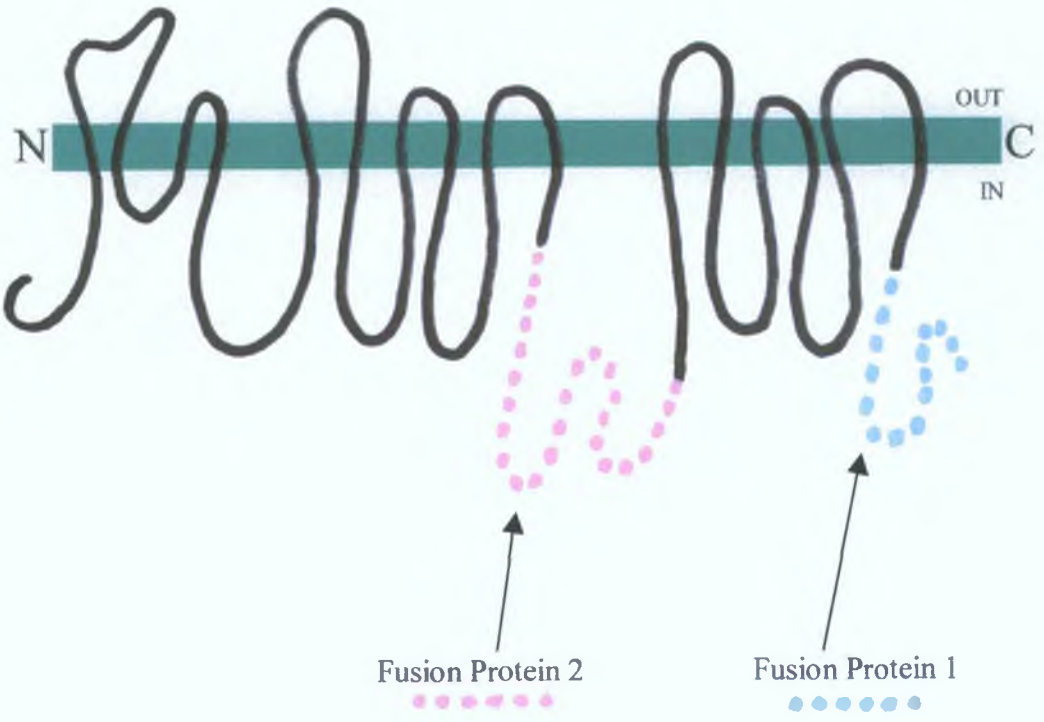


Diagram 3.5.2. Presumed location of fusion Proteins 1 and 2 based on the model of Bakos *et al.*, 1996. Fusion Protein 1 (amino acids 1294-1531) is situated intracellularly in the C-terminus of MRP1. Fusion Protein 2 (amino acids 647-912) is situated intracellularly towards the N-terminus of MRP1.

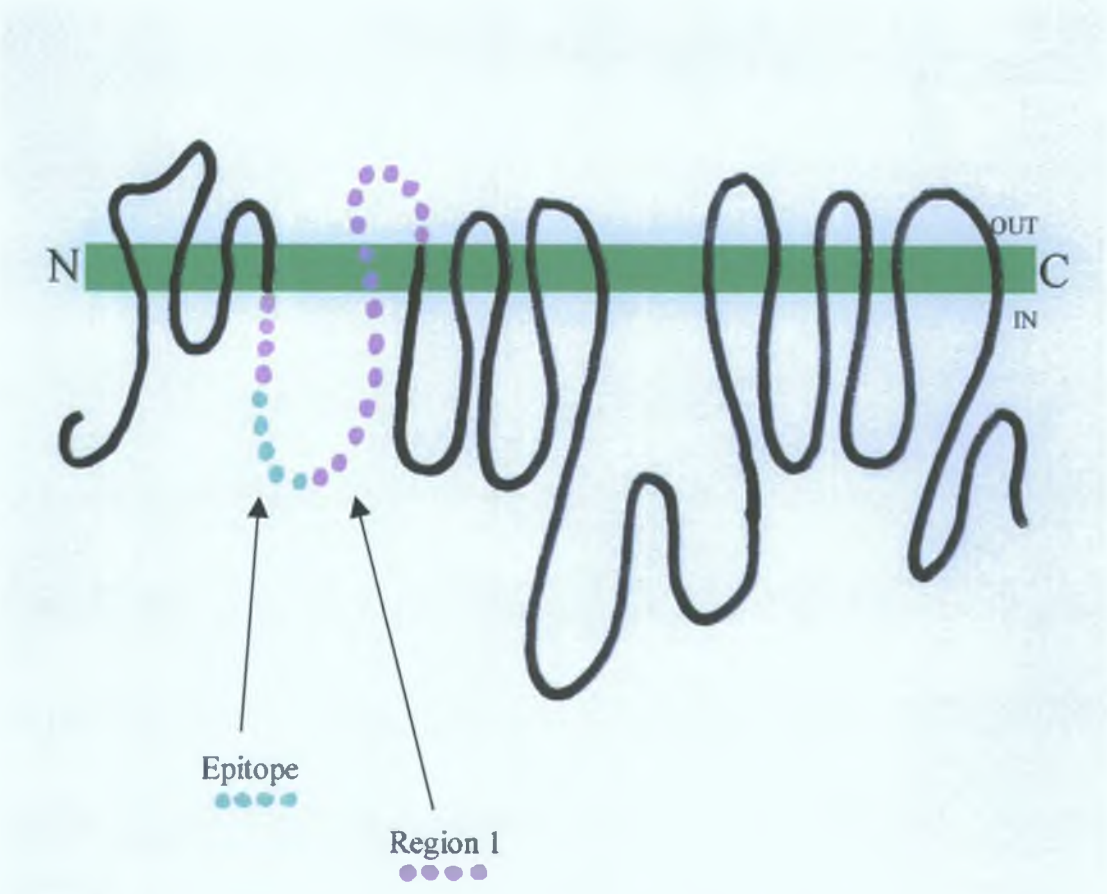


Diagram 3.5.3. Presumed location of the Fusion Protein (including amino acids 192-360 of human MRP1) used to produce the rat monoclonal antibody, MRPr1, based on the model of Bakos *et al.*, 1996. This region is mainly situated in the large cytoplasmic loop separating transmembrane domain 0 (TM0) and transmembrane domain 1 (TM1) in the N-terminus (see Region 1). The epitope recognised by MRPr1 has been mapped to amino acids 238 to 247.

3 6 POLYCLONAL ANTIBODY RESULTS

Immunisations

In order to generate polyclonal antibodies for the detection of MRP1, New Zealand White Rabbits underwent an immunisation regime with short synthetic peptides (see Diagram 3 5 1) taken from the deduced amino acid sequence of human MRP1 The resulting polyclonals were designated Polyclonal 1, Polyclonal 2 and Polyclonal 3

Screening of Polyclonal Serum

Serum collected from test bleeds after each immunisation were tested primarily by Western blotting on HL60ADR and HL60S whole cell lysates Further characterisation of each polyclonal was carried out on COR-L23R and COR-L23S cells and SF9 insect membranes expressing various fragments of MRP1 and controls (see Table 3 6 1)

Western blotting results

All three polyclonals were found to be suitable for Western blot analysis and reacted strongly with a 190kD band in the MRP1 over-expressing, drug-selected cell line, HL60ADR, and weakly or not at all with the parental HL60S cell line (see Figures 3 6 1 , 3 6 3 and 3 6 5) These results strongly suggested that the polyclonals were reacting with MRP1

Polyclonal 1, 2 and 3 detected a 150kD band in human MRP1-expressing SF9 insect membranes and a shift of the 190kD band to a 150kD band in tunicamycin treated HL60ADR cells None of the polyclonals reacted with WT-MDR expressing SF9 insect membranes All three polyclonals also reacted with a 140kD band in SF9 insect

membranes expressing DeltaMRP1 (Δ1), containing the MRPr1 rat antibody epitope (amino acids 192 - 360), while only polyclonal 1 detected a 120kD band in SF9 insect membranes expressing DeltaMRP2 (Δ2), which does not contain the MRPr1 epitope (see Figures 3 6 2 , 3 6 4 and 3 6 6)

Table 3 6 1 Explanation of various fragments used in the further characterisation of the MRP1 Polyclonal antibodies Carried out by Emese Sinkoe from The Institute of Enzymology, Biological Research Center, Hungarian Academy of sciences, H-1113 Budapest, Hungary

Various MRP1 controls	Description
CONTROL	Human HL60S cell membranes expressing no MRP1
MRP	Human HL60ADR cell membranes expressing fully glycosylated MRP1 at 190kD
MRP + TUNICAMYCIN	Human HL60ADR cells treated with tunicamycin, to block glycosylation, showing a shift in expression from 190kD to 150KD
WT-MDR	WT-MDR expressed in SF9 insect membranes as a negative control
hMRP	Unglycosylated human MRP1 expressed in SF9 insect membranes at 150kD (insect cells are unable to carry out glycosylation)
(TMO)	Region in the N-terminus of hMRP1 expressed in SF9 insect membranes (unglycosylated) at 30kD
Δ1	Delta MRP1 (full length MRP1, containing the MRPr1 fusion protein region) and expressed in SF9 insect membranes at 140kD
Δ2	Delta MRP2 (full length MRP1, with the MRPr1 fusion protein region deleted) and expressed in SF9 insect membranes at 120kD

One New Zealand White rabbit was immunised with Peptide 1 and is summarised in Table 3.6.2.

POLYCLONAL 1.

- AA 1520 - 1531 (Internal peptide / C-terminus).
- Sequence : CALFYSMAKDAGLV + KLH

IMMUNISATION REGIME : *IN VIVO* (NEW ZEALAND WHITE RABBITS).
SCREENING SYSTEM : WESTERN BLOTTING ON HL60ADR AND HL60S CELLS.
RESULTS : IDENTIFIES 190kD ON RESISTANT , MRP POSITIVE HL60ADR CELLS WITH NO STAINING ON SENSITIVE CELLS, HL60S (see Figure 3.6.1.).

TABLE 3.6.2.

Primary screening.

Primary screening of Polyclonal 1 by Western blot analysis on HL60ADR and HL60S cell lysates identified a 190kD band in HL60ADR cells but not in HL60S cells (see Figure 3.6.1.).

Characterisation.

Further screening of Polyclonal 1, raised to the C-terminus of MRP1, was carried out by Western Blot analysis using various MRP1 fragments and controls (see Figure 3.6.2.). As expected, Polyclonal 1 did not react with (TMO), HL60S cell lysates or WT-MDR. Polyclonal 1 detected MRP1 at 190kD in HL60ADR cell membranes, at 150kD in

tunicamycin treated cells and human MRP1 expressed in SF9 insect cells and at 140kD and 120kD respectively in Δ1 and Δ2 MRP1/2 which both express the C-terminus of MRP1

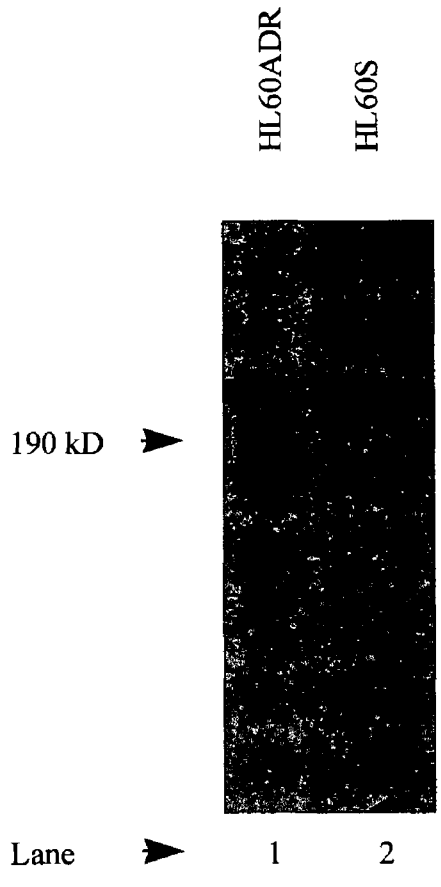


FIGURE 3.6.1. Western blot analysis of HL60ADR and HL60S cells with Polyclonal 1 Polyclonal 1 recognises a 190kD band in the MRP1 over-expressing cell line, HL6OADR but not the parental drug-sensitive HL60S cell line

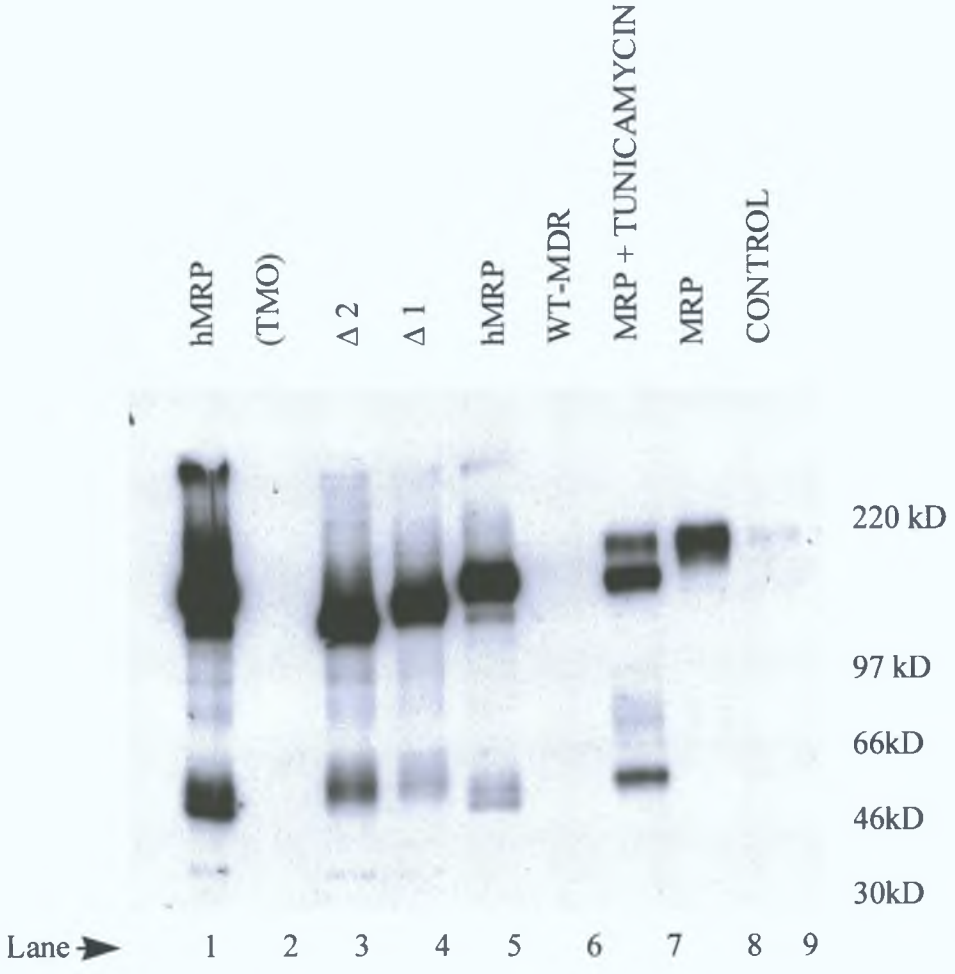


FIGURE 3.6.2. Western blot analysis of various MRP1 fragments and controls (see Table 3.6.1. for description of controls) with Polyclonal 1. As expected, Polyclonal 1 does not react with (TMO), CONTROL or WT-MDR because these controls do not contain any MRP1. Polyclonal 1 detects a 190kD band in MRP (fully glycosylated, full length MRP1), a 150kD band in MRP + TUNICAMYCIN and hMRP1 (unglycosylated form expressed at 150kD), and at 140kD and 120kD respectively in Δ1 and Δ2 (unglycosylated full length MRP1, resulting in lower molecular weight detection. The MRPr1 fusion protein region is deleted in Δ2.). All glycosylated MRP1 fragments were expressed in human HL60ADR cells (HL60) and all unglycosylated fragments were expressed in SF9 insect membranes (SF9) which are unable to undergo glycosylation.

One New Zealand White rabbit was immunised with Peptide 2 and is summarised in Table 3.6.3.

POLYCLONAL 2.

- AA 235 - 246 (Internal peptide (originally thought to be external) / N-terminus).
- Sequence : PLEGSDLWSLNK + BSA

IMMUNIZATION REGIME : *IN VIVO* (NEW ZEALAND WHITE RABBITS).
SCREENING SYSTEM : WESTERN BLOTTING ON HL60ADR AND HL60S CELLS.
RESULTS : IDENTIFIES 190kD ON RESISTANT , MRP POSITIVE HL60ADR CELLS WITH NO STAINING ON SENSITIVE CELLS, HL60S (see Figure 3.6.3.).

Table 3.6.3.

Primary screening.

Primary screening of Polyclonal 2 by Western blot analysis on HL60ADR and HL60S cell lysates identified a 190kD band in HL60ADR cells but not in HL60S cells (see Figure 3.6.3.).

Characterisation.

Further screening of Polyclonal 2, raised to a region in the N-terminus of MRP1, was carried out by Western blot analysis using various MRP1 fragments and controls (see Figure 3.6.4.). Polyclonal 2 did not detect any protein in HL60S cell lysates, WT-MDR or Δ2 MRP2 (MRPr1 fusion protein region deleted). As expected, Polyclonal 2 detected a 30kD band in (TMO), a 190kD band in HL60ADR cell membranes, a 150kD band in tunicamycin treated cells and human MRP1 expressed in SF9 insect cells and a 140kD band in Δ1 MRP1 which does express the MRPr1 fusion protein region.

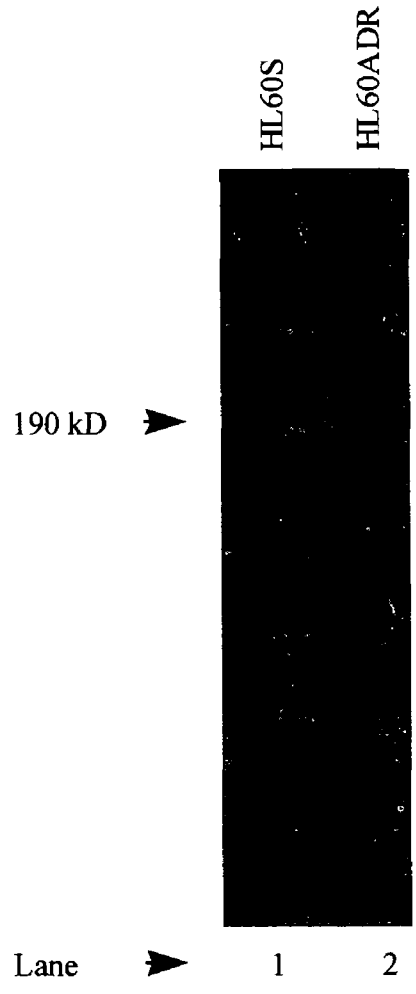


FIGURE 3.6.3. Western blot analysis of HL60ADR and HL60S cells with Polyclonal 2 Polyclonal 2 recognises a 190kD band in the MRP1 over-expressing cell line, HL6OADR but not the parental drug-sensitive HL60S cell line

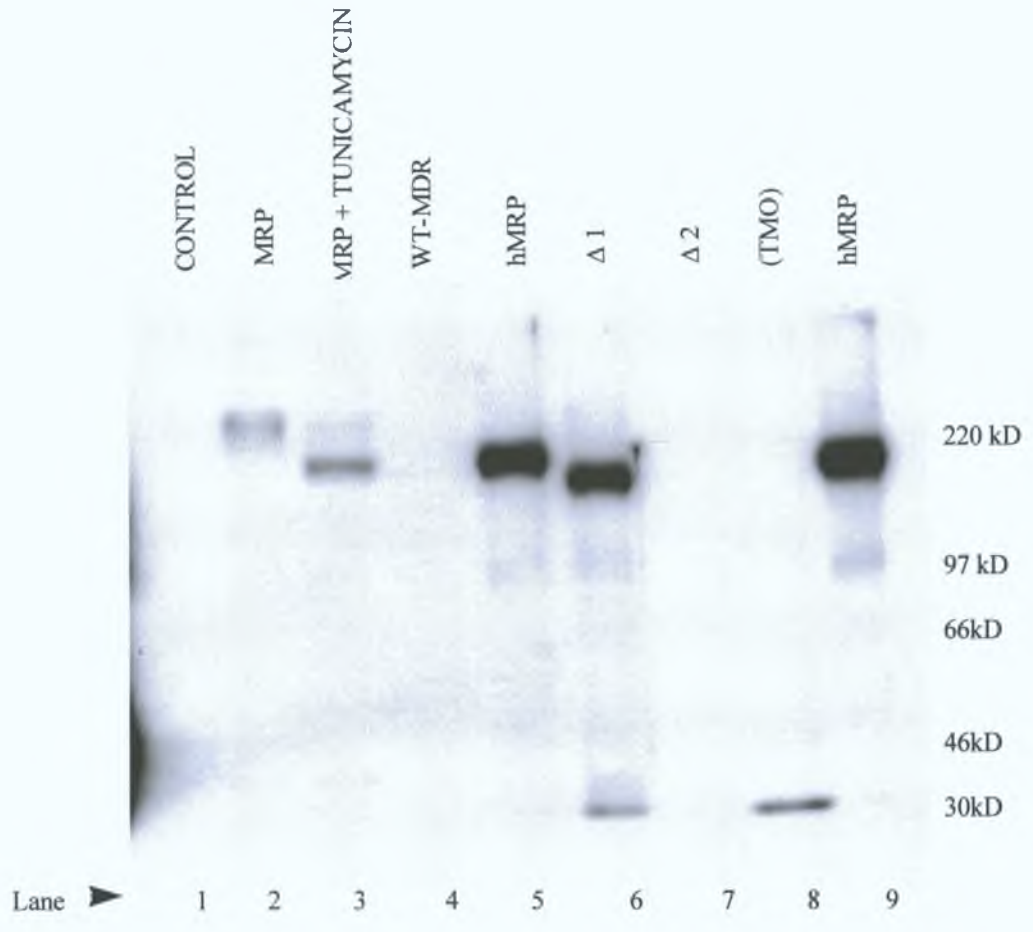


FIGURE 3.6.4. Western blot analysis of various MRP1 fragments and controls (see Table 3.6.1. for description of controls) with Polyclonal 2. As expected, Polyclonal 2 does not detect any protein in CONTROL, WT-MDR or Δ2 (MRPr1 fusion protein region deleted. This deleted region contains the amino acids to which Polyclonal 2 was raised). As expected, Polyclonal 2 detected a 30kD band in (TMO) which contains the region to which Polyclonal 2 was raised in the N terminus. Polyclonal 2 also detected a 190kD band in MRP (fully glycosylated, full length MRP1), a 150kD band in MRP + TUNICAMYCIN and hMRP1 (unglycosylated form expressed at 150kD), and a 140kD band in Δ1 (unglycosylated full length MRP1, resulting in lower molecular weight detection). All glycosylated MRP1 fragments were expressed in human HL60ADR cells (HL60) and all unglycosylated fragments were expressed in SF9 insect membranes (SF9) which are unable to undergo glycosylation.

One New Zealand White rabbit was immunised with Peptide 3 and is summarised in Table 3.6.4.

POLYCLONAL 3.

- AA 246 - 260 (Internal peptide / N-terminus)
- Sequence : KEDTSEQVVPVLVKN + KLH

IMMUNIZATION REGIME : *IN VIVO* (NEW ZEALAND WHITE RABBITS).
SCREENING SYSTEM : WESTERN BLOTTING ON HL60ADR AND HL60S CELLS.
RESULTS : IDENTIFIES 190kD ON RESISTANT , MRP POSITIVE HL60ADR CELLS WITH REDUCED STAINING ON SENSITIVE CELLS, HL60S (see Figure 3.6.5.).

Table 3.6.4.

Primary screening.

Primary screening of Polyclonal 3 by Western blot analysis on HL60ADR and HL60S cell lysates identified a 190kD band in HL60ADR cells with reduced staining in HL60S cells (see Figure 3.6.5.).

Characterisation.

Further screening of Polyclonal 3, raised to the N-terminus of MRP1, was carried out by Western blot analysis using various MRP1 fragments and controls (see Figure 3.6.6.). Polyclonal 3 did not detect any protein in HL60S cell lysates, WT-MDR or Δ2 MRP2 (MRPr1 fusion protein region deleted). As expected, Polyclonal 3 detected a 30kD band in (TMO), a 190kD band in HL60ADR cell membranes, a 150kD band in tunicamycin treated cells and human MRP1 expressed in SF9 insect cells and a 140kD band in Δ1 MRP1 which does express the MRPr1 fusion protein region.

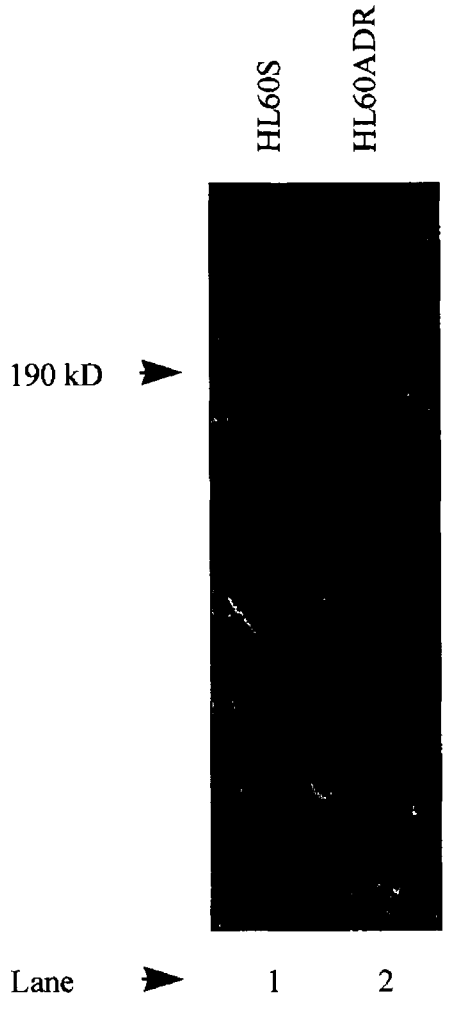


FIGURE 3.6.5. Western blot analysis of HL60S and HL60ADR cells with Polyclonal 3 Polyclonal 3 recognises a 190kD band in the MRP1 over-expressing cell line, HL6OADR Polyclonal 3 shows reduced staining but a high background in the parental drug-sensitive HL60S cell line

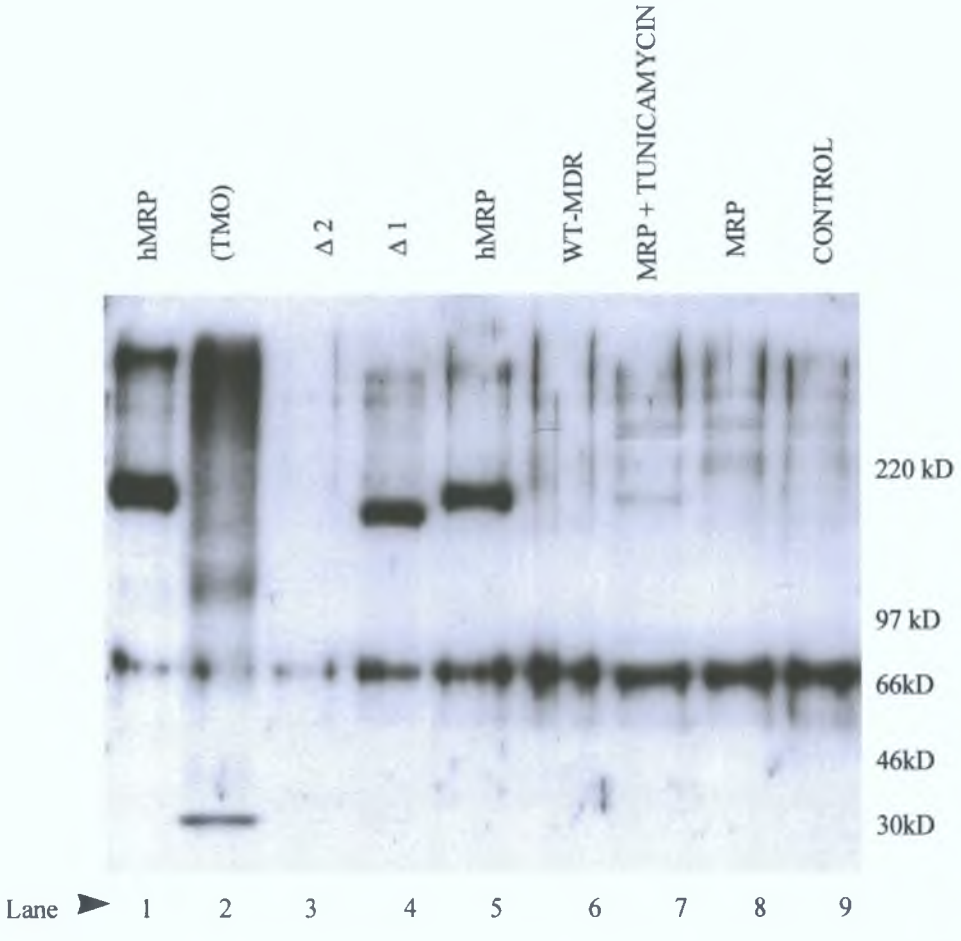


FIGURE 3.6.6. Western blot analysis of various MRP1 fragments and controls (see Table 3.6.1. for description of controls) with Polyclonal 3. As expected, Polyclonal 3 does not detect any protein in CONTROL, WT-MDR or Δ2 (MRPr1 fusion protein region deleted. This deleted region contains the amino acids to which Polyclonal 3 was raised). As expected, Polyclonal 3 detected a 30kD band in (TMO) which contains the region to which Polyclonal 3 was raised in the N terminus, a 190kD band in MRP (fully glycosylated, full length MRP1), a 150kD band in MRP + TUNICAMYCIN and hMRP1 (unglycosylated form expressed at 150kD), and a 140kD band in Δ1 (unglycosylated full length MRP1, resulting in lower molecular weight detection). All glycosylated MRP1 fragments were expressed in human HL60ADR cells (HL60) and all unglycosylated fragments were expressed in SF9 insect membranes (SF9) which are unable to undergo glycosylation.

3 7 MOUSE MONOCLONAL ANTIBODY PRODUCTION RESULTS

Immunisations and fusions

In order to generate monoclonal antibodies for the detection of MRP1, BALB/c mice underwent a regime of combined *in-vivo / in-vitro* or purely *in-vivo* immunisations The immunogens used included, short synthetic peptides (see Diagram 3 5 1) or fusion proteins (see Diagram 3 5 2) taken from the deduced amino acid sequence of human MRP1, and whole cell lysates or cell membrane preparations of the MRP1 over-expressing COR-L23R cell line Fusion rates for all the immunisations were approximately 95%

Screening of hybridomas

Screening of confluent supernatants was carried out by ELISA, against the relevant peptide or by Western blot analysis, against whole cell lysates or cell membrane preparations of the MRP1 over-expressing COR-L23R cell line All antibodies chosen by the ELISA screening method identified MAbs which reacted with the linear peptide by ELISA but failed to identify the native protein on Western blots Antibodies chosen by the Western blot screening method identified MAbs which detected a 190kD band in various cell lines, including cell lines which do not over-express MRP1, by Western blot analysis Results for each fusion and screenmg regime are dealt with individually in their respective section

Immunocytochemical analysis of the mouse MAbs using the ABC/HRP method

Supernatants from positive hybridomas were examined by immunocytochemical analysis on various cell lines

Glycosylation studies

MRP1 undergoes N-linked Glycosylation when expressed in mammalian cells To determine if the epitopes recognised by the MAbs chosen by the ELISA screening method were masked by glycosylation sugars when expressed in mammalian cells, COR-L23R MRP1 over-expressing cells were grown in the presence of tunicamycin (preventing N-hnked glycosylation) and the cell lysates were examined by Western blot analysis The rat monoclonal MRPr1 was included as a control

The MAbs chosen by the Western blotting method identified 190kD bands on various cell lines including cell lmes which do not over-express MRP1 The detection of the 190kD protein in the cell lines which do not over-express MRP1 suggested cross-reaction of the antibodies with another protein, possibly another isoform of MRP1 To determine if the MAbs chosen by the Western blotting method were recognising MRP1 and another protein or another MRP isoform, MRP1 over-expressing COR-L23R and MRP1 non-over-expressing COR-L23S cells were grown the presence of tunicamycin (preventing N-linked glycosylation) and analysed by Western blot analysis The subsequent detection of a 150kD band or an increase in the 190kD band in the COR-L23R cells but not the COR-L23S cells would indicate that the MAbs were reacting with MRP1

Heat treatment of protein samples prior to Western Blot analysis

Heat treatment of MRP1 protein samples @ 100^0C for 3 minutes prior to Western blot analysis destroys the epitope recognised by the rat monoclonal antibody MRPr1 (see Figure 3 2 8) but not the epitope in the MRP1 transfected 2008 cell line (see Figure 3 2 9) This indicates that MRPr1 recognises an epitope which is heat labile when expressed by drug inducement but not when it is induced by transfection To determine if the epitopes recognised by the MAbs identified by the Western blotting screening method were heat labile, heat treatment of COR-L23R and COR-L23S cells was carried out prior to Western blot analysis

Two immunisations/fusions were carried out using Peptide 1 (see Diagram 3 5 2) and are summarised in Table 3 7 1

Peptide 1

- AA 1520 - 1531
- Internal peptide / C-terminus
- Sequence CALFYSMAKDAGLV + KLH

Fusion 1.	Immunisation regime	*IN VIVO/IN VITRO* (BALB/c MICE)
	Screening system	ELISA, AGAINST PEPTIDE & PEPTIDE/KLH
	Selected MAbs	POSITIVE AGAINST PEPTIDE BY ELISA DO NOT IDENTIFY MRP ON WESTERN BLOTS
	Positive clone	58C
Fusion 2.	Immunisation regime	*IN VIVO* (BALB/c MICE)
	Screening system	WESTERN BLOTTING ON COR-L23R CELLS
	Selected MAbs	IDENTIFY BAND AT 190kD ON COR-L23R AND A LARGER OR DOUBLET BAND ON COR-L23S CELLS POSITIVE AGAINST PEPTIDE BY ELISA
	Positive clone	1A2

Table 3.7.1.

Primary screening.

Primary screening of Fusion 1 (Peptide 1) by ELISA identified the monoclonal antibody 58C However, MAb 58C did not detect protein on Western Blots

Primary screening of Fusion 2 (Peptide 1) by Western Blotting identified the monoclonal antibody 1A2 MAb 1A2 identified a 190kD band on COR-L23R cells, a larger or doublet 190kD band on COR-L23S cells (see Figure 3 7 1) and a 150kD band on the un-glycosylated MRP1 expressing SF9 insect cell membranes (see Figure 3 7 2)

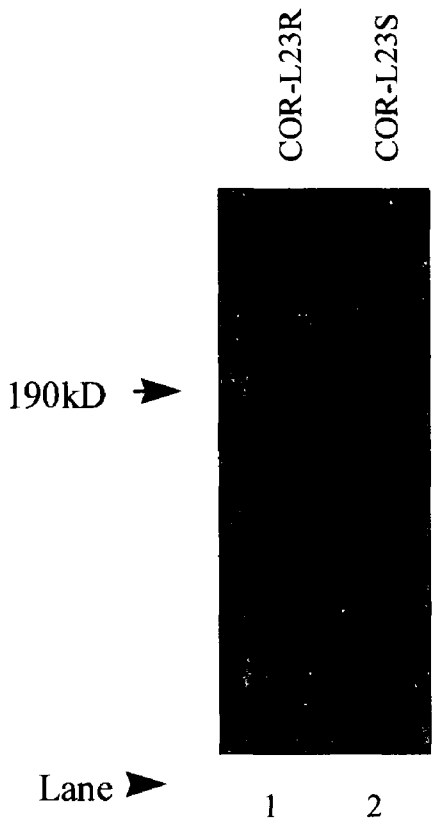


FIGURE 3.7.1. Western blot analysis of COR-L23R and COR-L23S cells with MAb 1A2 MAb 1A2 recognises a 190kD band in the COR-L23R cell lines and a larger band in the COR-L23S cell line

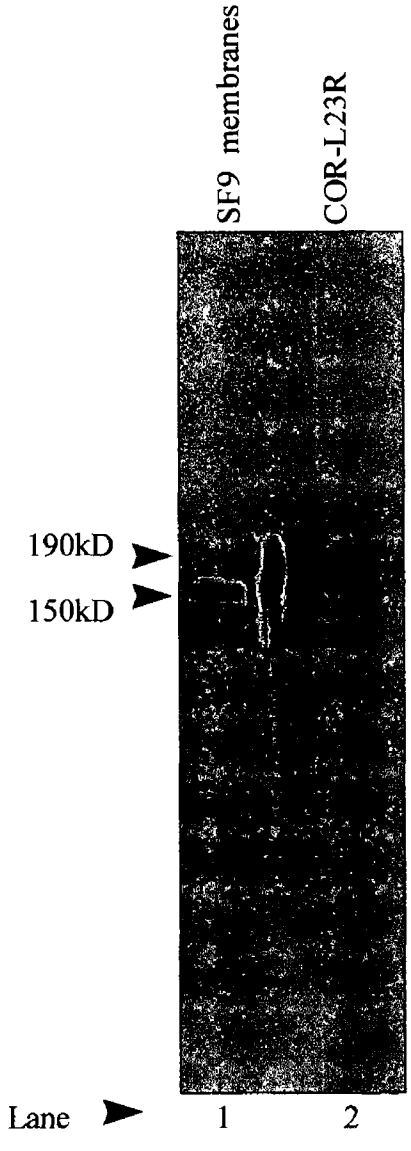


FIGURE 3.7.2. Western blot analysis of un-glycosylated MRP1-expressing SF9 membranes and COR-L23R cells with MAb 1A2 MAb 1A2 detects a 190kD band in the COR-L23R cell line and the un-glycosylated SF9 MRP1 at the lower molecular weight of 150kD

MRP isoform cross-reactivity studies

Cross-reactivity studies of the 1A2 antibody with the MRP1, MRP2 and MRP3 isoforms were carried out using the 2008 parental and 2008 MRP transfectants by Western blot analysis 1A2 detects a 190kD doublet band in the 2008 parental cell line and a larger doublet band in the MRP1, MRP2 and MRP3 2008 transfected cell lines

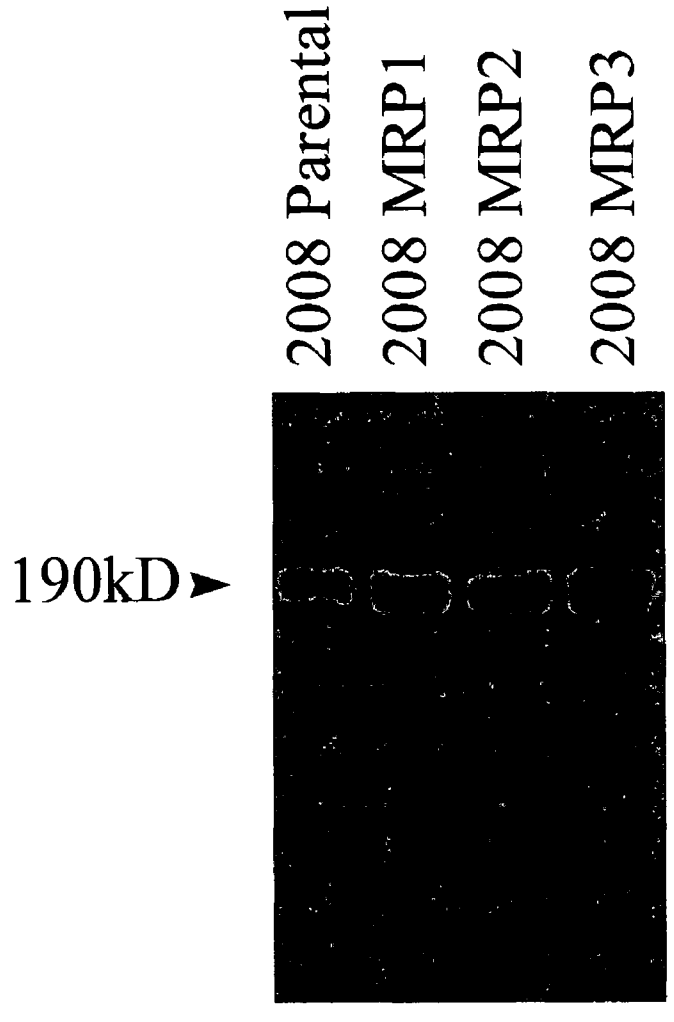


Figure 3.7.2.1. Cross reactivity studies in various MRP transfected cell lines by Western blot analysis with the mouse MAb, 1A2 1A2 detects a 190kD doublet band in the 2008 parental cell line and a larger doublet band in the MRP1, MRP2 and MRP3 2008 transfected cell lines

Heat treatment of cell lysates prior to Western Blot analysis

Heat treatment of cell preparations destroys the epitope recognised by the rat monoclonal antibody MRPr1 in the drug selected COR-L23R cell line but not the 2008 MRP1-transfected cell line (see section 3 2 Figure 3 2 8 and 3 2 9) The monoclonal antibody 1A2 detects a 190kD band in the COR-L23R cell line and a larger or doublet 190kD band in the parental COR-L23S cell line (see Figure 3 7 1) Heat treatment of COR-L23R and COR-L23S cell lysates @ 100°C for 3 minutes does not destroy the epitope recognised by MAb 1A2 (see Figure 3 7 3 , 3 7 3 1 and 3 7 3 2)

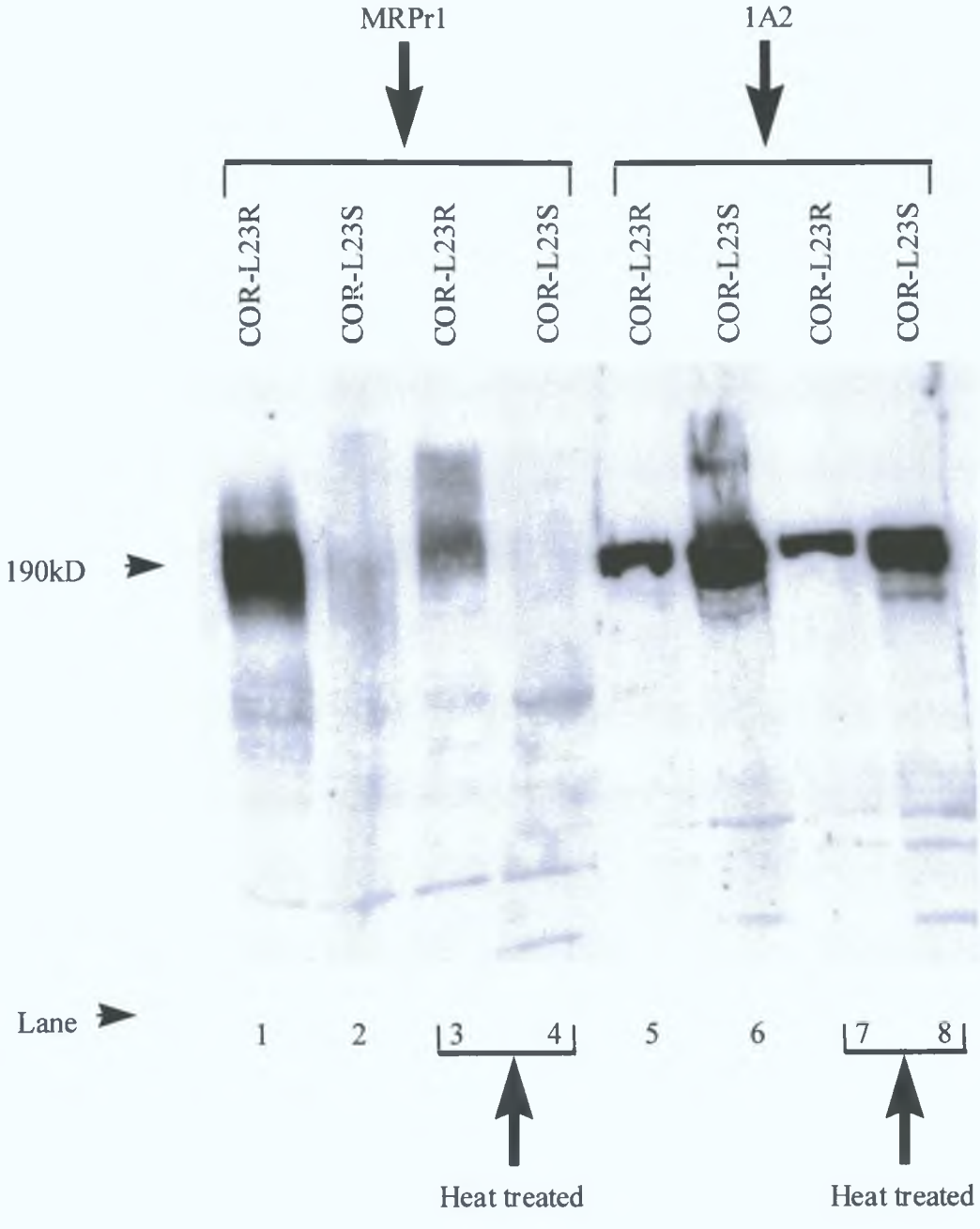


FIGURE 3.7.3. Western blot analysis of COR-L23R and COR-L23S cells with MRPr1 and MAb 1A2. Protein samples in Lanes 3, 4, 7 and 8 were heat treated @ 100^0C for 3 minutes prior to loading. The epitope recognised by MRPr1 in COR-L23R cells is destroyed by the heat treatment but the epitope recognised by MAb 1A2 is not.

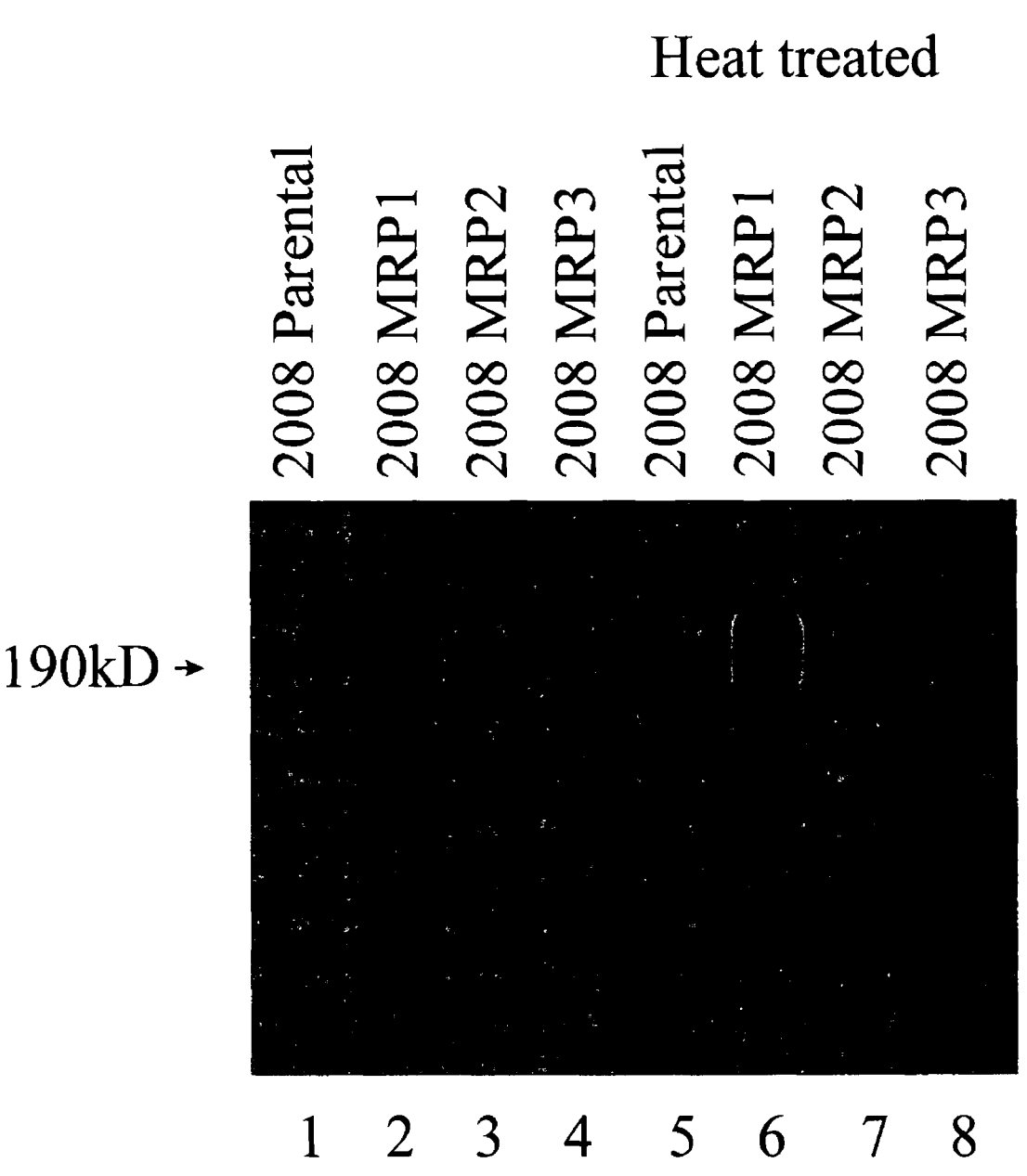


Figure 3.7.3.1. Western blot analysis of 2008 parental and MRP transfected cells with MRPr1 Protein samples in Lanes 5 to 8 were heat treated @ 100^0C for 3 minutes prior to loading The epitope recognised by MRPr1 in the 2008 MRP1 transfected cells is not destroyed by the heat treatment

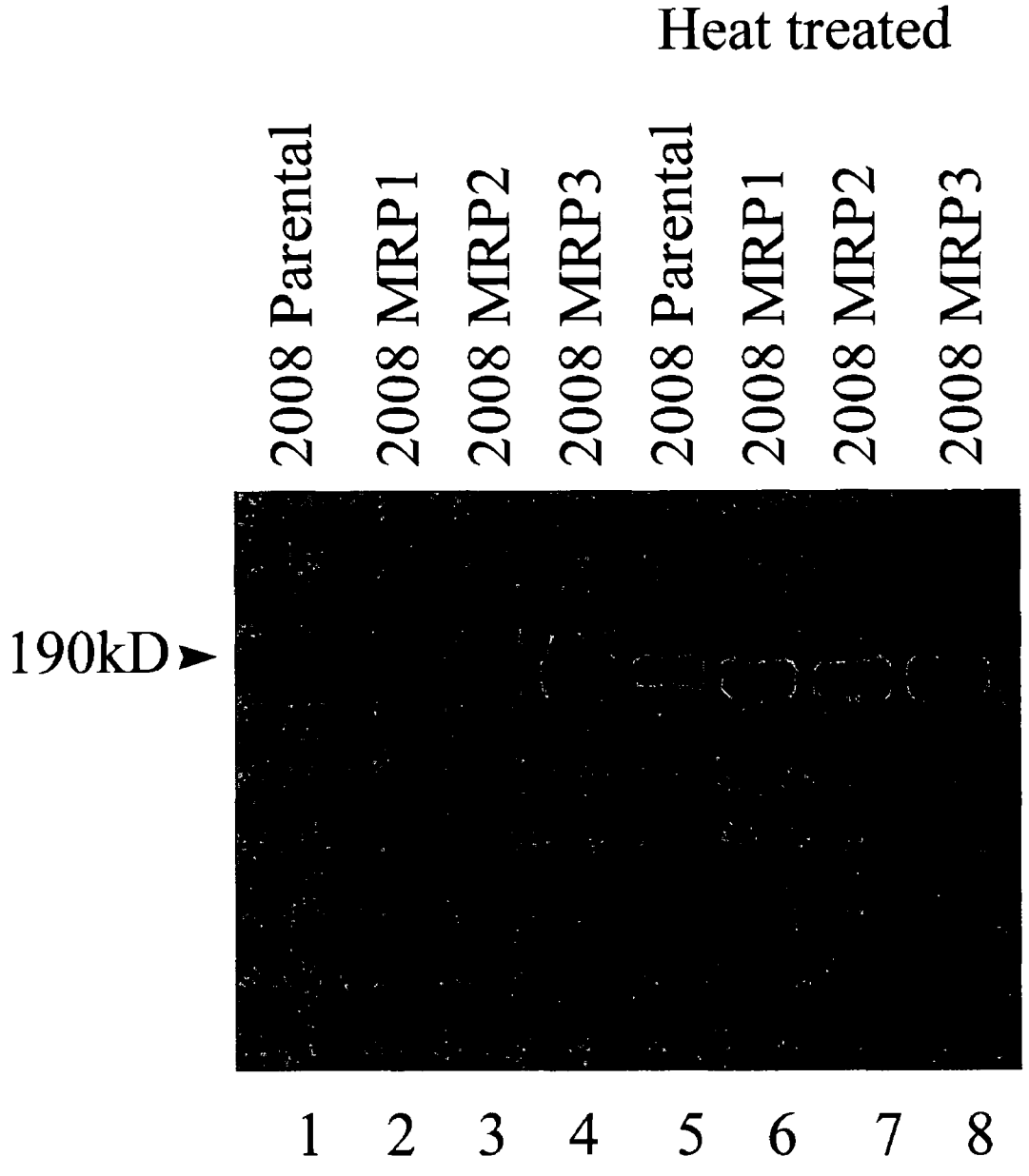


Figure 3.7.3.2. Western blot analysis of 2008 parental and MRP transfected cells with 1A2 Protein samples in Lanes 5 to 8 were heat treated @ 100^0C for 3 minutes prior to loading The epitope recognised by 1A2 in the 2008 parental and transfected cells is not destroyed by the heat treatment

Glycosylation studies

The COR-L23R and COR-L23S cells were grown in the presence of tunicamycin to prevent N-linked glycosylation The cells were harvested at various time-points and analysed by Western Blotting with MAb 1A2 An increase in the 190kD band and an increase in a lower band was observed in COR-L23R cells from 0 to 9 hours The increase of a lower band was also observed in the COR-L23S cells but the 190kD band remained constant (see Figure 3 7 4) The MRPr1 antibody detected the disappearance of the 190kD band and an increase in a lower band in COR-L23R cells from 0 to 24 hours The MRPr1 antibody did not detect any change in the COR-L23S cells The 1A2 antibody detected a doublet band at time 0 hours, a faint lower band at time 4 hours and increasing to a lower doublet band with increasing time However, both MRPr1 and 1A2 showed a disappearance of all bands in the COR-L23S cells at 20 hours (see Figure 3 7 5) This may be due to the half-life of the protein recognised by MRPr1 or 1A2 (MRP1 = 19 5 hours) or loss of protem sample It may be possible that 1A2 is recognising a protein which undergoes glycosylation or another protein also which is masked by glycosylation sugars This protein may be more highly glycosylated in the COR-L23R cells than in the COR-L23S cells

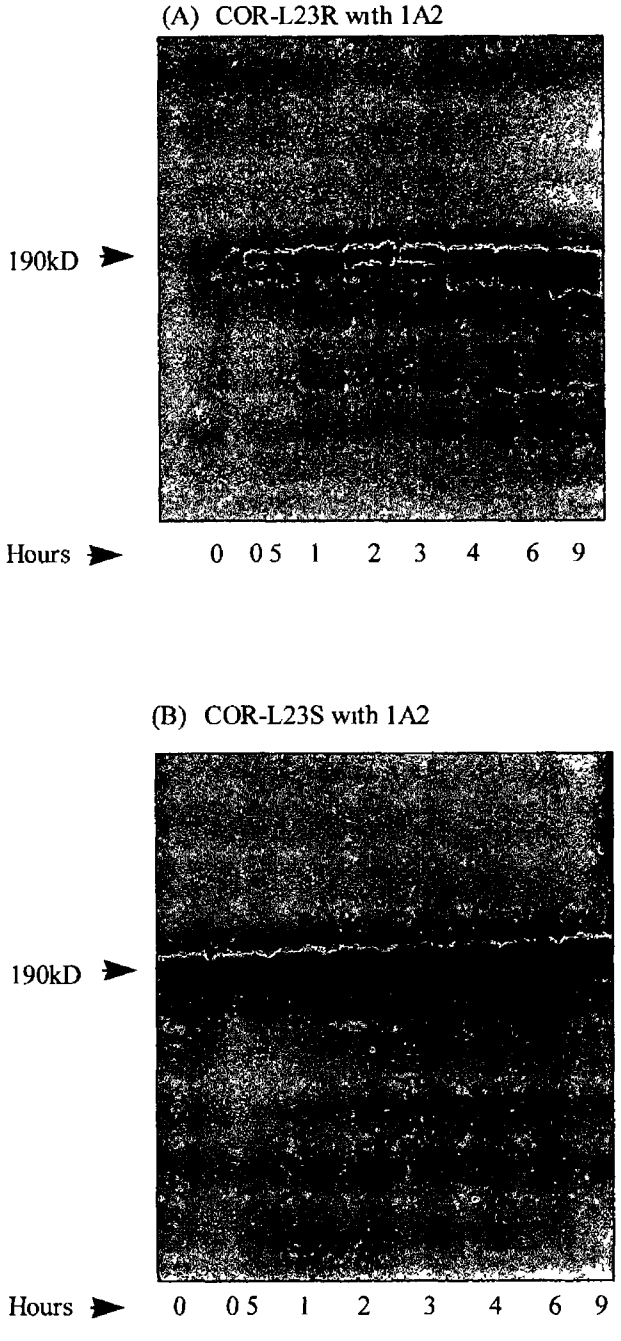


FIGURE 3.7.4. Western blot analysis of (A) COR-L23R and (B) COR-L23S cells grown in medium containing 8ug/ml of Tunicamycin from 0 to 9 hours with MAb 1A2 MAb 1A2 detects an increase in a lower band in both (A) COR-L23R and (B) COR-L23S cells with increasing exposure in time to Tunicamycin It is possible that 1A2 is recognising another protein in this second band which is masked by glycosylation

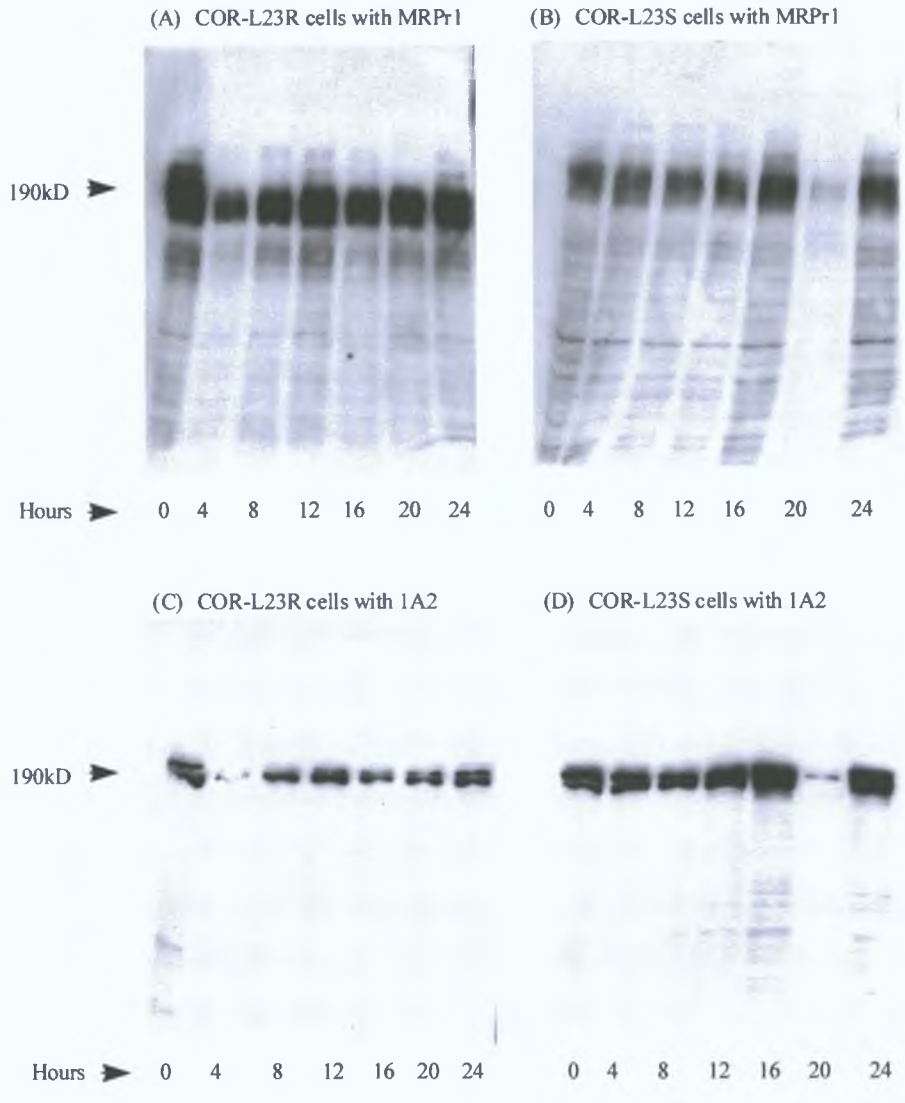


Figure 3.7.5. Western blot analysis of COR-L23R and COR-L23S cells with MRPr1 (A+B) and MAb 1A2 (C+D). The cells were grown in medium containing 8ug/ml of Tunicamycin from 0 to 24 hours. MRPr1 detects a lower 150kD band in the treated (A) COR-L23R cells as expected but detects no change in the faintly expressed band in the (B) COR-L23S cells. Like MRPr1, 1A2 detects a shift to a lower band in the treated (C) COR-L23R cells no change in the (D) COR-L23S cells. At time 20 hours, the protein recognised by 1A2 and MRPr1 show decreasing levels. This may be due to the half-life of the protein (MRP1 = 19.5 hours) or loss of protein sample.

One immunisation/fusion was carried out using Peptide 2 (see Diagram 3 5 2) and is summarised in Table 3 7 2

Peptide 2.

- AA 235 - 246
- Internal peptide (originally thought to be external) / N-terminus
- Sequence PLEGSDLWSLNK + BSA

Fusion 1.	Immunisation regime	*IN VIVO/IN VITRO* (BALB/c MICE)
	Screening system	ELISA, AGAINST PEPTIDE & PEPTIDE/BSA
	Selected MAbs	POSITIVE AGAINST PEPTIDE BY ELISA DO NOT IDENTIFY MRP ON WESTERN BLOTS
	Positive clone	76F

Table 3.7.2.

Primary screening:

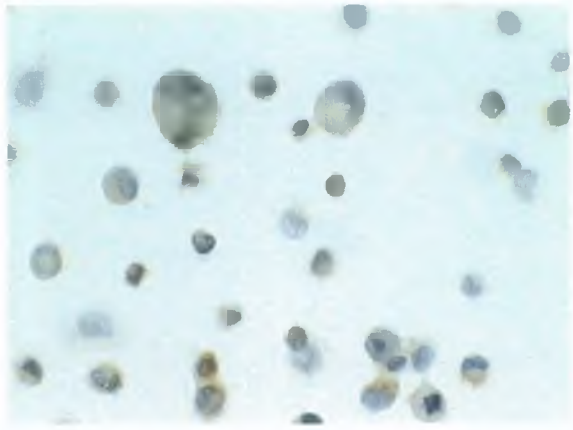
Primary screening of Fusion 1 (Peptide 2) by ELISA identified the monoclonal antibody 76F

Western blot analysis.

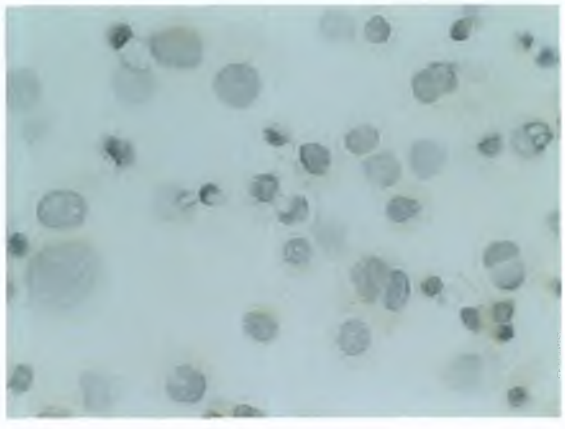
MAb 76F did not identify any protein on Western Blots

Immunocytochemical characterisation:

MAb 76F stained COR-L23R cells faintly but not the COR-L23S cells (see Figure 3 7 6)



A. COR-L23R cells stained with 76F



B. COR-L23S cells stained with 76F

FIGURE 3.7.6. Immunocytochemical characterisation of (A) COR-L23R cells and (B) COR-L23S cells, with the monoclonal antibody 76F. MAb 76F stained the (A) COR-L23R cells very faintly (40X magnification).

Two immunisations were carried out using Peptide 3 (see Diagram 3.5.2) and are summarised in Table 3.7.3.

Peptide 3.

- AA 246 - 260
- Internal peptide / N-terminus
- Sequence : KEDTSEQVVPVLVKN + KLH

Fusion 1.	Immunisation regime:	*IN VIVO/IN VITRO* (BALB/c MICE)
	Screening system:	ELISA, AGAINST PEPTIDE & PEPTIDE/KLH
	Selected MAbs:	POSITIVE TO PEPTIDE BY ELISA DO NOT IDENTIFY MRP ON WESTERN BLOTS
	Positive clone:	2B5
Fusion 2.	Immunisation regime:	*IN VIVO* (BALB/c MICE)
	Screening system:	WESTERN BLOTTING ON COR-L23R CELLS
	Selected MAbs:	IDENTIFY BAND AT 190kD ON COR-L23R AND A LARGER OR DOUBLET BAND ON COR-L23S CELLS POSITIVE AGAINST PEPTIDE
	Positive clones:	3A7

Table 3.7.3.

Primary screening.

Primary screening of Fusion 1 (Peptide 3) by ELISA identified the monoclonal antibody 2B5. MAb 2B5 did not detect protein on Western Blots.

Primary screening of Fusion 2 (Peptide 3) by Western Blotting identified the monoclonal antibody 3A7. MAb 3A7 identified a 190kD band on COR-L23R cells and

a 170-190kD doublet band on COR-L23S cells (see Figure 3 7 8), a large or doublet 190kD band on both DLKP and DLKPA cells (see Figure 3 7 9), a 190kD doublet band on both HL60ADR and HL60S cells and a 150kD band on un-glycosylated MRP1-expressing SF9 membranes (see Figure 3 7 10)

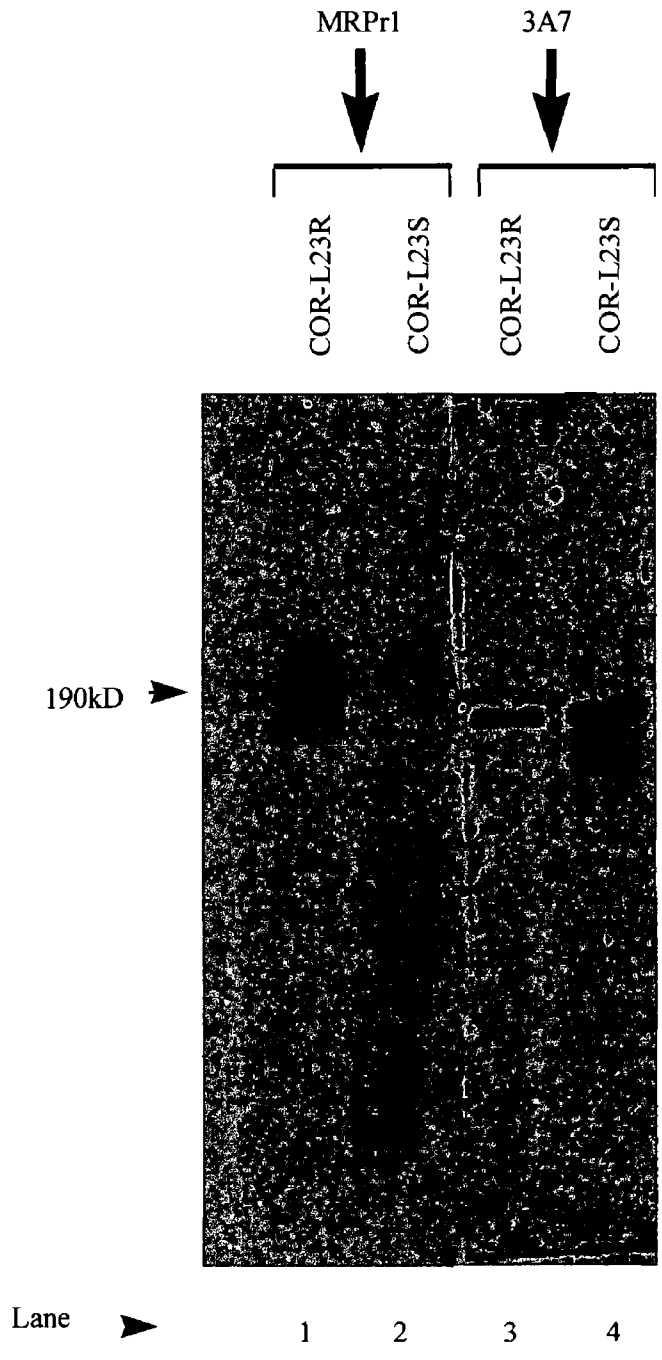


FIGURE 3.7.8. Western blot analysis of COR-L23R and COR-L23S cells with the rat monoclonal antibody MRPr1 (lanes 1 and 2) and MAb 3A7 (lanes 3 and 4) MAb 3A7 recognises a 190kD band in the COR-L23R cells and a 190kD to 170kD doublet band in the COR-L23S cells

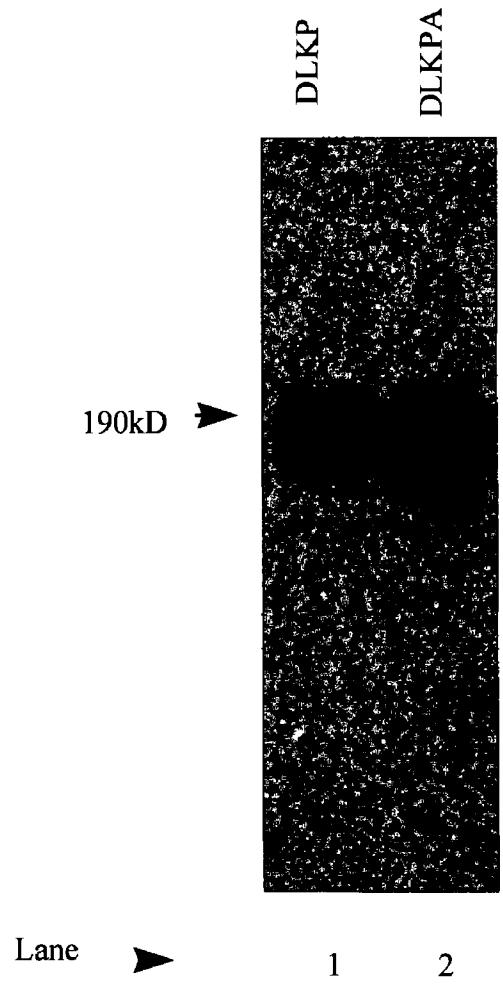


FIGURE 3.7.9. Western blot analysis of DLKP (lane 1) and DLKPA (lane 2) cells with MAb 3A7 MAb 3A7 recognises a large 190kD band in both the DLKP and DLKP-A cells

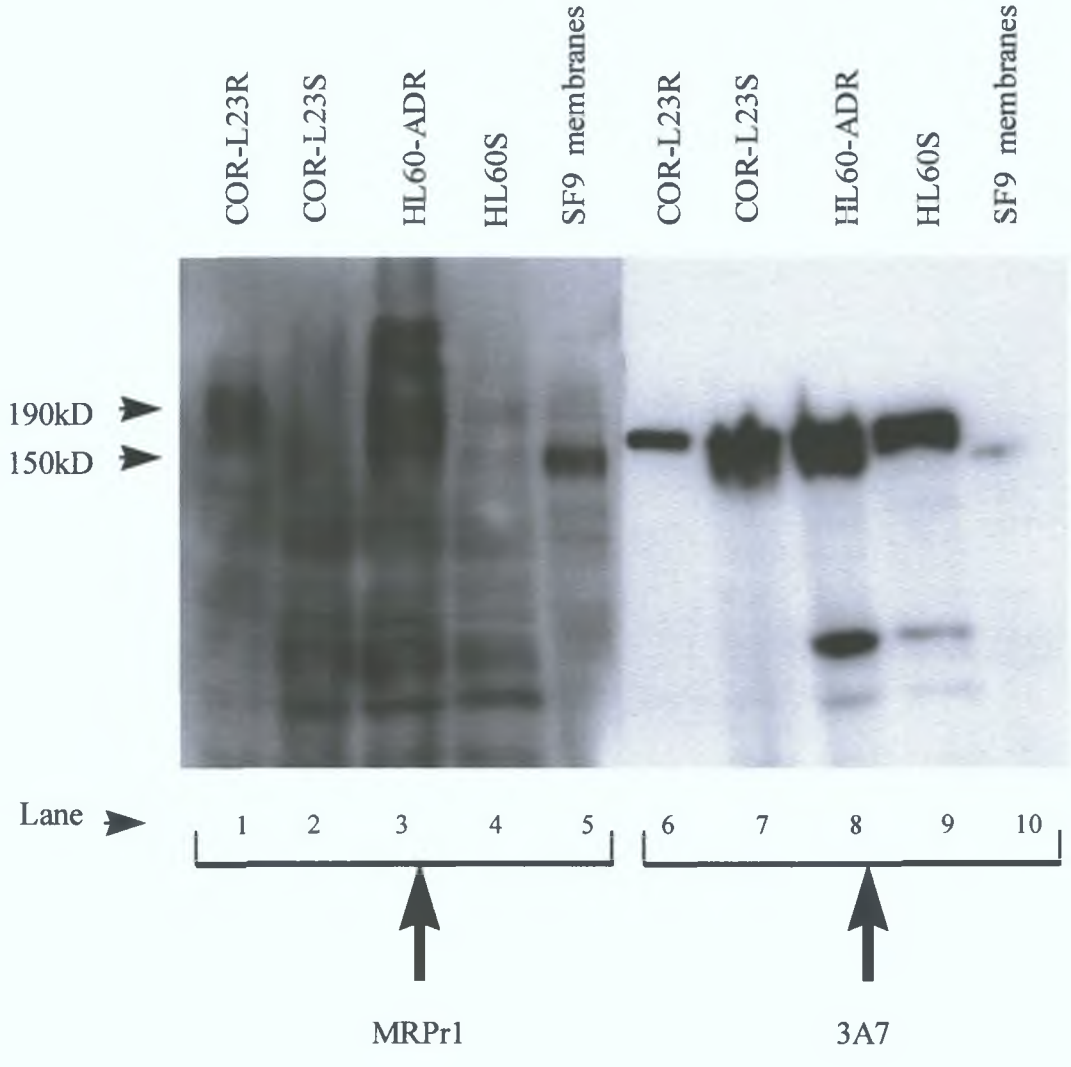
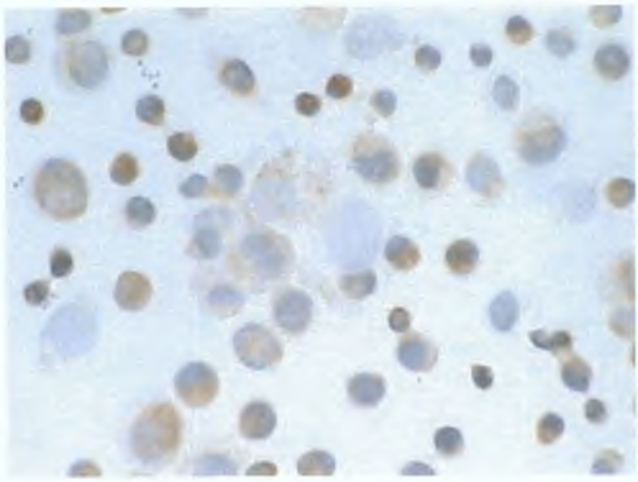


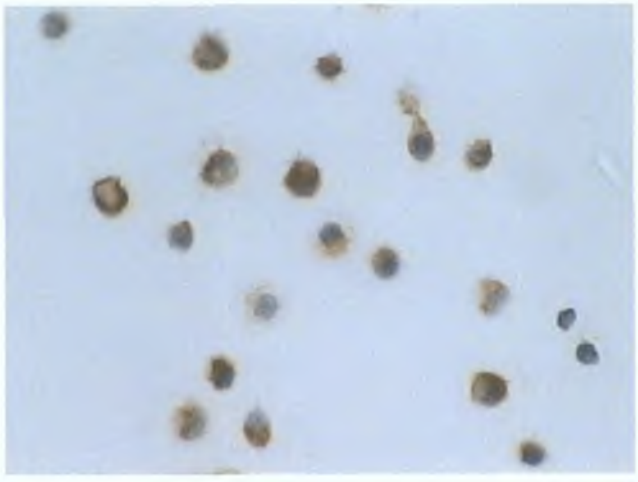
FIGURE 3.7.10. Western blot analysis of various cell lines (COR-L23R, COR-L23S, HL60ADR and HL60S) and MRP1-expressing SF9 membranes with the rat monoclonal MRPr1 (lanes 1 to 5) and MAb 3A7 (lanes 6 to 10). MAb 3A7 detects a single 190kD band in the COR-L23R cells, a 150kD band in the SF9 MRP1-expressing membranes and a larger or doublet 190kD band in the COR-L23S, HL60ADR and HL60S cells.

Immunocytochemical characterisation

Immunocytochemical analysis of COR-L23R and COR-L23S cells with MAb 3A7 showed staining on both cell lines (see Figure 3 7 11)



A. COR-L23R cells stained with 3A7



B. COR-L23S cells stained with 3A7

FIGURE 3.7.11. Immunocytochemical staining of (A) COR-L23R cells and (B) COR-L23S cells with MAb 3A7. MAb 3A7 stains both the COR-L23R and COR-L23S cells (40X magnification).

Heat treatment of cell lysates prior to Western Blotting

Heat treatment of COR-L23R whole cell lysates @ 100^0C for 3 minutes destroys the epitope recognised by MRPr1 (see Figure 12, lane 3) The monoclonal antibody 3A7 detects a 190kD band in the COR-L23R cell line and a larger or doublet 190kD band in the parental COR-L23S cell line Heat treatment of COR-L23S cell lysates @ 100^0C for 3 minutes does not destroy the epitope recognised by MAb 3A7 (see Figure 3 7 12)

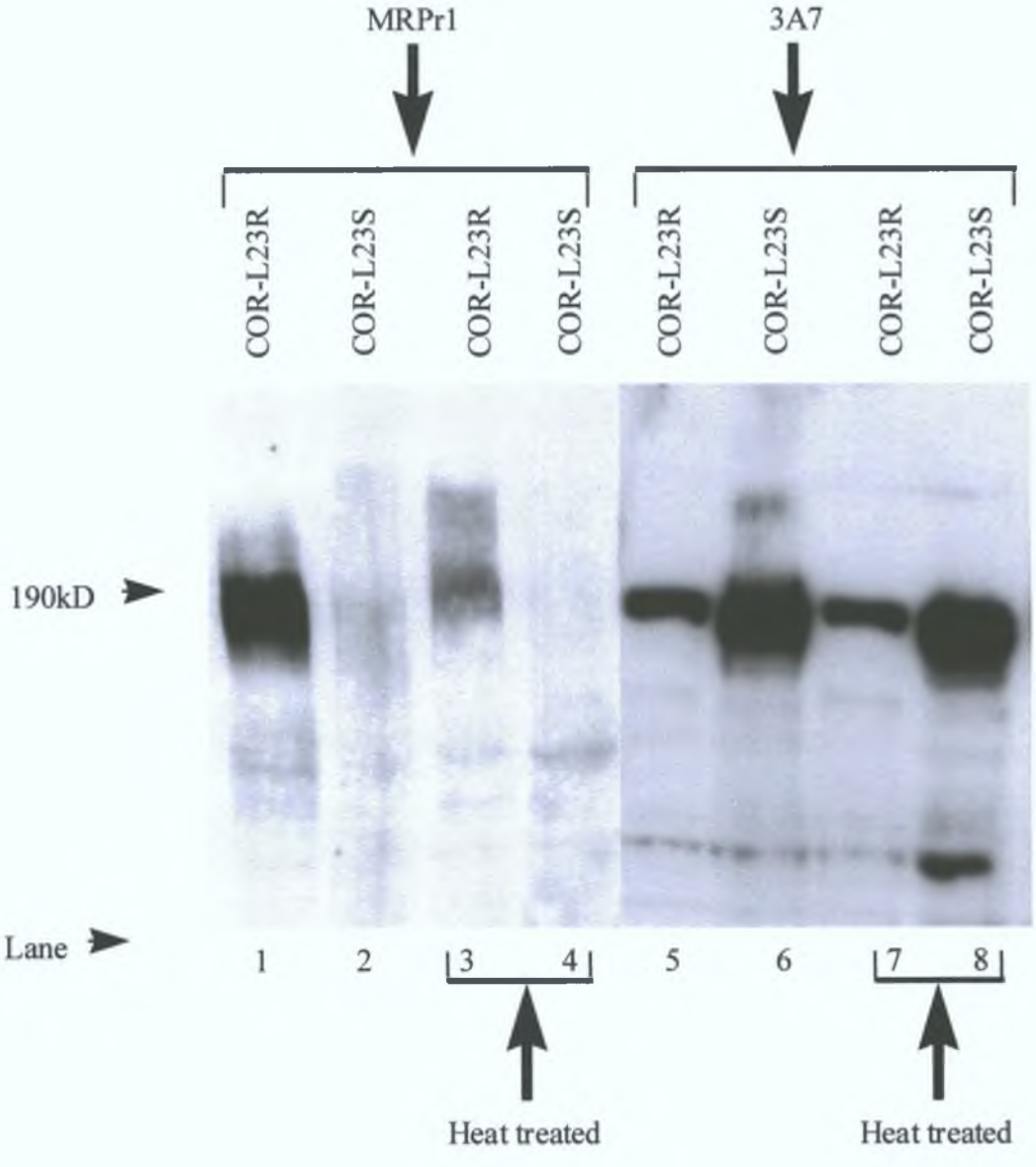


FIGURE 3.7.12. Western blot analysis of COR-L23R and COR-L23S cells, with the rat monoclonal MRPr1 (lanes 1 to 4) and MAb 3A7 (lanes 5 to 9). Protein samples in lanes 3, 4, 7 and 8 were heat treated @ 100^0C for 3 minutes prior to loading. The epitope recognised by MAb 3A7 is not destroyed by heat treatment.

Immunoprecipitation studies

To determine that the monoclonal antibodies produced recognised MRP1, immunoprecipitation studies were carried out (see Figure 3 7 13) COR-L23R and COR-L23S cells were immunoprecipitated with the MRP1 specific, MRPr1 antibody The precipitates were run out on Western Blots and re-probed with MAb 3A7, MRPr1 and control mouse IgG MAb 3A7 identified a 190kD band in the COR-L23R cells and a slightly stronger 190kD band in the COR-L23S cells The control mouse IgG was negative

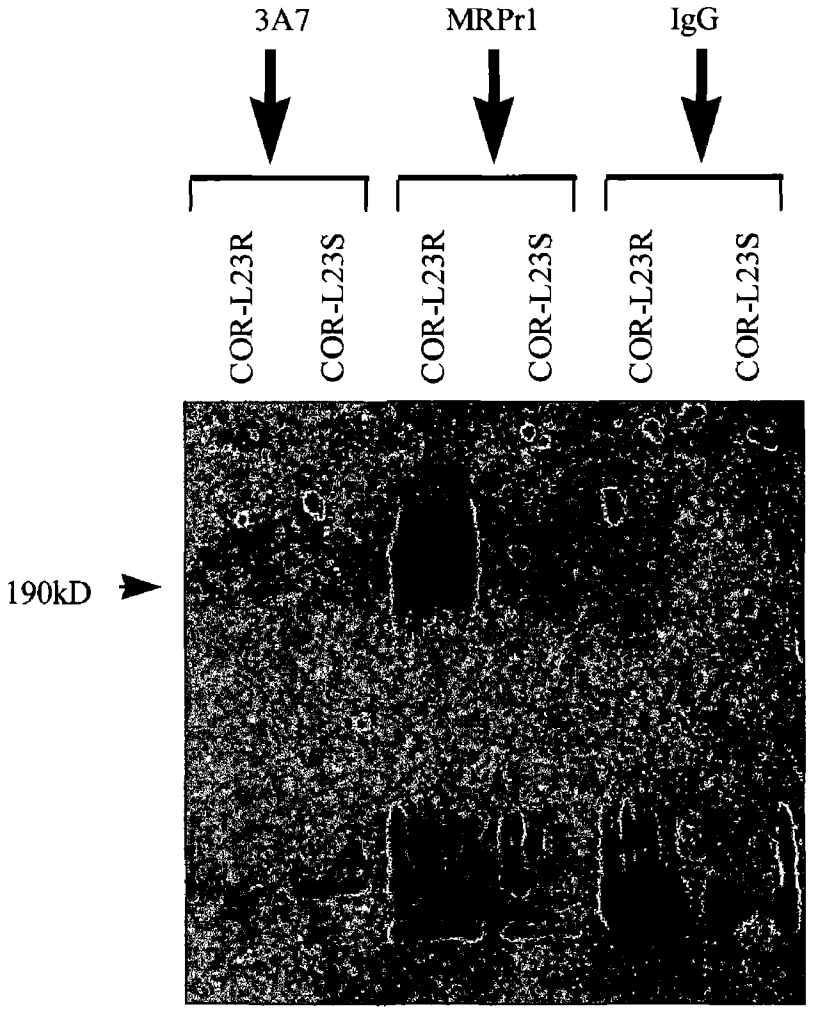


FIGURE 3.7.13. Immunoprecipitation of COR-L23R and COR-L23S cells with MRPr1 The immunoprecipitates were re-probed with MAb 3A7, the rat monoclonal MRPr1 and control mouse IgG MRPr1 detects a strong 190kD band in the COR-L23R immunoprecipitates and a faint 190kD band in the COR-L23S immunoprecipitates MAb 3A7 detects a 190kD band in the COR-L23R immunoprecipitates and a stronger 190kD band in the COR-L23S immunoprecipitates The control mouse IgG did not detect a 190kD band in any of the immunoprecipitates

Glycosylation studies.

Glycosylation studies with tunicamycin showed that the epitope recognised by MAb 3A7 was destroyed after 40 hours treatment Unexpectedly, MAb 3A7 failed to detect a band in the MRP1 expressing SF9 membranes (see Figure 3 7 14) Previously MAb 3A7 recognised a faint 150kD band in unglycosylated MRP1 expressing SF9 insect membranes (Figure 3 7 10) The 3A7 MAb showed a tendency to give varying results and became unstable, eventually failing to secrete any antibody

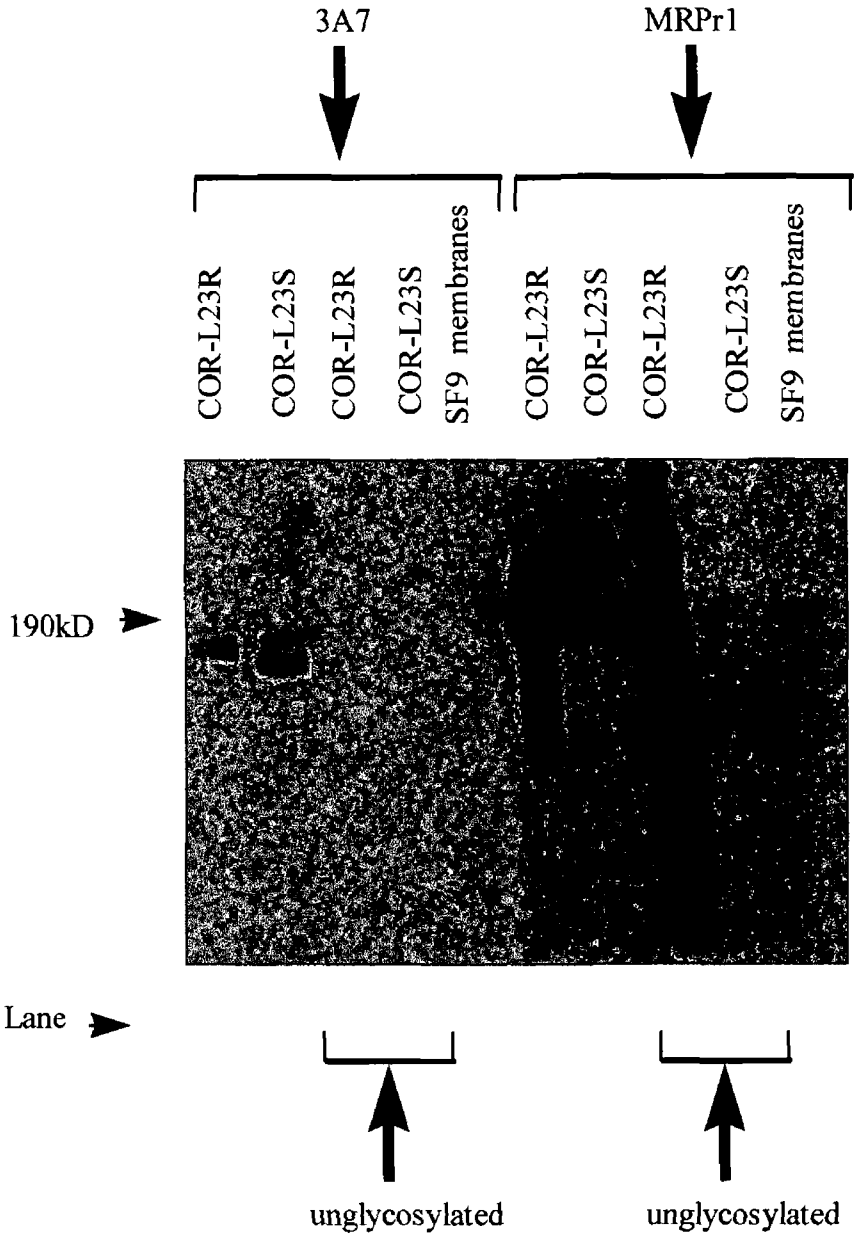


FIGURE 3.7.14. Western blot analysis of COR-L23R cells, COR-L23S cells and MRP1-expressing SF9 membranes with MAb 3A7 and the rat monoclonal MRPr1 Samples loaded onto lanes 3, 4, 8 and 9 were grown in medium containing 8ug/ml Tunicamycin for 40 hours The epitope recognised by 3A7 is destroyed after 40 hours Unexpectedly, MAb 3A7 failed to detect MRP1 expressed in the SF9 insect membranes (as it had done previously Figure 3 7 10) possibly because it was becoming an unstable antibody secretor

One immunisation/fusion was carried out using Fusion protein 1 (see Diagram 3 5 3) and is summarised in Table 3 7 4

Fusion protein 1.

- AA 1294 - 1531 (This immunisation/fusion and screening was carried out by Irene Cleary)
- C-terminus

Fusion 1.	Immunisation regime	*IN VIVO/IN VITRO* (BALB/c MICE)
	Screening system	WESTERN BLOTTING ON COR-L23R CELLS
	Selected MAbs	IDENTIFY BAND AT 190kD ON COR-L23R AND COR-L23S CELLS
	Positive clone	5E7

Table 3.7.4.

Primary screening

Primary screening of Fusion 1 (Fusion Protein 1) by Western Blotting identified the monoclonal antibody 5E7 MAb 5E7 identified a 190kD band on COR-L23R cells and a larger 190kD band on COR-L23S cells (see Figure 3 7 15)

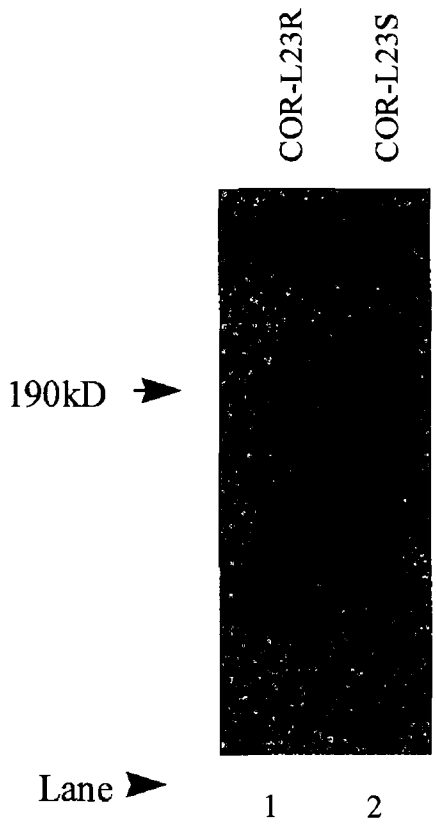


FIGURE 3.7.15. Western blot analysis of COR-L23R (lane 1) and COR-L23S (lane 2) cells with MAb 5E7 MAb 5E7 identifies a 190kD band on COR-L23R cells and a larger or doublet 190kD band on COR-L23S cells

One immunisation/fusion was carried out using Fusion protein 2 (see Diagram 3 5 3) and is summarised in Table 3 7 5

Fusion protein 2.

- AA 647 - 912
- N-terminus

Fusion 1	Immunisation regime	*IN VIVO/IN VITRO* (BALB/c MICE)
	Screening system	ELISA, AGAINST FUSION PROTEIN & MBP
	Selected MAbs	POSITIVE TO PEPTIDE & MBP
		DO NOT IDENTIFY MRP ON WESTERN BLOTS
	Positive clone	2A6

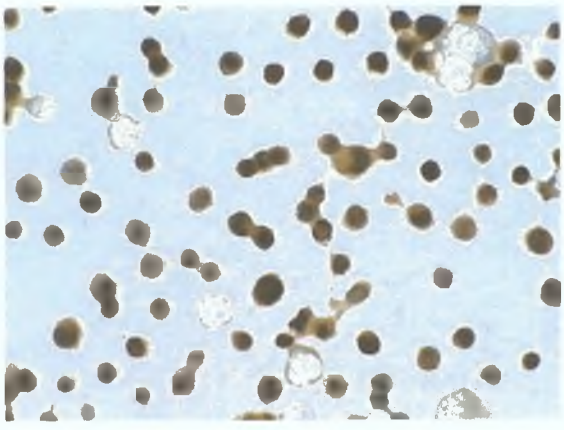
Table 3.7 5

Primary screening.

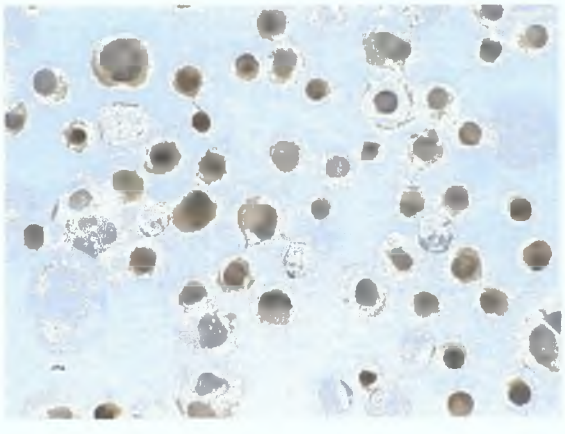
Primary screening of Fusion 1 (Fusion Protein 2) by ELISA identified the monoclonal antibody 2A6 MAb 2A6 did not identify any protein on Western Blots However, 2A6 also reacted positively by ELISA with an irrelevant fusion protein, which also contained the Maltose Binding Protein (MBP) expression tag This indicated that 2A6 was reacting with MBP and not the peptide immunogen

Immunocytochemical charaterisation

MAb 2A6 stained both COR-L23R and COR-L23S cells by immunocytochemistry but staining was reduced on the COR-L23S cells (see Figure 3 7 16)



A. COR-L23R cells stained with 2A6



B. COR-L23S cells stained with 2A6

FIGURE 3.7.16. Immunocytochemical staining of COR-L23R (A) and COR-L23S (B) cells with MAb 2A6. MAb 2A6 stained the COR-L23R cells more intensely than the COR-L23S cells. 40X magnification.

One immunisation/fusion was carried out using COR-L23R whole cell lysates and is summarised in Table 3.7.6.

COR-L23R WHOLE CELL LYSATE FUSION.

- Immunogen : COR-L23R whole cell lysates

Fusion 1.	Immunisation regime:	*IN VIVO/IN VITRO* (BALB/c MICE)
	Screening system:	WESTERN BLOTTING ON COR-L23R CELLS
	Selected MAbs:	IDENTIFY BAND AT 190kD ON COR-L23R, COR-L23S, HL60ADR AND HL60S CELLS. STAIN COR-L23R CELLS BUT NOT THE SENSITIVE PARENTAL COR-L23S CELLS BY IMMUNOCYTOCHEMISTRY.
	Positive clone:	4F6.

Table 3.7.6.

Primary screening.

Primary screening by Western Blotting identified the monoclonal antibody 4F6. MAb 4F6 identified a 190kD band on COR-L23R, COR-L23S, HL60ADR and HL60S cells (see Figure 3.7.16.).

Immunocytochemical characterisation.

4F6 also reacted strongly with COR-L23R cells but not with COR-L23S cells by immunocytochemistry (see Figure 3.7.17.). However, this hybridoma was unstable and stopped secreting antibody despite cloning.

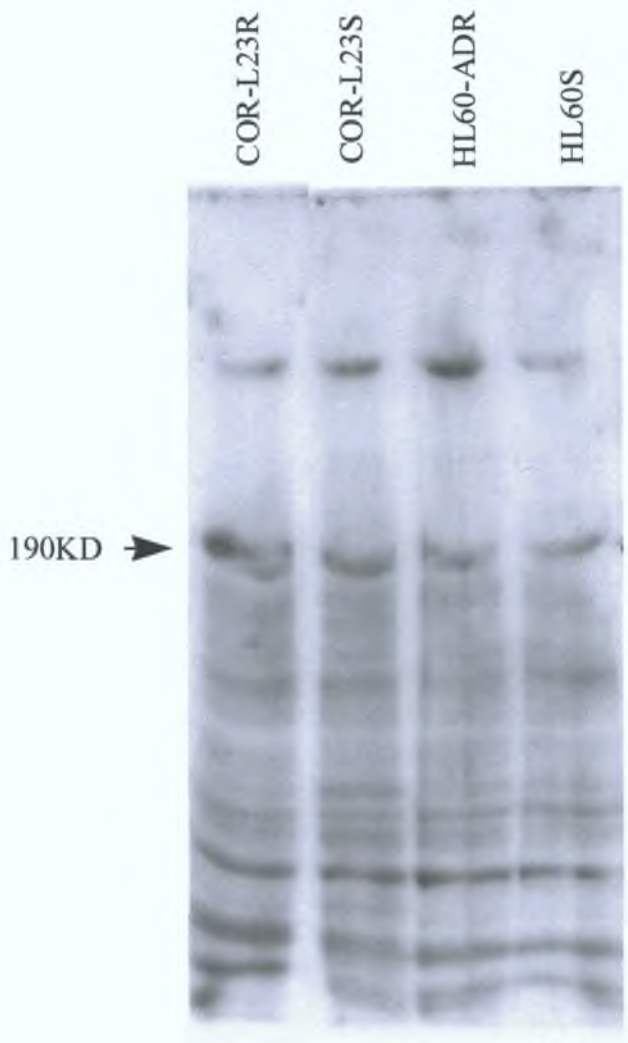
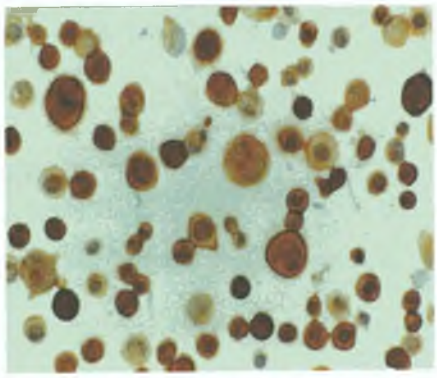
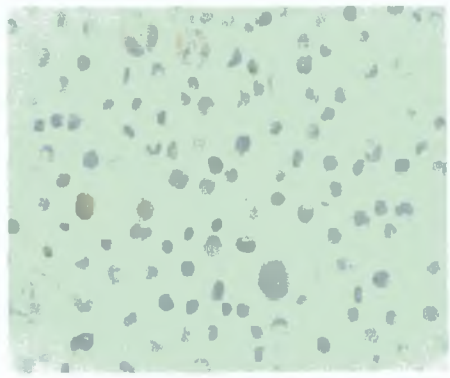


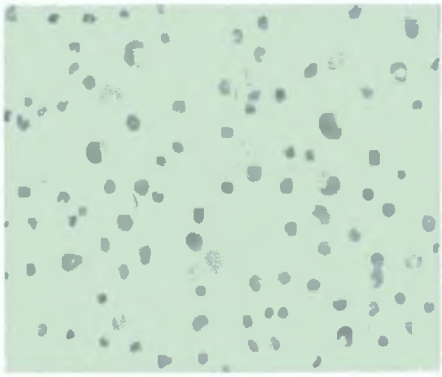
Figure 3.7.16. Western blot analysis of COR-L23R, COR-L23S, HL60ADR and HL60S cells with MAb 4F6. MAb 4F6 detects a very faint 190kD band in the COR-L23R, COR-L23S, HL60ADR and HL60S cell lines.



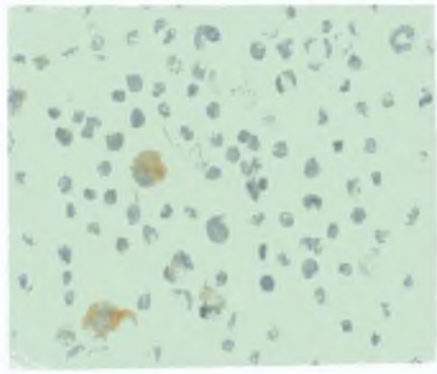
COR-L23R cells stained with the rat monoclonal antibody MRPr1



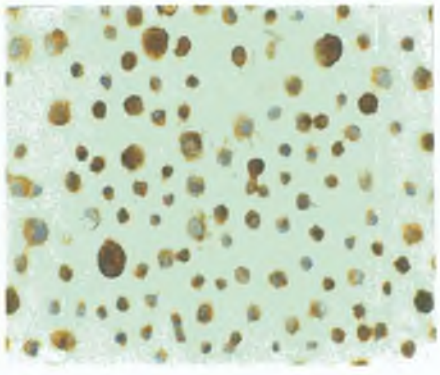
COR-L23S cells stained with the rat monoclonal antibody MRPr1



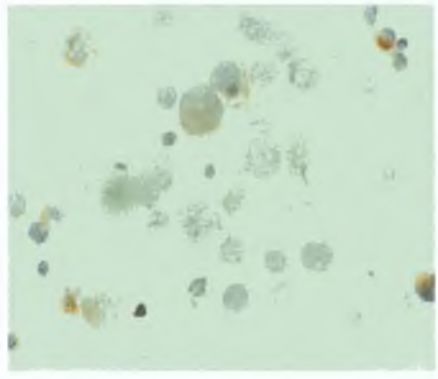
COR-L23R cells stained with negative control



COR-L23S cells stained with negative control



COR-L23R cells stained with Mab 4F6



COR-L23S cells stained with Mab 4F6

FIGURE 3.7.17. Immunocytochemical analysis of COR-L23R and COR-L23S cells with the rat monoclonal antibody MRPr1 and MAb 4F6. Like the MRPr1 MAb, MAb 4F6 shows plasma membranous staining in the MRP1 over-expressing COR-L23R cells but not the drug-sensitive COR-23S cells (40X magnification).

One immunisation/fusion was carried out using COR-L23R cell membrane preparations and is summarised in Table 3 7 7

COR-L23R CELL MEMBRANE FUSION.

- Immunogen COR-L23R cell membrane preparations

Fusion 1	Immunisation regime	*IN VIVO* (BALB/c MICE)
	Screening system	WESTERN BLOTTING ON COR-L23R CELL MEMBRANES
	Selected MAbs	IDENTIFY BAND AT 190kD ON COR-L23R CELLS
	Positive clones	6C6 and 2A7

Table 3.7.7.

Primary screening

Primary screening by Western blotting identified the monoclonal antibodies 2A7 and 6C6 Both MAb 2A7 and MAb 6C6 recognised a 190kD band in COR-L23R and COR-L23S whole cell lysates and cell membrane preparations (see Figure 3 7 18) MAb 2A7 detected two lower bands in the MRP1-over-expressing HL60ADR cells and a faint single lower band in the MRP1 negative HL60S cell line at a 1 15 concentration However, MAb 2A7 detected only a single lower band in the HL60ADR cells and no bands in the HL60S cells at a 1 20 concentration (see Figure 3 7 19) MAb 6C6 did not recognise any bands on the HL60ADR or HL60S cells by Western blotting (see Figure

3 7 20) MAb 2A7 or MAb 6C6 did not detect un-glycosylated MRP1 in MRP1-expressing SF9 membranes by Western blotting (see Figures 3 7 21 , 3 7 22 and 3 7 23)

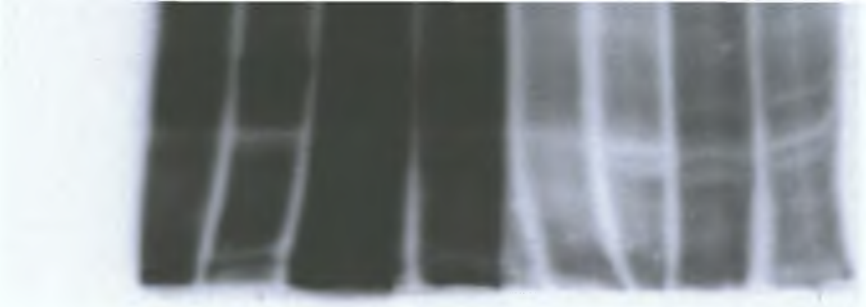


FIGURE 3.7.18. Western blot analysis of COR-L23R and COR-L23S membranes and whole cell lysate preparations with MAb 2A7 and MAb 6C6 and the rat monoclonal MRPr1.

190kDa
COR-L23R membranes
COR-L23S membranes
MRPr1
COR-L23R membranes
COR-L23S membranes
COR-L23R whole cell preps
COR-L23S whole cell preps
2A7
COR-L23R membranes
COR-L23S membranes
COR-L23R whole cell preps
COR-L23S whole cell preps
6C6

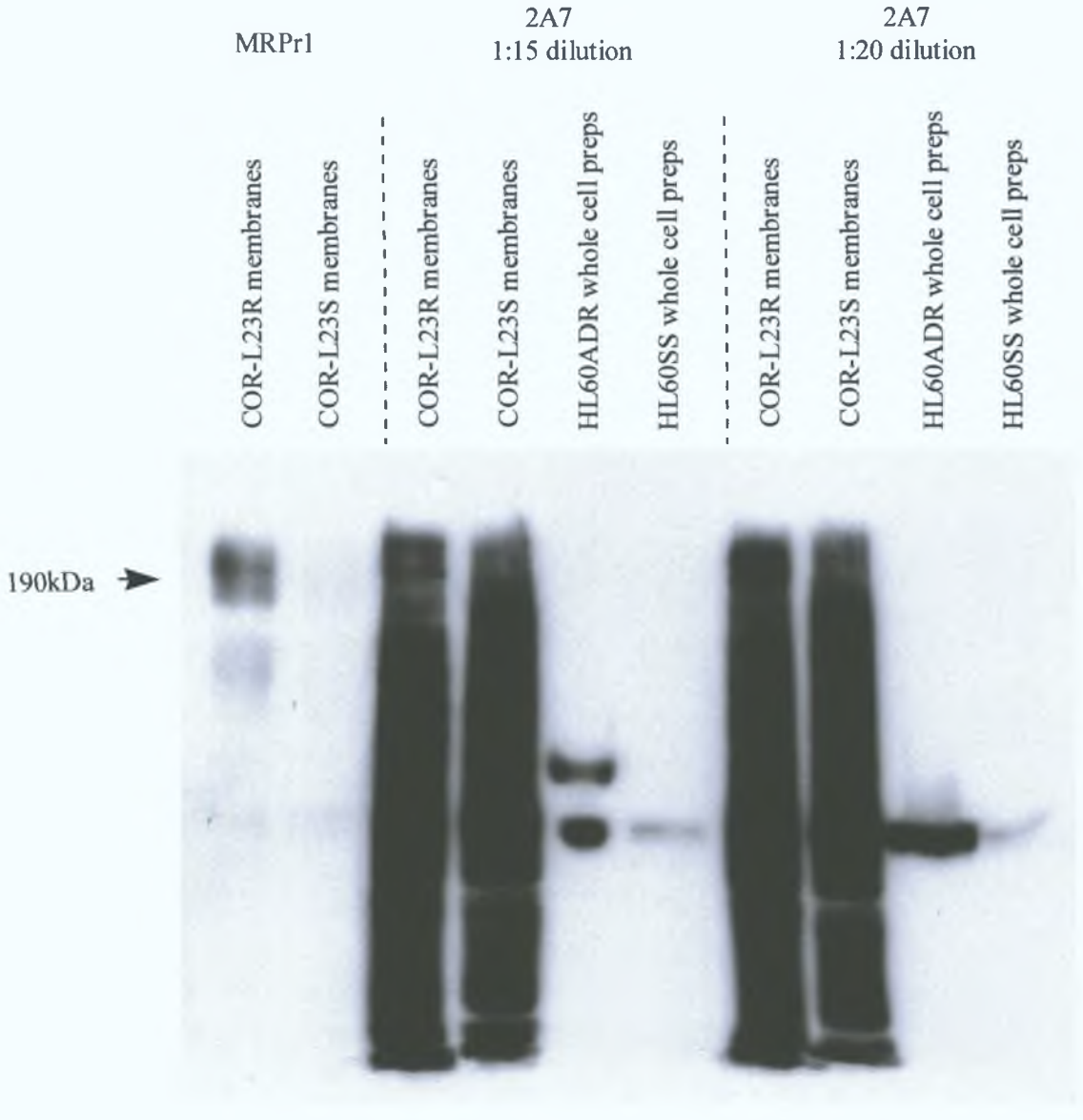


FIGURE 3.7.19. Western blot analysis of COR-L23R and COR-L23S membranes with MAb 2A7 and the rat monoclonal MRPr1 and analysis of HL60ADR and HL60S whole cell lysate preparations with MAb 2A7.

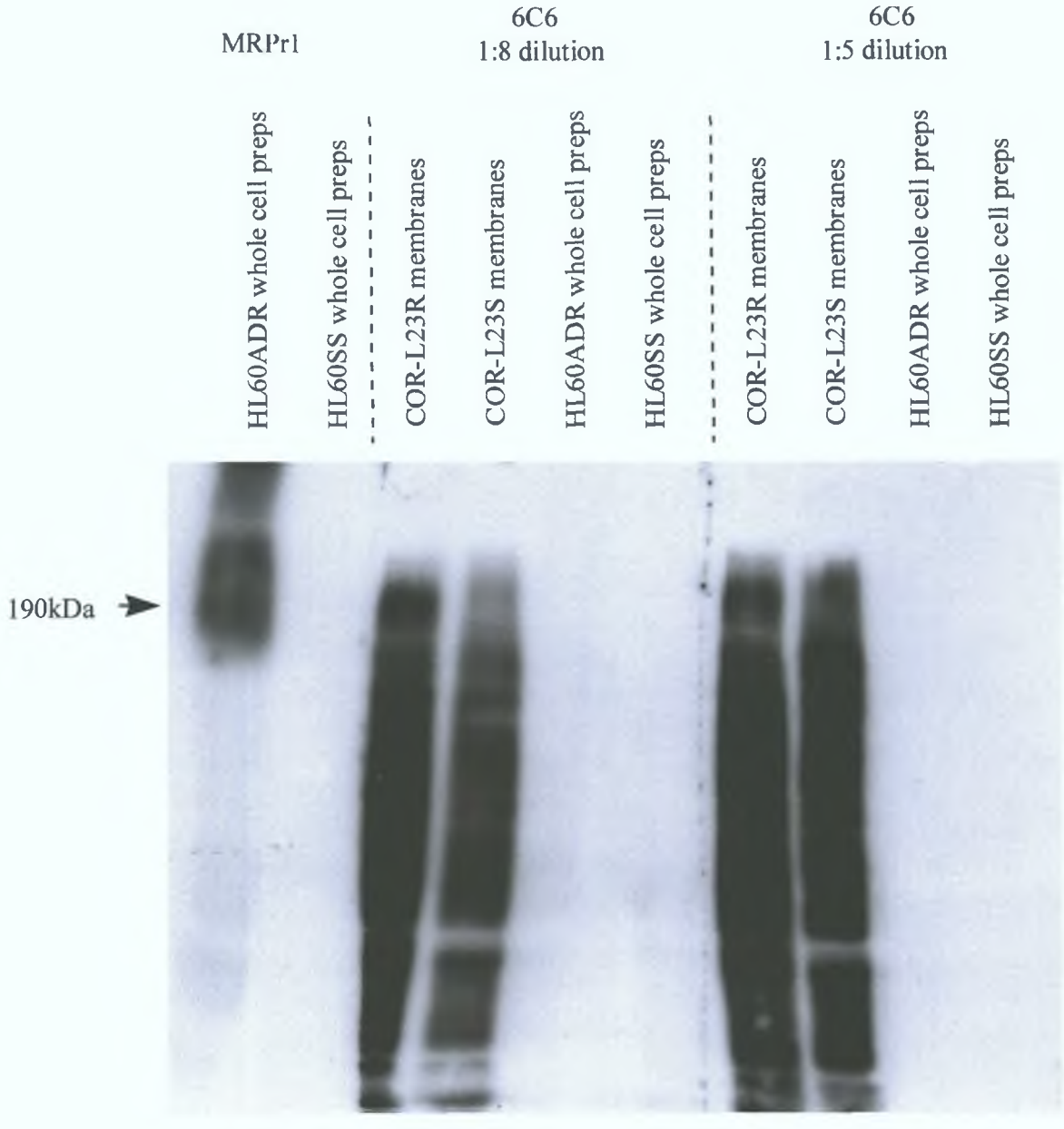


FIGURE 3.7.20. Western blot analysis of HL60ADR and HL60S whole cell lysate preparations with MAb 6C6 and the rat monoclonal MRPr1 and analysis of COR-L23R and COR-L23S membranes with MAb 6C6.

FIGURE 3.7.21. Western blot analysis of COR-L23R and COR-L23S cell membranes and whole cell lysate preparations and MRP1-expressing SF9 membranes with MRPr1.

190kDa
COR-L23R membranes
COR-L23S membranes
COR-L23R whole cell preps
COR-L23S whole cell preps
MRP1 expressing SF9 membranes
MRPr1
COR-L23R membranes
COR-L23S membranes
COR-L23R whole cell preps
COR-L23S whole cell preps
MRP1 expressing SF9 membranes
TBS (negative control)

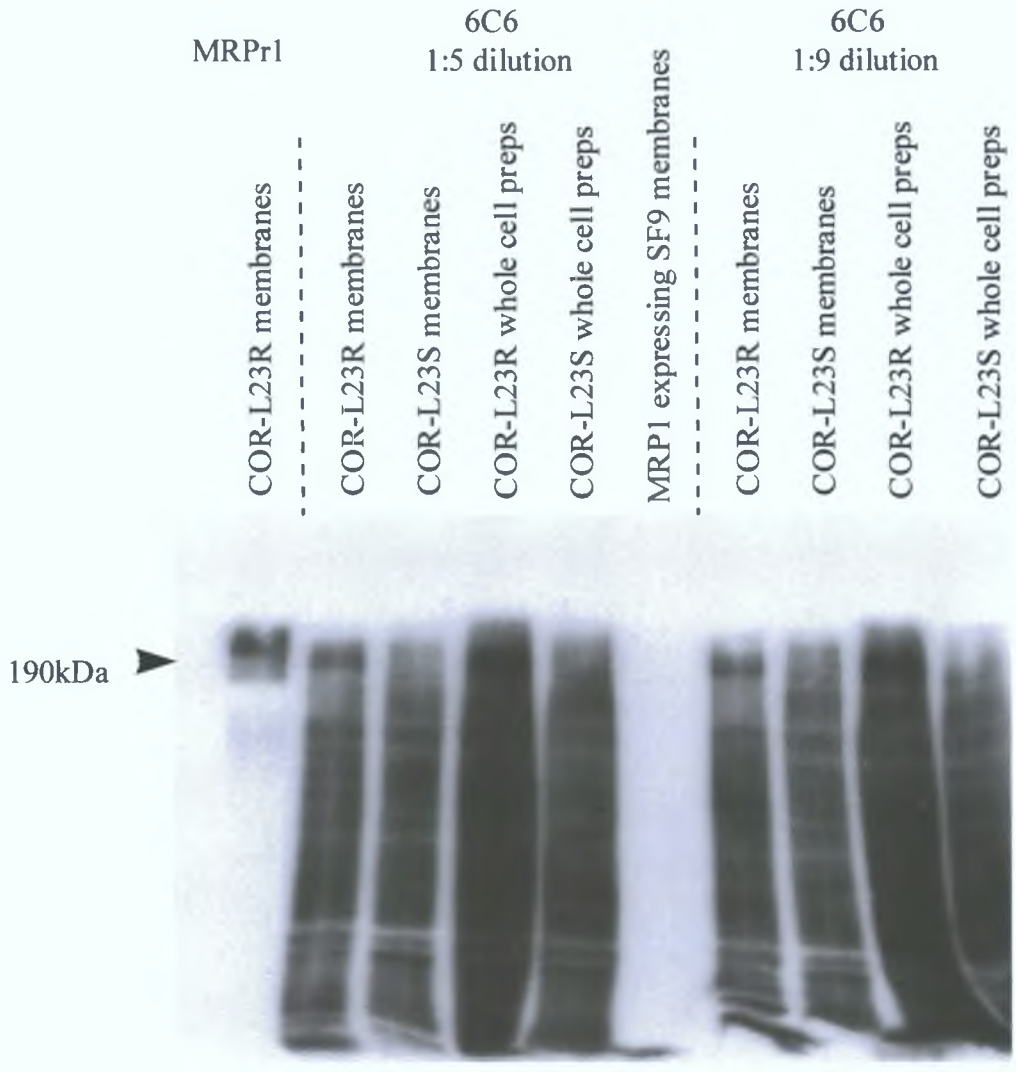


FIGURE 3.7.22. Western Blot analysis of COR-L23R and COR-L23S membrane and whole cell lysate preparations and MRP1 expressing SF9 membranes with the hybridoma supernatant 6C6.

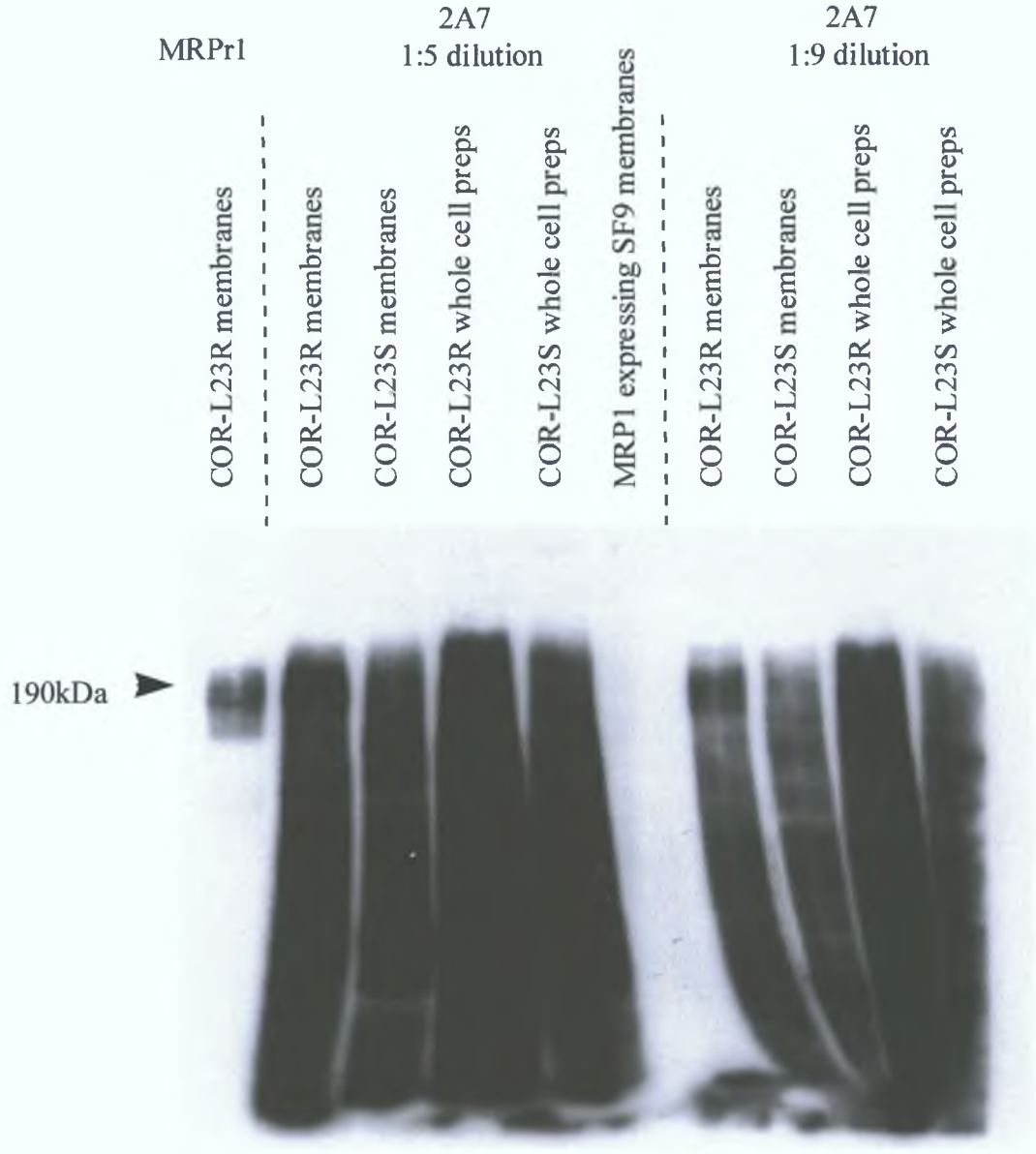
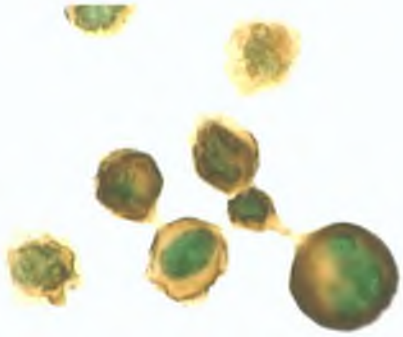


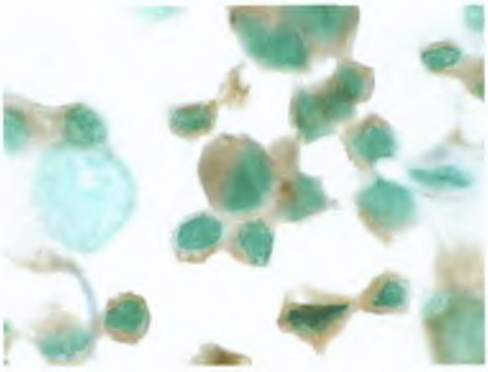
FIGURE 3.7.23. Western Blot analysis of COR-L23R and COR-L23S membrane and whole cell lysate preparations and MRP1 expressing SF9 membranes with the hybridoma supernatant 2A7.

Immunocytochemical characterisation

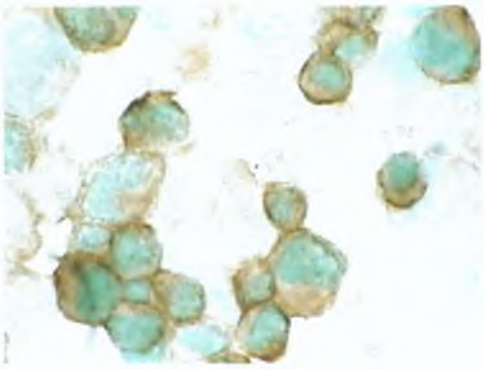
MRPr1 consistently stains the plasma membrane of COR-L23R and HL60ADR cells more intensely than COR-L23S and HL60S cells (see Figure 3 7 24) MAb 2A7 stained COR-L23R and COR-L23S cells in the plasma membrane region but stained the HL60ADR and HL60S cells in their nucleoli (see Figure 3 7 25) MAb 6C6 stained COR-L23R and COR-L23S cells in the plasma membrane region also but stained HL60ADR and HL60S cells in the cytoplasmic region (see Figure 3 7 26) The negative controls can be viewed in Figure 3 7 27



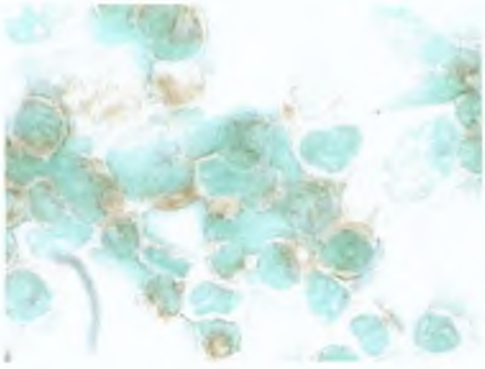
(A) COR-L23R cells stained with MRPr1



(B) COR-L23S cells stained with MRPr1

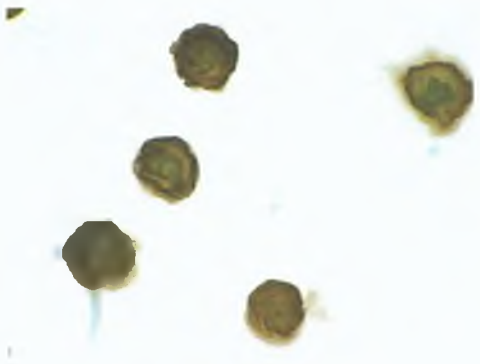


(C) HL60ADR cells stained with MRPr1

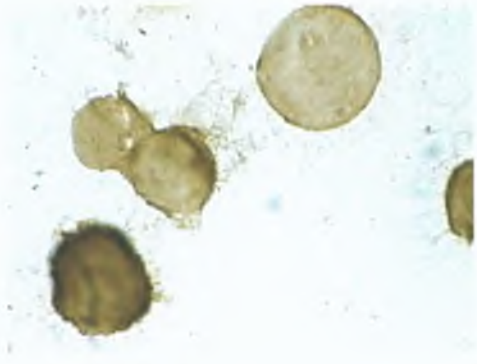


(D) HL60S cells stained with MRPr1

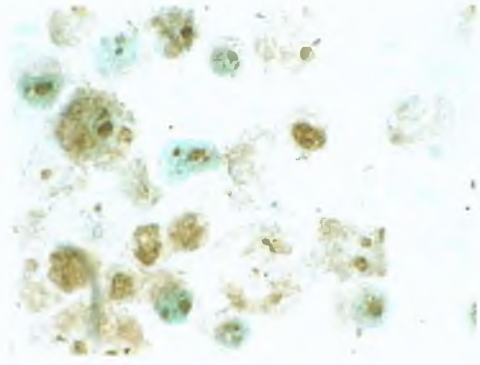
FIGURE 3.7.24. Immunocytochemical analysis of COR-L23R, COR-L23S, HL60ADR and HL60S cells with the rat monoclonal antibody MRPr1. MRPr1 intensely stains the COR-L23R and HL60ADR cells in the plasma membrane region but does not stain the drug sensitive COR-L23S or HL60S cells. (100X magnification).



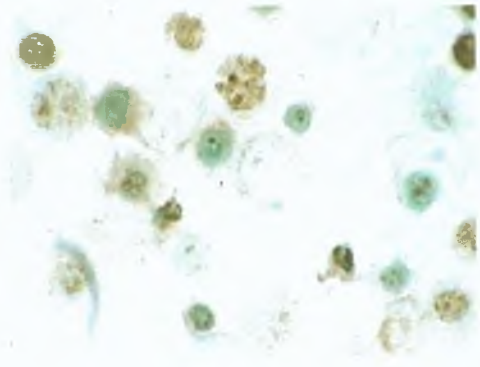
(A) COR-L23R cells stained with 2A7



(B) COR-L23S cells stained with 2A7

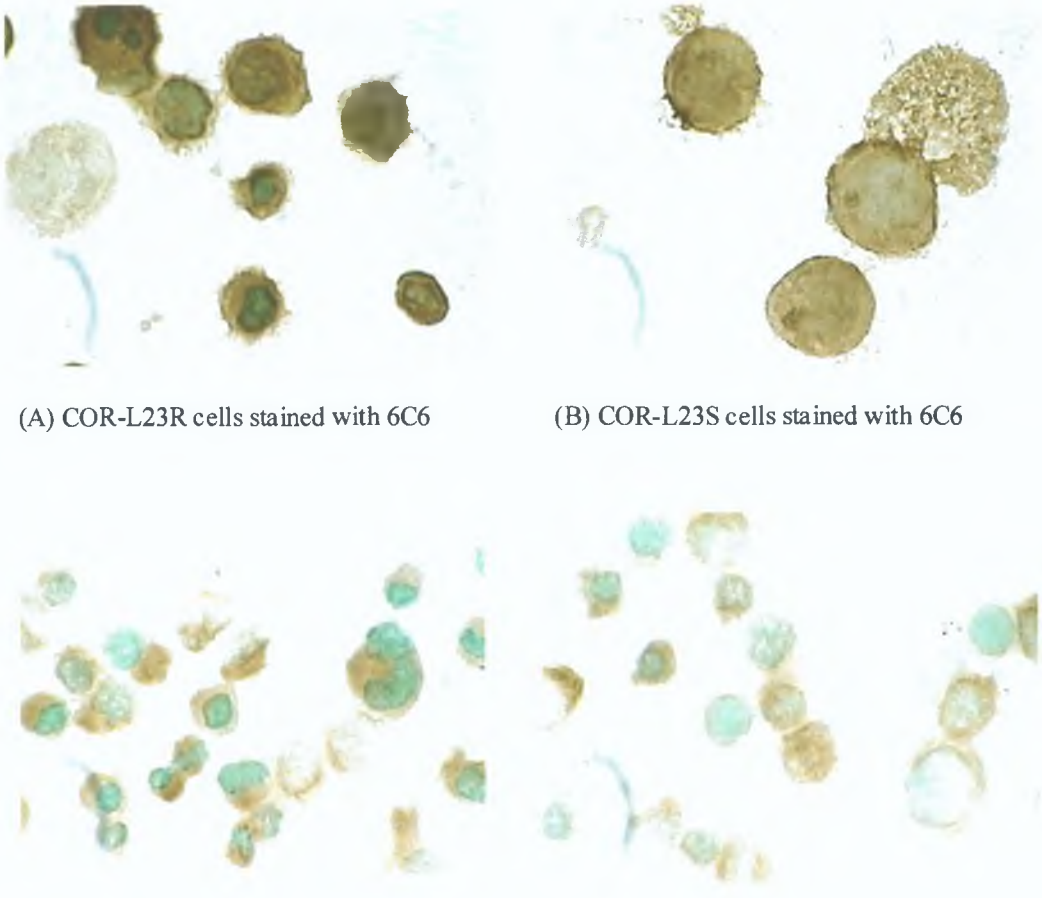


(C) HL60ADR cells stained with 2A7



HL60S cells stained with 2A7

FIGURE 3.7.25. Immunocytochemical analysis of COR-L23R, COR-L23S, HL60ADR and HL60S cells with MAb 2A7. MAb 2A7 stains the COR-L23R and COR-L23S cells in the plasma membrane region but stains the Hl60ADR and HL60S cells in the nuclei. (100X magnification).



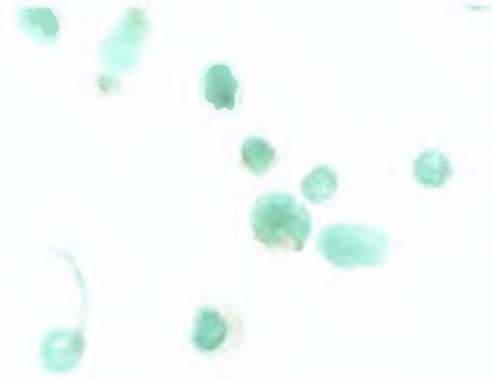
(A) COR-L23R cells stained with 6C6

(B) COR-L23S cells stained with 6C6

(C) HL60ADR cells stained with 6C6

(D) HL60S cells stained with 6C6

FIGURE 3.7.26. Immunocytochemical analysis of COR-L23R, COR-L23S, HL60ADR and HL60S cells with MAb 6C6. MAb 6C6 stains the COR-L23R and COR-L23S cells in the plasma membrane region and the HL60ADR and HL60S cells in the cytoplasmic region. (100X magnification).



(A) COR-L23R cells stained with TBS

(B) COR-L23S cells stained with TBS

(C) HL60ADR cells stained with TBS

(D) HL60S cells stained with TBS

FIGURE 3.7.27. Immunocytochemical analysis of COR-L23R, COR-L23S, HL60ADR and HL60S cells with negative control. (100X magnification).

3 8 RAT MONOCLONAL ANTIBODY PRODUCTION

Immunisations

In order to generate rat monoclonal antibodies for the detection of MRP1, female Wistar rats were immunised with either a double peptide combination or MRP1 over-expressing COR-L23R membranes Two synthetic peptides corresponding to the deduced amino acid sequence of human MRP1 (Peptide 2, amino acids 235-246 and Peptide 3, ammo acids 246-260) (see Diagram 3 5 2) were used in the double peptide immunisation The epitope recognised by the MRP1 specific rat antibody, MRPr1, has been mapped to amino acids 238 to 247 of the deduced ammo acid sequence of human MRP1 This amino acid sequence is similar to that of Peptide 2 and was used as a positive control The COR-L23R membranes were used as an immunogen in the hope of obtaining an antibody recognising an external epitope The rats received footpad injections of 10-25ug of protein initially emulsified in freunds complete, followed two weeks later by a booster emulsified in freunds incomplete and finally 4 days before fusion a booster in PBS without adjuvant

Fusions.

Lymphocytes were isolated from the popliteal nodes (see Diagram 2 3 2) of the immunised rats and fused with mouse myeloma Sp2/0 cells using polyethylene glycol Prior to this, the popliteal lymph nodes were identified and removed in practice runs by injecting Isosulfan blue into the footpads of non-immunised rats Isosulfan blue is selectively picked up by the lymphatic vessels and is drained into the popliteal nodes making them discernible from the surrounding tissue Fusion rates for all the immunisations were approximately 95%

Screening of hybridomas

Screening of confluent supernatants from the double peptide immunisation was carried out by ELISA against the relevant peptides or by Western blot analysis, against cell membrane preparations of the MRP1-over-expressing COR-L23R cell line Screening of confluent supernatants from the COR-L23R cell membrane immunisation was carried out by live cell immunofluoresence against the COR-L23R and COR-L23S cells

Results

Antibodies resulting from the COR-L23R membrane immunisation reacted with both the COR-L23R and COR-L23S cell lines by live cell immunofluoresence and therefore were not characterised any further Approximately 10% of hybridomas analysed from the double peptide immunisation by ELISA were positive to the linear peptides Western blot analysis showed that a number of these hybridomas secreted antibodies that reacted with the COR-L23R but not the COR-L23S cells Further characterisation showed that these antibodies detected a 190KD band in the COR-L23R, HL60ADR and MRP1-transfected cell line, 2008 MRP1, by Western blotting These antibodies were designated P2A8, P1A6 and P2E4 Of these antibodies P2A8 was cloned and designated P2A8(6) Immunocytochemical analysis of these antibodies identified one antibody, P2A8(6), which like the rat monoclonal antibody MRPr1, stained all cell populations in the COR-L23R and MRP1 2008 transfectants (see Figure 3 8 10) However, immunocytochemical analysis also identified an antibody, P1D7, which stained a subpopulation of cells in the COR-L23R and 2008 MRP1 transfected cell lines, unlike the MRP1 specific rat monoclonal antibody MRPr1 which stains all the cell population in these cell lines Further characterisation of P1D7 by Western blot

analysis revealed that it detected a 190kD band in the MRP1 2008 transfectants but not the drug selected COR-L23R cell line P1D7 did not cross react with the 2008 parental, 2008 MRP2 or 2008 MRP3 transfectants by Western blot analysis or immunocytochemical analysis In summary, these antibodies did not react with COR-L23S, HL60S, 2008 Parental, 2008 MRP2 or 2008 MRP3 cell lines by Western blotting or immunocytochemistry indicating that these antibodies are specific for MRP1 over-expressing cell lines (See following results)

3 8 5 Rat Isotyping ELISA Results

Isotyping of each antibody was carried out using a rat antibody isotyping kit (Pharmmgen, 04117K) All uncloned antibodies gave a range of isotypes However, the subclone P2A8(6) consisted mainly of IgG_{2a} κ chain with a slight amount of IgG_1 The MRP1 rat monoclonal antibody, MRPr1, is IgG_{2a} κ chain See Table 3 8 1

Table 3.8.1.

ANTIBODY	ISOTYPE	CHAIN
MRPr1	IgG2a	κ
P2A8(6) clone	IgG2a mainly, some IgG1	κ
P2A8	Various	κ and λ
P1D7	Various	κ and λ
P1A6	Various	κ and λ
P2E4	Various	κ and λ

3 8 6 Reactive Peptide ELISA results

Both Peptide 2 and Peptide 3 were used in separate ELISA screenings to determine which peptide each antibody recognised Results are outlined in Table 3 8 2

Table 3.8.2.

ANTIBODY	REACTIVE PEPTIDE
MRPr1	Peptide 2
P2A8	Peptide 3
P2A8(6) clone	Peptide 3
P1D7	Peptide 3
P1A6	Peptide 3
P2E4	Peptide 3

3 8 7 Peptide Inhibition Studies

Peptide inhibition studies were carried out with each antibody by Western blot analysis to further confirm their peptide specificity Antibody supernatants were incubated overnight with either of the two immunisation peptides and a control supernatant containing neither peptide These supernatants were then analysed by Western blot analysis with MRP1 transfected 2008 cells The resulting blots confirmed results obtained by the Reactive Peptide ELISA (see following blots)

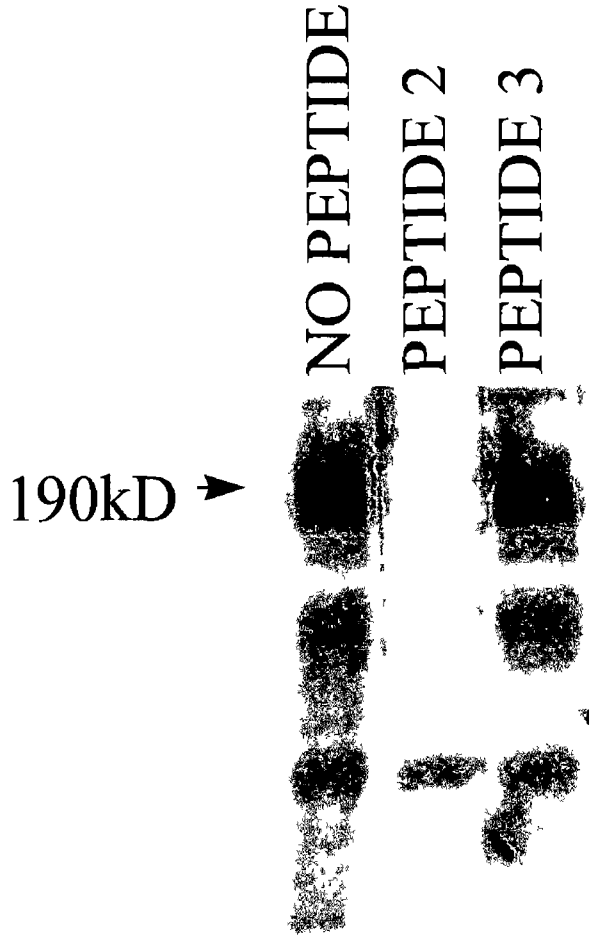


Figure 3.8.1. Peptide-inhibition studies using the 2008 MRP1 transfected cell line, by Western blot analysis with the rat MAb, MRPr1 Peptide 2 containing the MRPr1 epitope inhibits the binding of the MRPr1 antibody

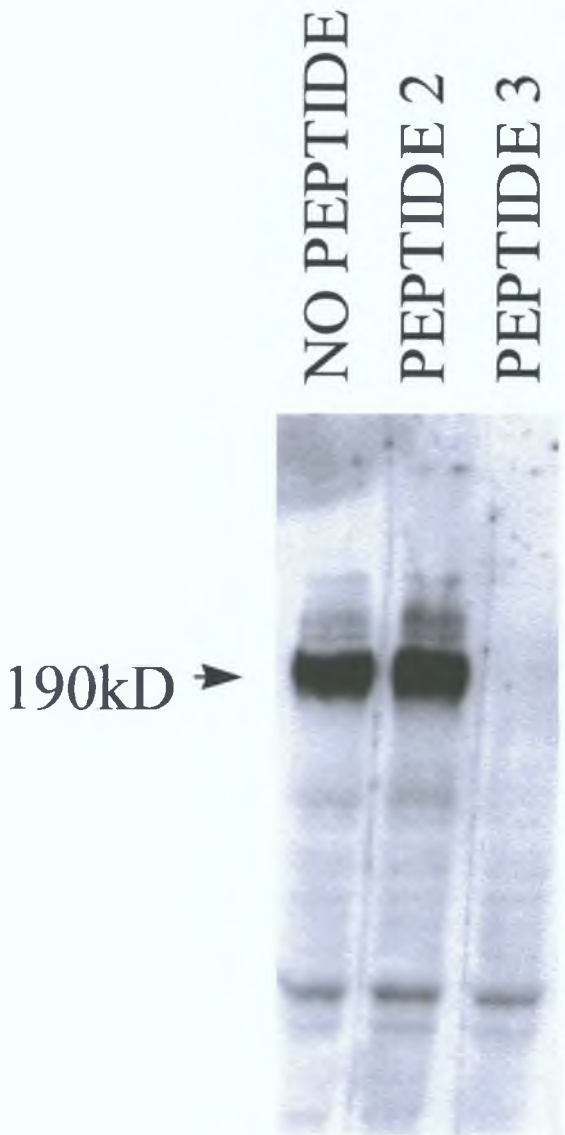


Figure 3.8.2. Peptide-inhibition studies in the MRP1 transfected cell line 2008 MRP1, by Western blot analysis with the rat MAb, P2A8(6). Peptide 3 inhibits the binding of the P2A8 antibody determining that Peptide 3 contains the P2A8(6) epitope.

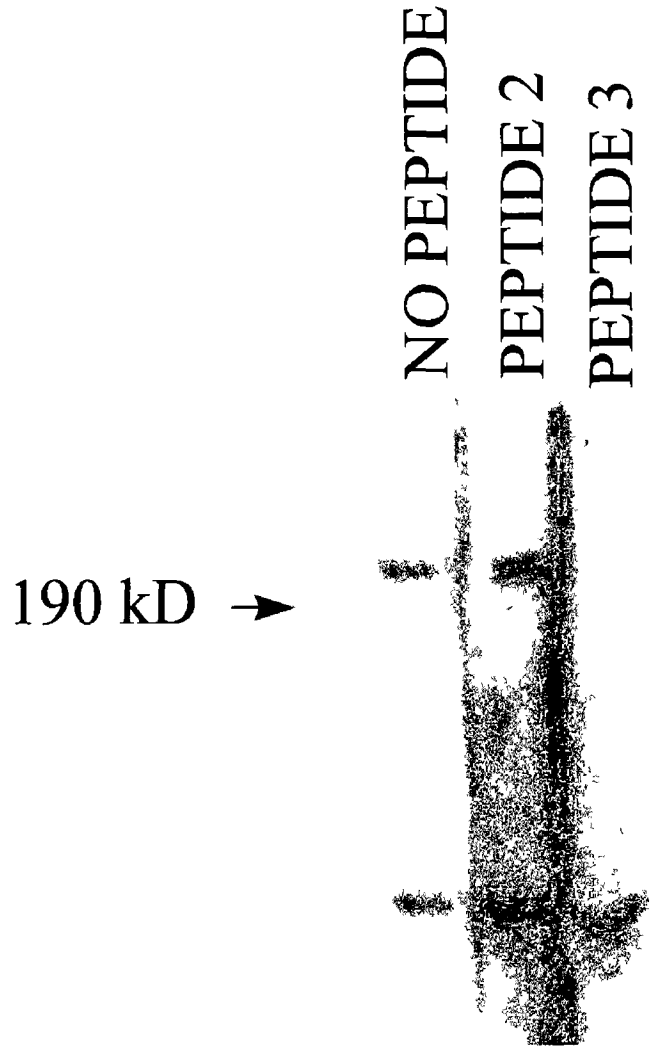


Figure 3.8.3. Peptide-inhibition studies in the MRP1 transfected cell line 2008 MRP1, by Western blot analysis with the rat MAb, P1D7 Peptide 3 inhibits the binding of the P1D7 antibody determining that Peptide 3 contains the P1D7 epitope

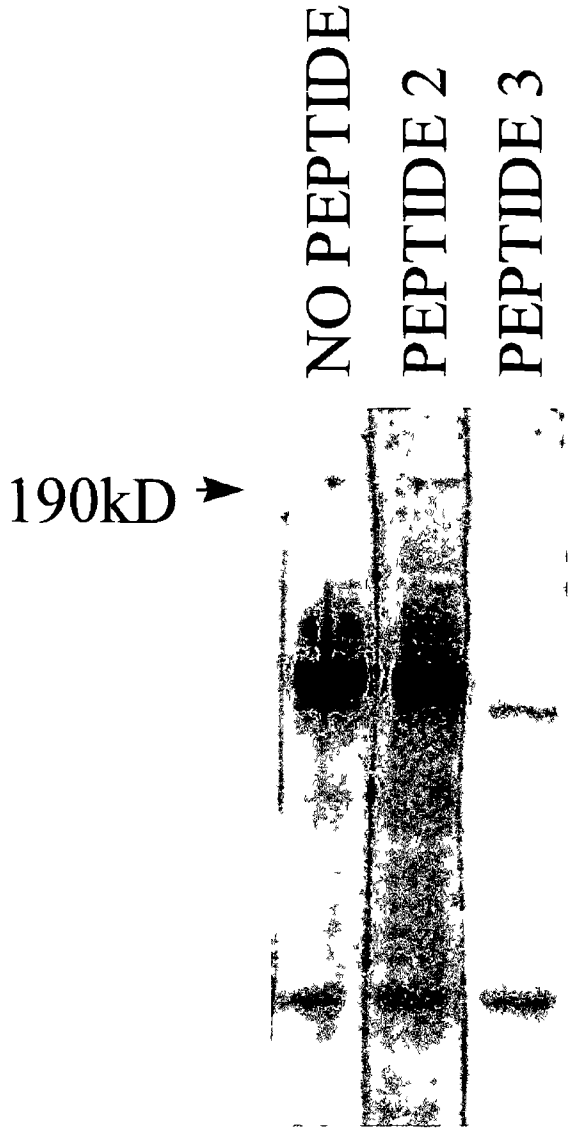


Figure 3.8.4. Peptide-inhibition studies in the MRP1 transfected cell line 2008 MRP1, by Western blot analysis with the rat MAb, P1A6 Peptide 3 inhibits the binding of the P1A6 antibody determining that Peptide 3 contains the P1A6 epitope

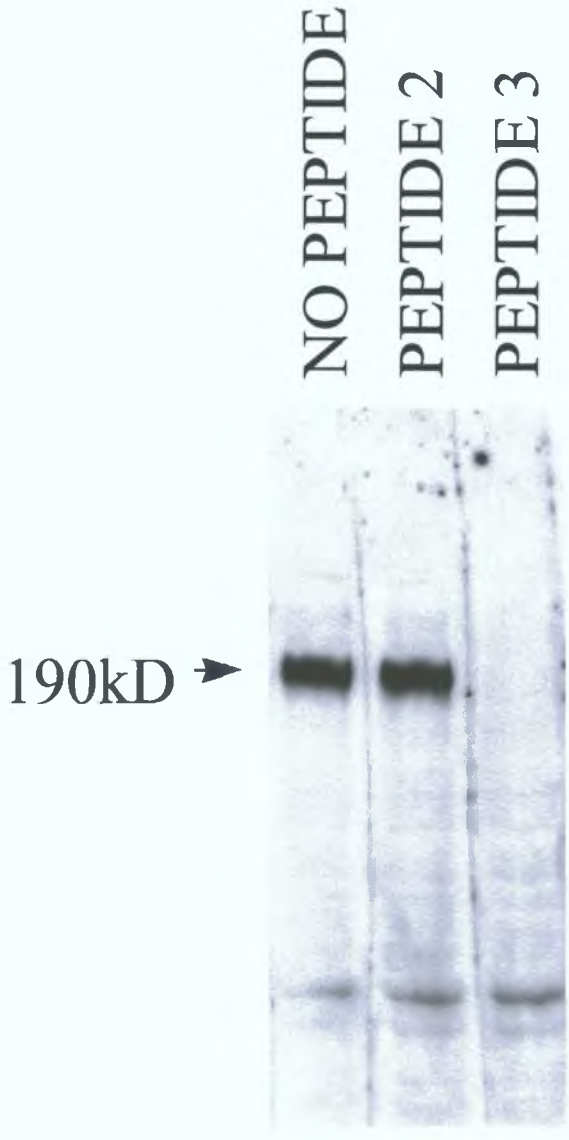


Figure 3.8.5. Peptide-inhibition studies in the MRP1 transfected cell line 2008 MRP1, by Western blot analysis with the rat MAb, P2E4. Peptide 3 inhibits the binding of the P2E4 antibody determining that Peptide 3 contains the P2E4 epitope.

3 8 8 Western Blot analysis

P2A8(6)

The MAb P2A8(6) was cloned from the MAb P2A8 Western blot analysis of this MAb revealed that it is specific for a 190kD band in MRP1 over-expressing cells P2A8(6) detects a 190kD band in drug selected MRP1 over-expressing COR-L23R cells and MRP1 transfected 2008 MRP1 cells (see Figure 3 8 6) P2A8(6) also detects a 190kD band in the drug selected MRP1 over-expressing HL60ADR cells (see Figure 3 8 7)

P1D7

The MAb P1D7 detects a 190kD band in the 2008 MRP1 transfectants (see Figure 3 8 8) However, P1D7 does not detect a 190kD band in the drug-selected MRP1-over-expressing COR-L23R cell line suggesting that this MAb is specific for the transfected MRP1 and not the drug-induced MRP1 (see Figure 3 8 9)

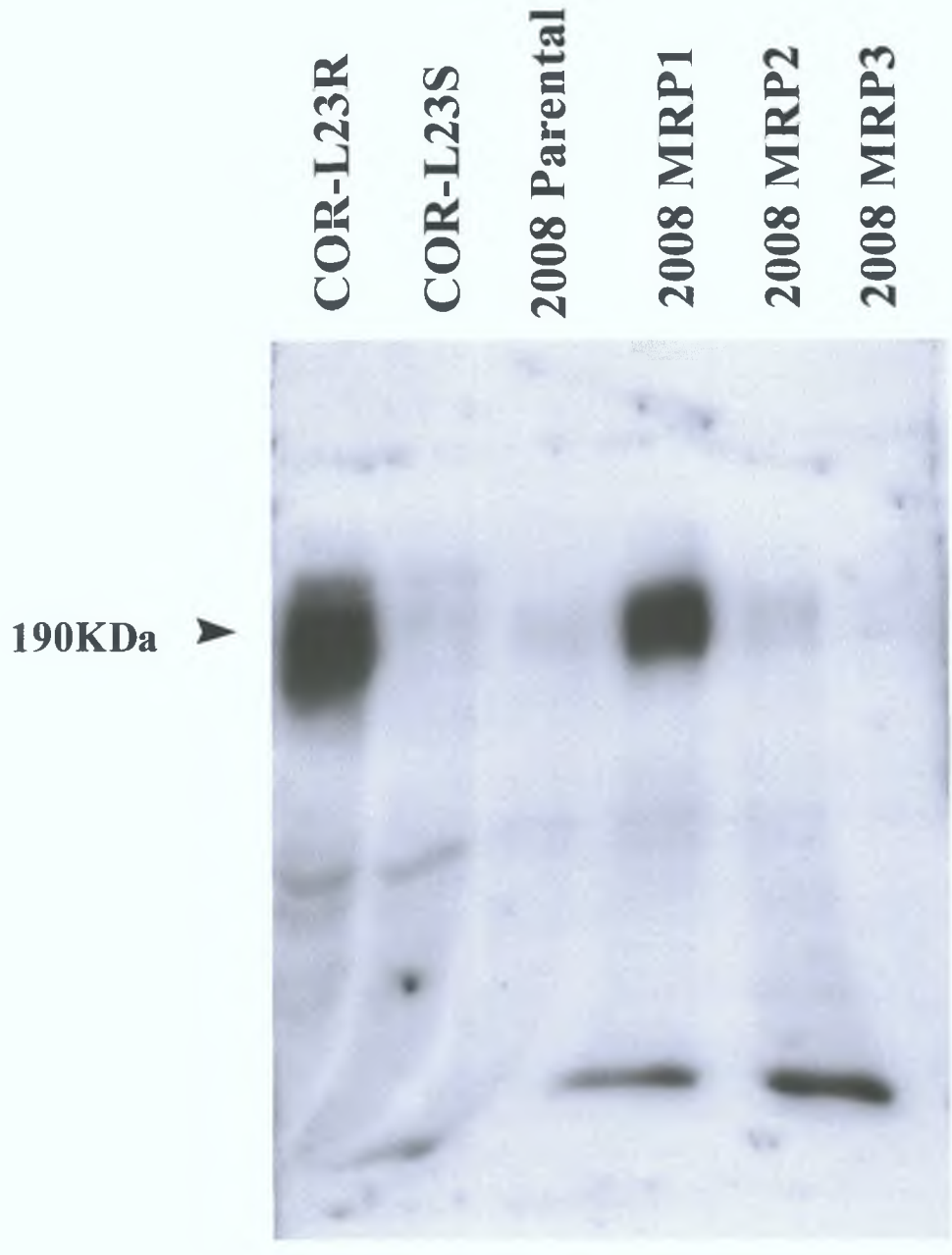


Figure 3.8.6. Western blot analysis of COR-L23R, COR-L23S, 2008 Parental, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells with MAb P2A8(6). MAb P2A8(6) detects a 190KD band in the MRP1 over-expressing COR-L23R and MRP1 transfected 2008 MRP1 cell lines.

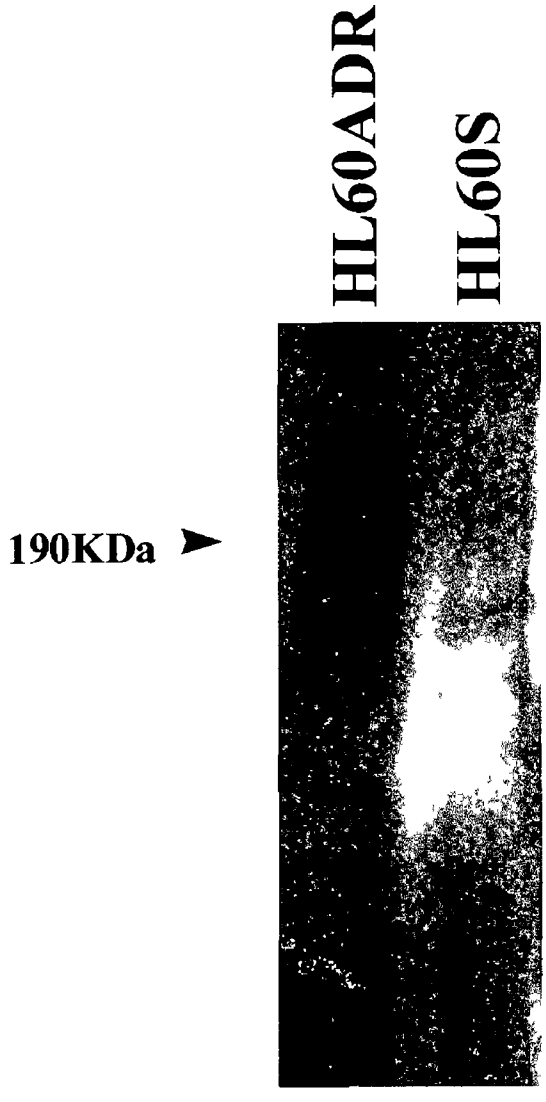


Figure 3.8.7. Western blot analysis of HL60ADR and HL60S cells with MAb P2A8(6) MAb P2A8(6) detects a 190KD band in the MRP1-over-expressing HL60ADR cell line

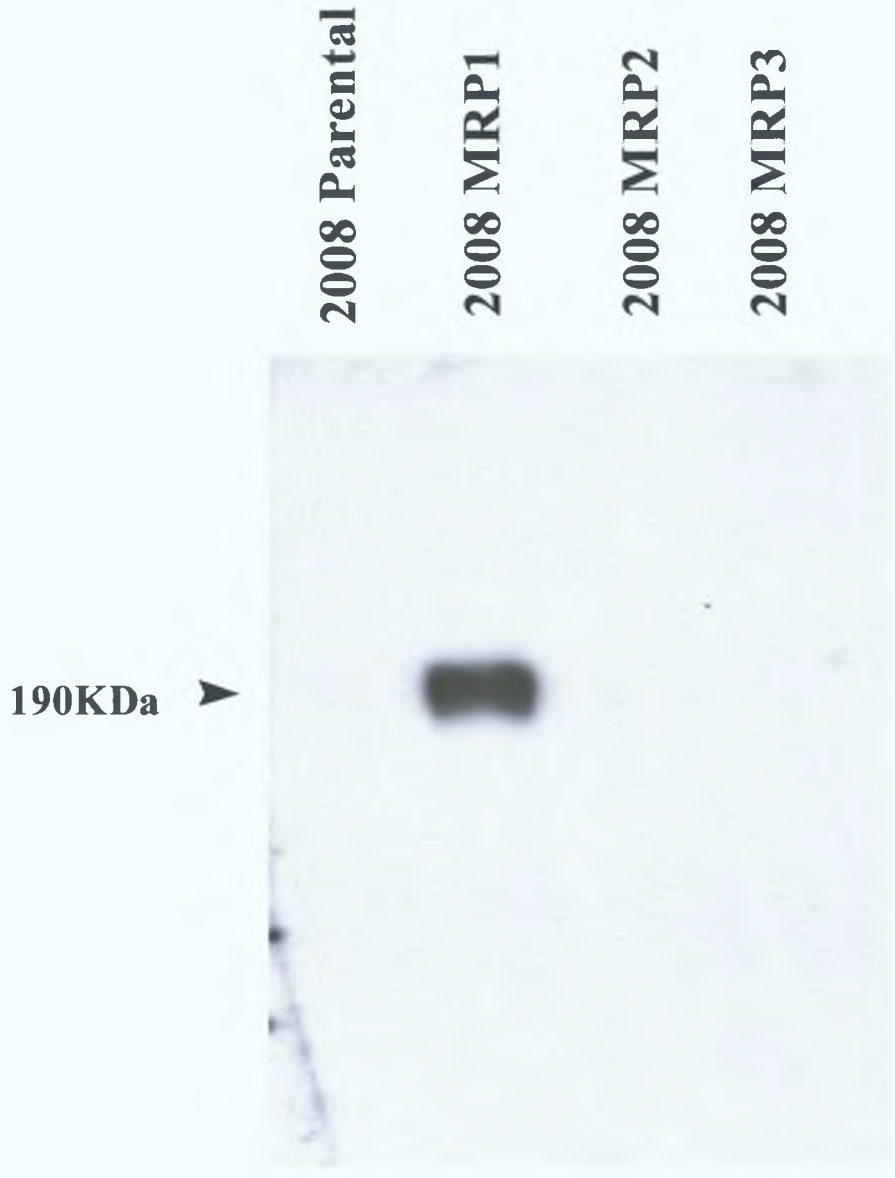


Figure 3.8.8. Western blot analysis of 2008 Parental, 2008 MRP1, 2008 MRP2 and 2008 MRP3 cells with the MAb P1D7. MAb P1D7 detects a 190KD band in the 2008 MRP1 cell line.

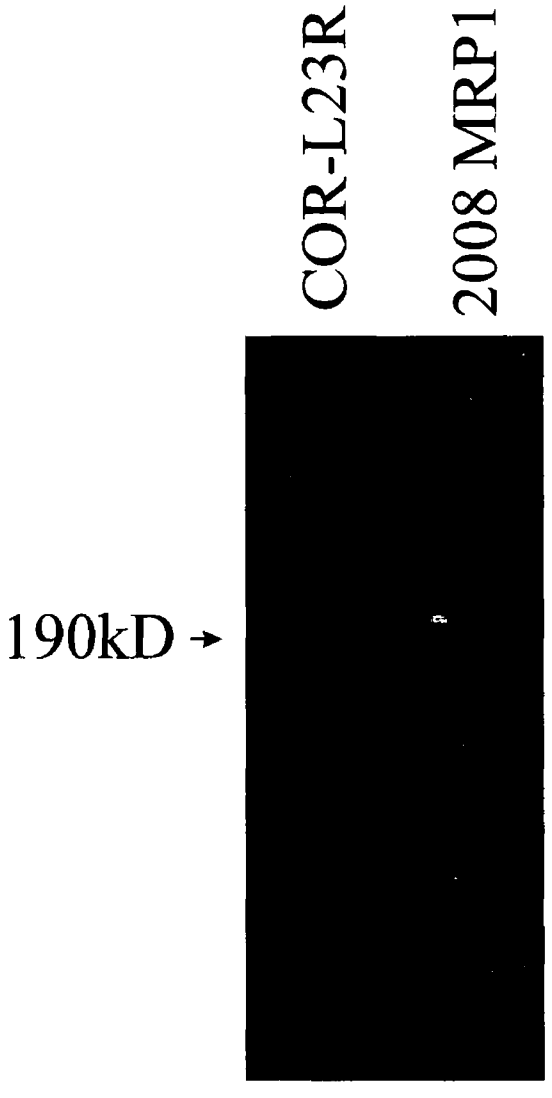


Figure 3.8.9. Western blot analysis of COR-L23R and 2008 MRP1-transfected cells with the MAb P1D7 MAb P1D7 does not detect a 190KD band in the COR-L23RR cell line whereas P1D7 detects a strong 190KD band in the 2008 MRP1-transfected cell line

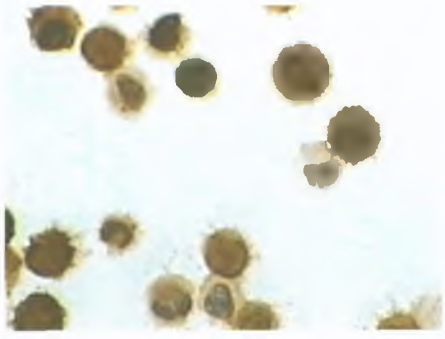
3 8 9 Immunocytochemical analysis·

P2A8(6)

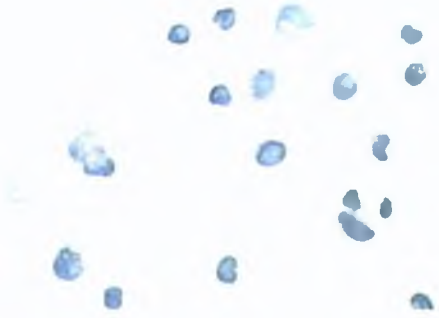
Immunocytochemical analysis of various cell lines with P2A8(6) shows strong plasma-membranous staining in every cell population of the COR-L23R and 2008 MRP1 cell lines A basal level of staining was detected in the Parental and MRP2 and MRP3 2008 transfectants (see Figure 3 8 10) The staining by P2A8(6) of these cell lines is similar to the staining of these same cell lines by the rat monoclonal antibody, MRPr1, in Figures 3 2 6 and 3 2 7

P1D7

Immunocytochemical of various cell lines with P1D7 shows strong plasma-membranous staining in a sub-population of cells in the COR-L23R and 2008 MRP1 cell lines P1D7 does not stain non or low MRP1-expressing cells (see Figure 3 8 11)



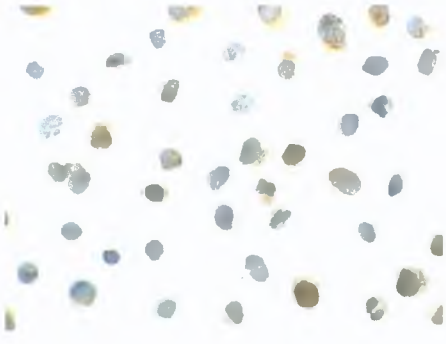
1. COR-L23R



2. COR-L23S

3. 2008 Parental.

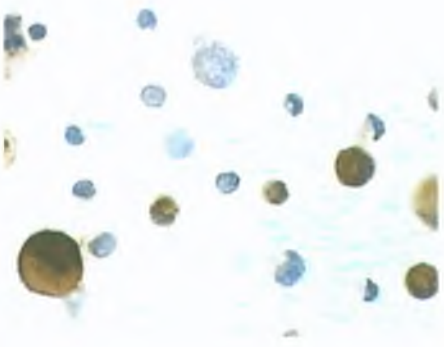
4. 2008 MRP1



5. 2008 MRP2

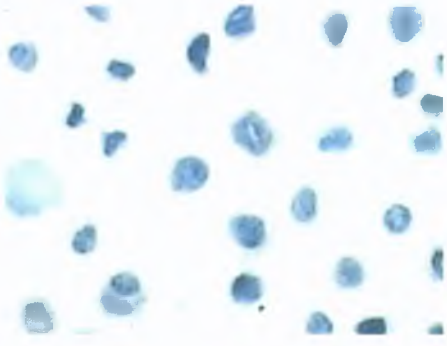
6. 2008 MRP3

Figure 3.8.10. Immunocytochemical analysis of various cell lines with the rat MAb P2A8(6). MAb P2A8(6) shows strong plasma-membranous staining in every cell population of the COR-L23R and 2008 MRP1 cell lines. A basal level of staining can be seen on the Parental and MRP2 and MRP3 2008 transfectants. The staining by P2A8(6) of these cell lines is similar to the staining of these same cell lines by the rat monoclonal antibody, MRPr1, in Figures 3.2.6. and 3.2.7.

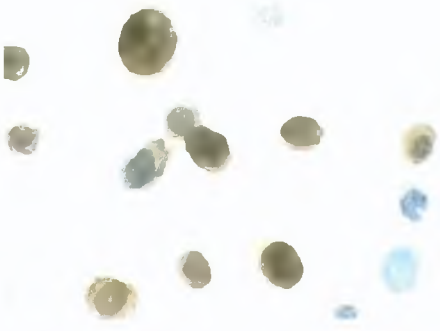


1. COR-L23R

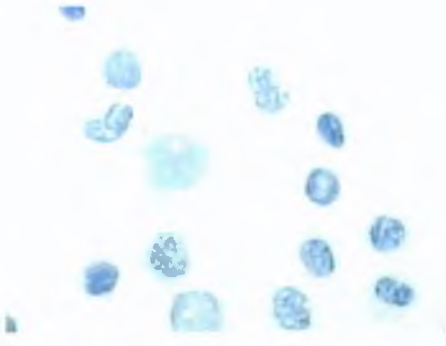
2. COR-L23S



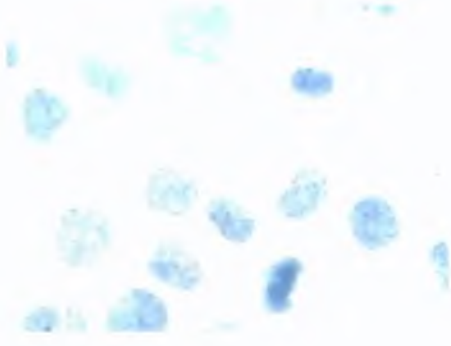
3. 2008 Parental



4. 2008 MRP1



5. 2008 MRP2



6. 2008 MRP3

Figure 3.8.11. Immunocytochemical analysis of various cell lines with the rat monoclonal antibody P1D7. MAb P1D7 shows strong plasma-membranous staining in a sub-population of cells in the COR-L23R and 2008 MRP1 cell lines but does not stain any of the non or low MRP1-expressing cell lines.

3 9 SUMMARY OF MOST INTERESTING ANTIBODIES

The antibodies below were chosen because they are specific, consistent, stable and are applicable in immunocytochemical and Western blotting techniques

Table 3 9 1.

ANTIBODY	IMMUNOGEN	SPECIES	PROTEIN SPECIFICITY
Polyclonal 1	Peptide 1	Rabbit	MRP1
Polyclonal 2	Peptide 2	Rabbit	MRP1
Polyclonal 3	Peptide 3	Rabbit	MRP1
1A2	Peptide 1	Mouse	MRP1 and/or another protein or MRP form
P2A8(6)	Peptide 2 and Peptide 3	Rat	MRP1
P1D7	Peptide 2 and Peptide 3	Rat	Transfected MRP1

3 10 CELL LINE SURVEY

A cell line survey was carried out on a small number of cell lines by Western blot analysis The most interesting MAbs (listed in Table 3 9 1) and MRPr1, were surveyed

Table 3.10.1. Summary of cell line survey.

	PROTEIN EXPRESSION LEVELS (as determined by Western blotting)					
CELL LINE	**1A2**	**P2A8(6)**	**MRPr1** (MRP1)	**M2III-6** (MRP2)	**M3II-21** (MRP3)	**M5II-54** (MRP5)
COR-L23R	+	+	+	—	—	—
COR-L23S	+	+/-	+/-	—	—	—
HL60ADR	+	+	+	—	—	—
HL60S	—	—	—	—	—	—
DLKP	+	—	—	—	—	—
DLKPA	+/-	—	—	—	—	—
A549	+	+	+	+	—	—
RPMI 2650 Parental	+	—	+/-	—	—	—
RPMI 2650 Taxol	—	—	—	—	—	—
RPMI 2650 Melphalan	+	+	+	+	—	—

Table **3.10.1.** KEY:

-	undetectable levels
+	Over-expression
+/-	Very low basal levels

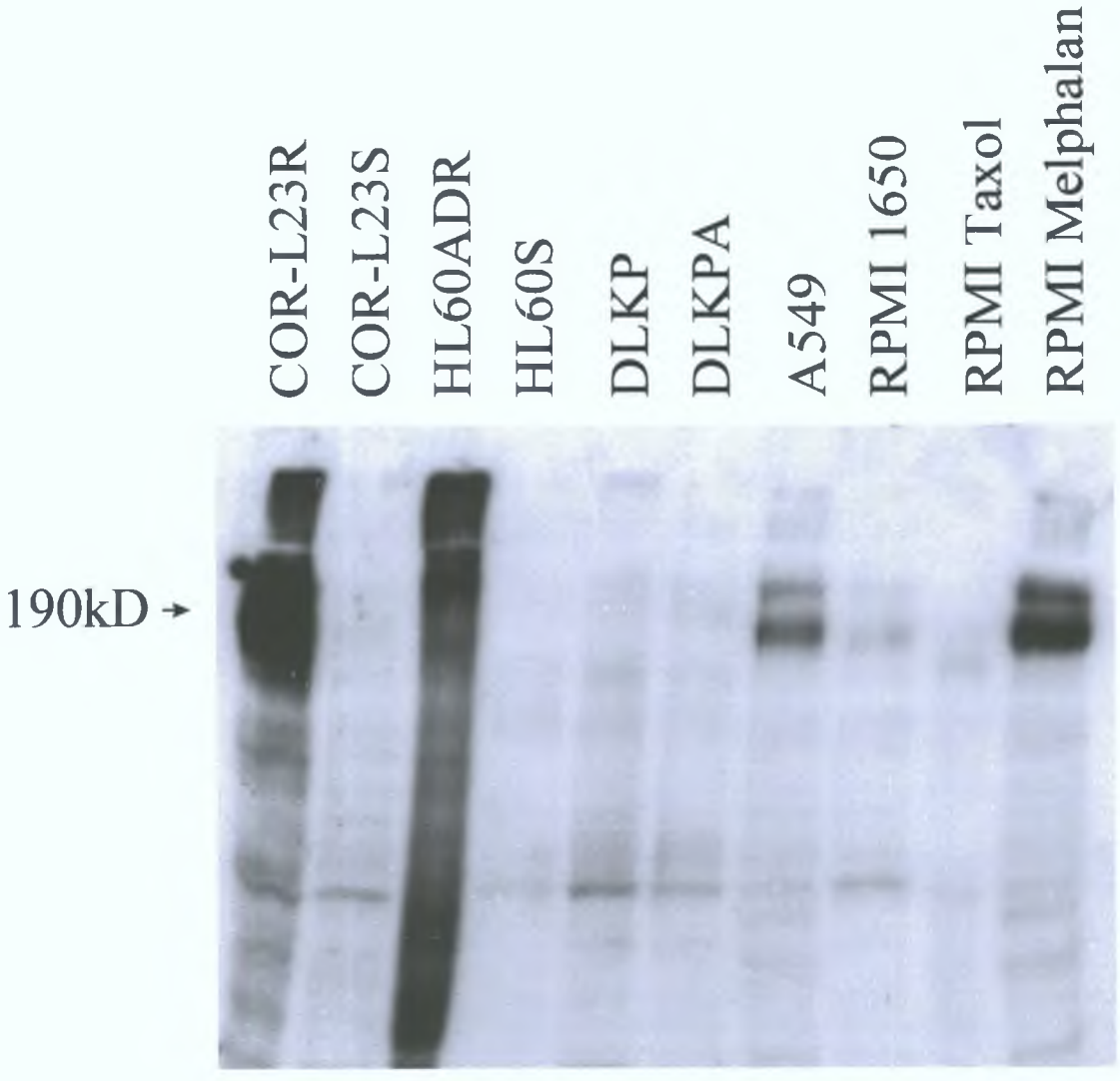


Figure 3.10.1. Survey by Western blot analysis, of various cell lines using the MRP1 specific MRPr1 MAb. Cell lines which express high levels of MRP1 are; COR-L23R, HL60ADR, A549 and RPMI Melphalan. Cell lines which express very low levels of MRP1 are; COR-L23S and RPMI 1650.

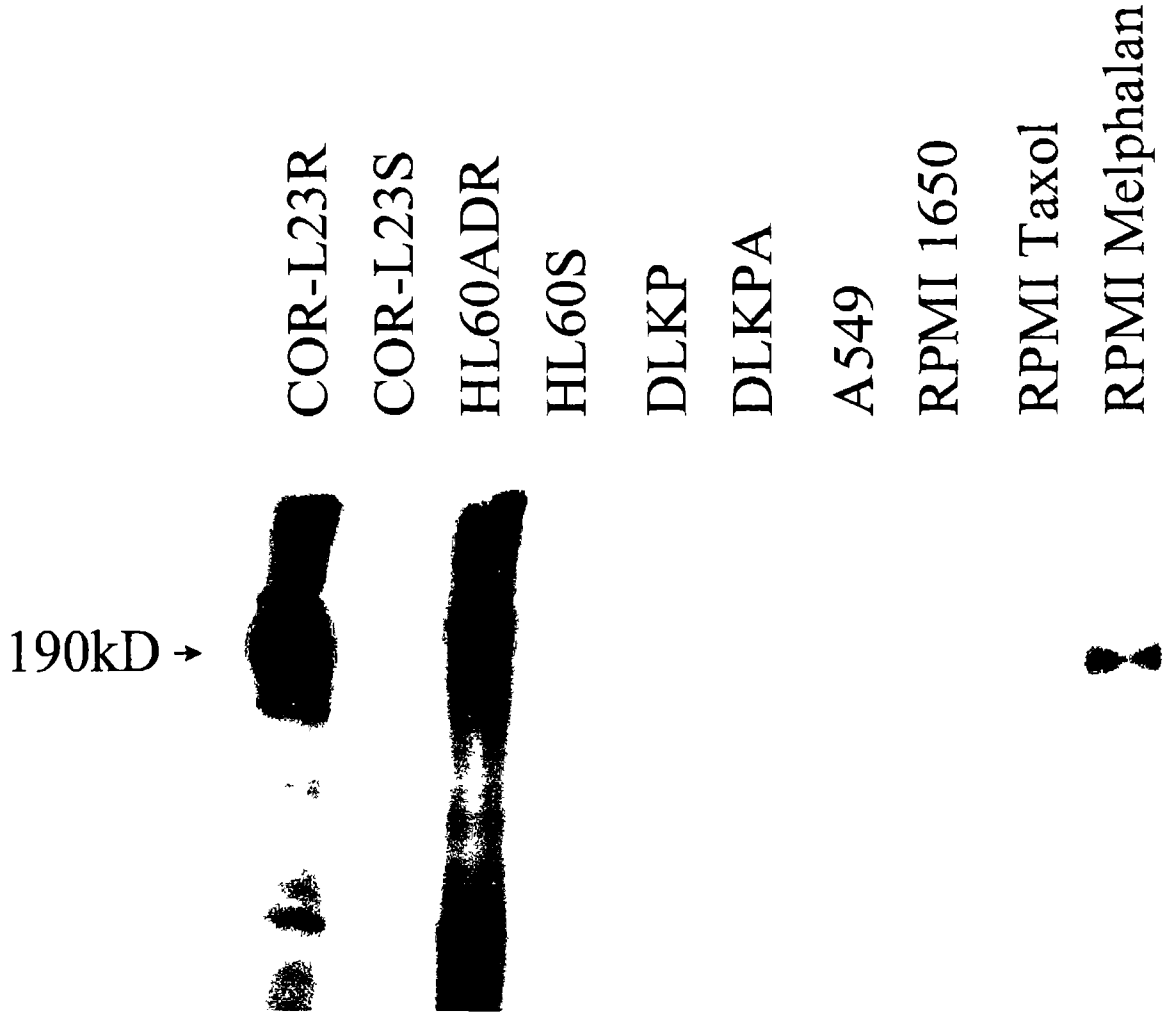


Figure 3.10.2. Survey by Western blot analysis, of various cell lines using the P2A8(6) MAb Cell lines which express high levels of P2A8(6) are, COR-L23R, HL60ADR, A549 and RPMI Melphalan

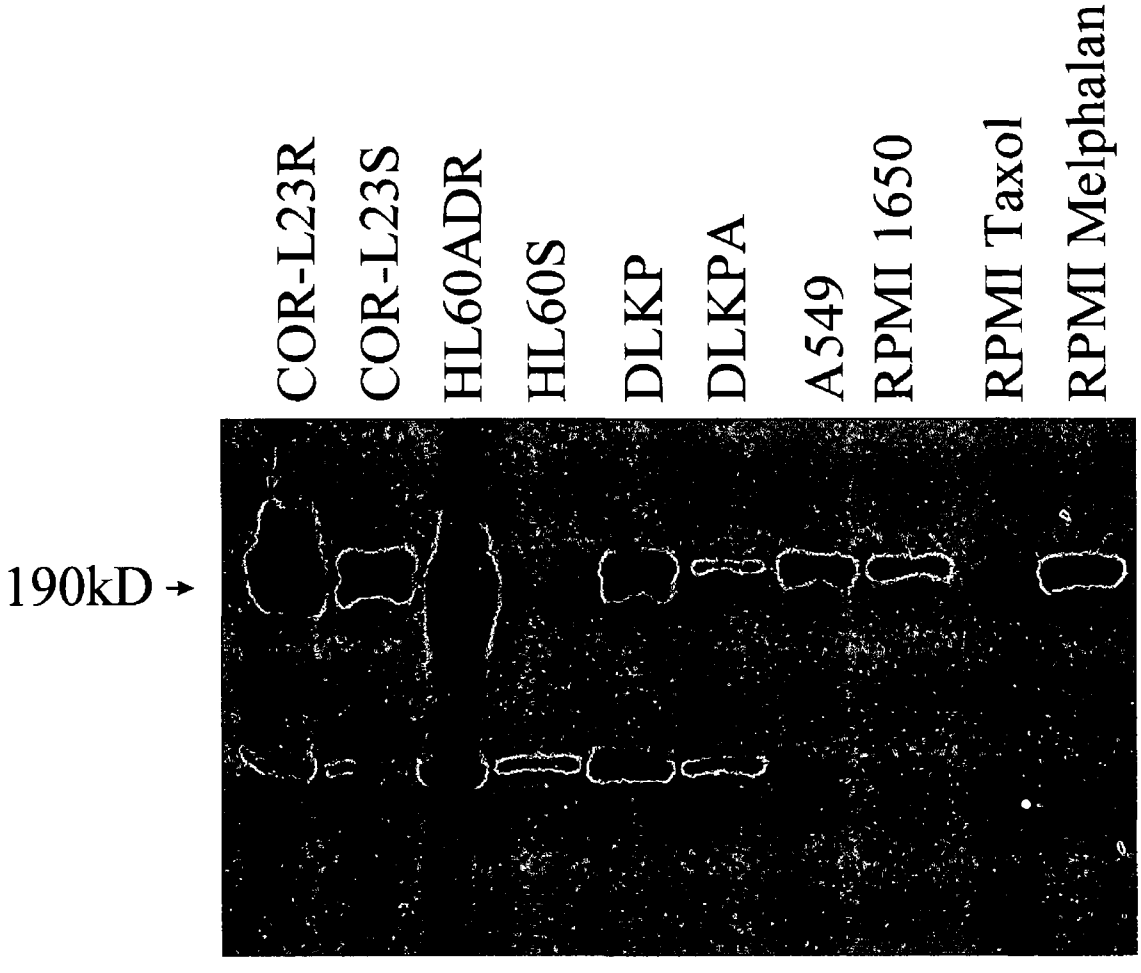


Figure 3.10.3. Survey by Western blot analysis, of various cell lines using the IA2 MAb Cell lines which express high levels of MAb IA2 are, COR-L23R, COR-L23S, HL60ADR, DLKP, A549, RPMI 2650 Parental and RPMI Melphalan Cell lines which express low levels of MRP1 are, DLKPA

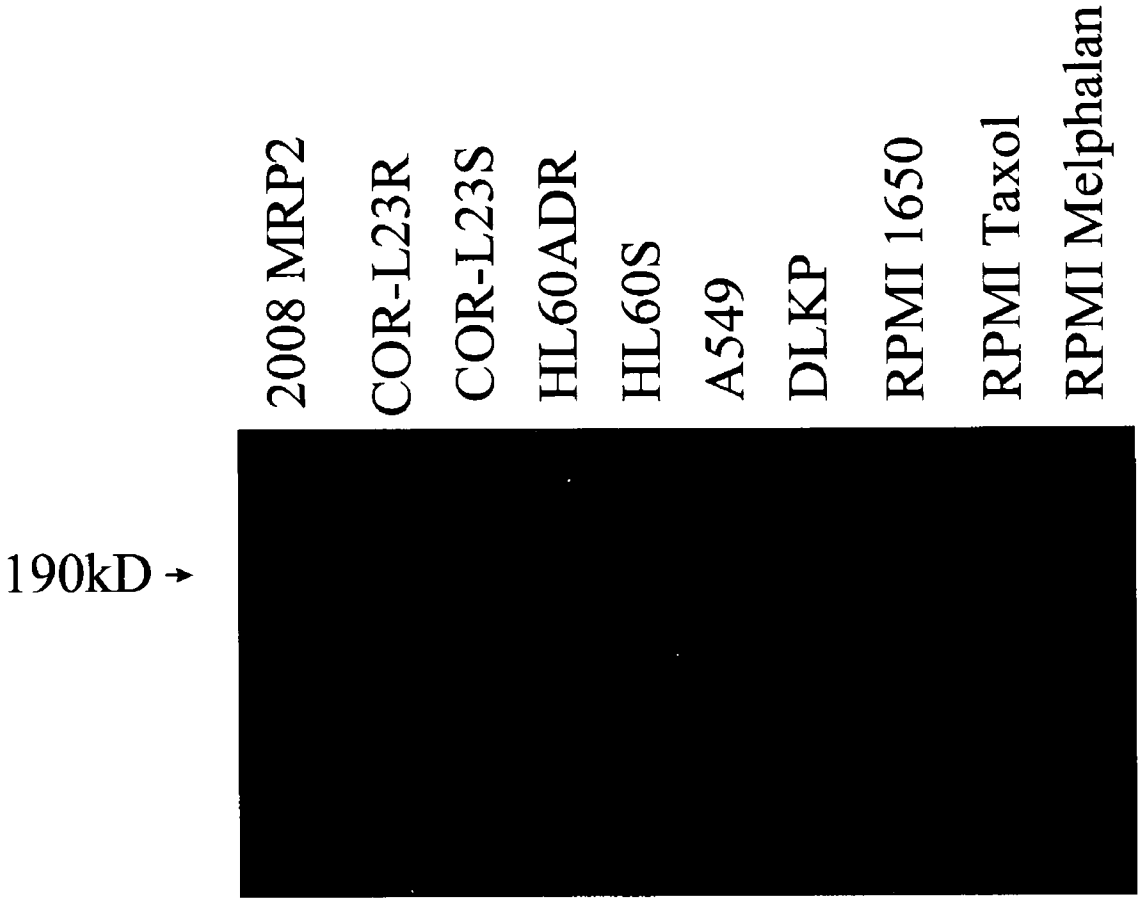


Figure 3.10.4. Survey by Western blot analysis, of various cell lines using the MRP2 specific MAb, M2III-6 Cell lines which express high levels of MRP2 are, 2008 MRP2 transfectants, A549 and RPMI 2650 Melphalan

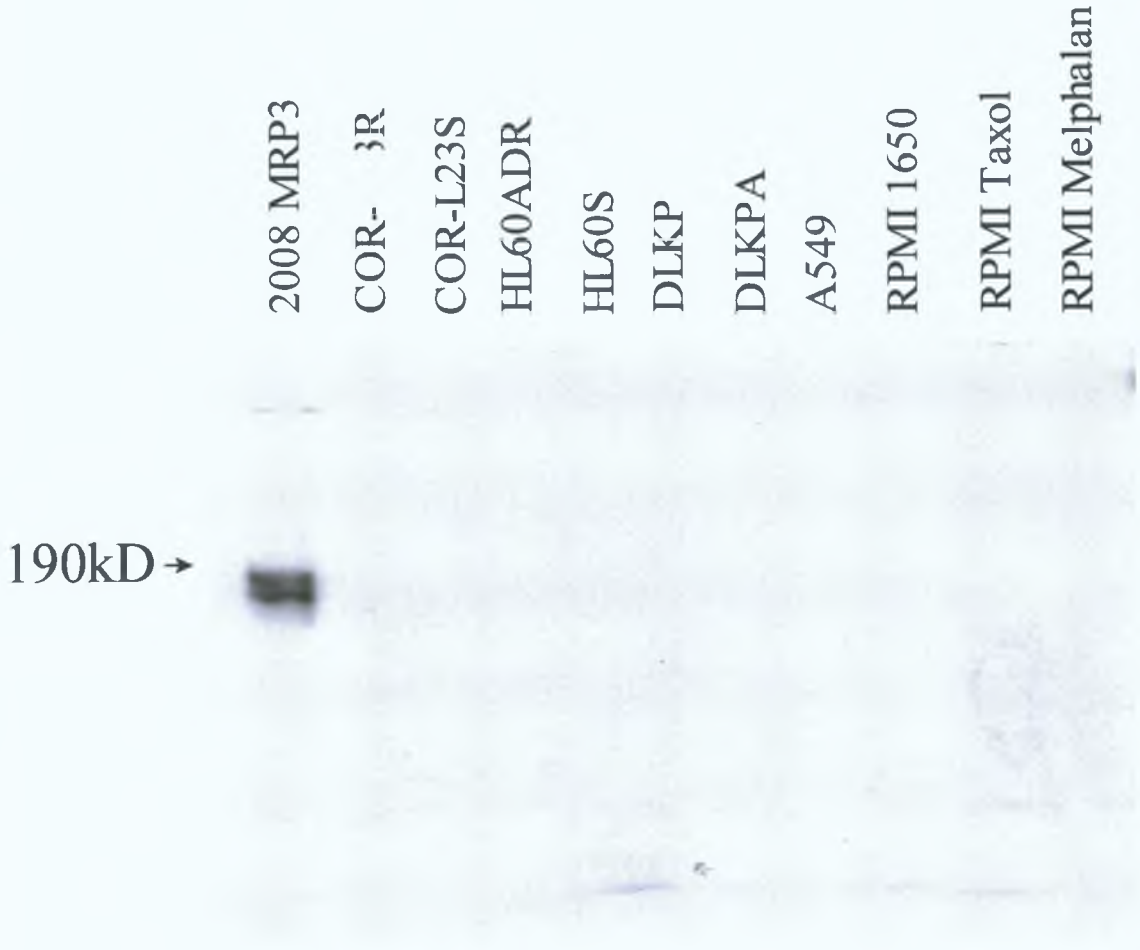


Figure 3.10.5. Survey by Western blot analysis, of various cell lines using the MRP3 specific MAb, M3II-21. None of the cell lines surveyed were found to express high levels of MRP3. However, the 2008 MRP3 transfectants did express a strong 190-170 kD doublet band.

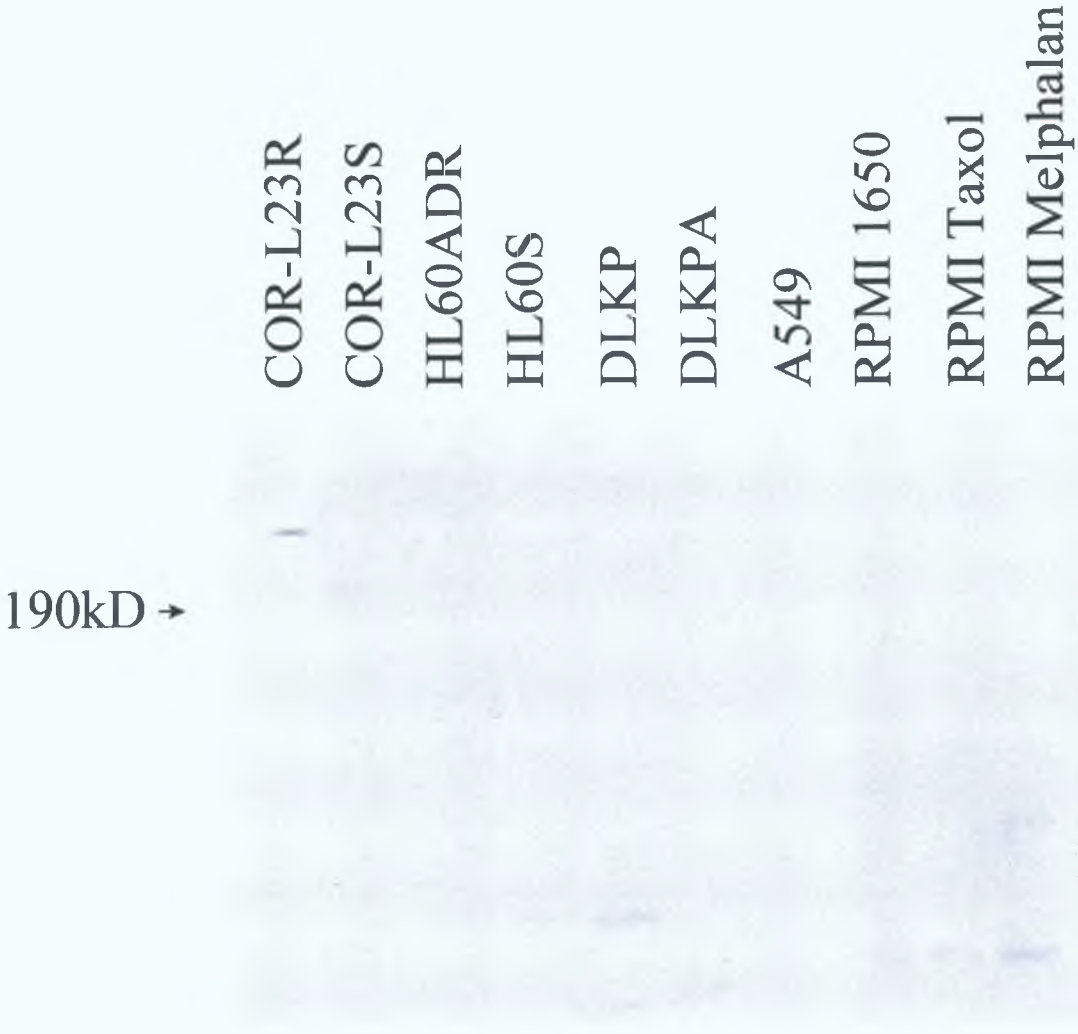


Figure 3.10.6. MRP5 cell line survey.

Survey by Western blot analysis, of various cell lines using the MRP5 specific MAb, M5II-54. None of the cell lines surveyed were found to express high levels of MRP5.

4.0. DISCUSSION

4 0 DISCUSSION

4 1 General

The choice and synthesis of short synthetic peptides for the production of specific monoclonal antibodies in our laboratory has proven highly successful Using short synthetic peptides our laboratory has produced a range of monoclonal antibodies specific for proteins such as the MDR-1 gene product, P-gp, MDR-3, and Topo II alpha (Moran et al , 1997, Larkin et al , 1999b)

However, many factors may limit the utility of short synthetic peptides Antibodies recognise conformational structures (epitopes) consisting of amino acid sequences which interact with the antigen-binding site of the antibody Epitopes may be either continuous in the primary amino acid sequence (linear epitopes), or discontinuous, resulting from juxtaposition of distant linear sequences There is no strict consensus as to what might be the minimal size of an antigenic determinant recognisable by antibodies Amino acid sequences of 7-10 amino acids may represent such a determinant As a consequence, synthetic linear peptides can be used to generate antibodies which cross-react with the native protein This offers the advantage that antibodies can be raised against antigenic structures to which antibodies would not easily be generated otherwise (for example, where a source of pure protein is unavailable) Therefore, when short peptides are utilised for immunisation, anti-peptide responses are generally obtained However, the recognition of the native protein in these cases is not always assured (Claassen *et al* , 1993) Induction of anti-native proteins by synthetic peptides is dependent on many parameters The successful application of the synthetic peptide approach involves a number of not entirely predictable elements, including the

selection of a sequence, immunisation regime and screening system

Therefore, the employment of other forms of immunogens may be required in order to gain a specific MAb which works under various conditions For example, longer peptides, such as fusion proteins, may be used to increase the chances of obtaining an antibody which recognises the native protein by increasing the chances of presenting unique linear amino acid sequences which are present in the native protein Immunising with the native protein itself greatly increases the chances of obtaining a MAb which recognises the native protein by presenting the antigen in its native conformation In this case, antibodies should be produced which are specific for the conformation of the native protein The pitfall in this scenario is that the location of the epitope is unknown and undirected For example, specific epitopes may be advantageous in functional assays and studies Short synthetic peptides are therefore useful for producing MAbs which recognise these specific sites The most abundant source of a cellular protein in its native form is obviously the cell itself Whole cell lysates or purer sources of the native protein extracted from the cell, depending on the location of the protein in the cell, may be used for immunisation In the case of MRP1, cell membranes are a rich source of this protein and can be purified from whole cell lysates to give a concentrated source of the native MRP1 protein

An important consideration for the successful production of a specific MAb is the choice of species or strain of an animal for immunisation A number of species are useful for hybridoma production including rats, and mice However, individual species of animals may elicit various immune responses to the same immunogen Importantly some antigens from origins other than mouse or rat species can induce much stronger

responses in rats than in mice and evidently the reciprocal could be true for other antigens. Immunogens must also be compared with individual species for homology to any proteins expressed by that species. The less homology found between the immunogen and species, the more likely that an immune response will be elicited against the immunogen.

The route of immunisation is extremely important for the presentation of the immunogen (Harlow and Lane 1988). Various routes such as subcutaneous, intraperitoneal and the lymphatic system present different environments for presentation of the antigen and may structurally change the conformation of the antigen presented to immune cells (Claassen *et al.*, 1993). Therefore, antibodies produced from the same antigen in various environments may detect various conformational forms of the antigen and may not be suitable for all assays.

Therefore, in our experience the production of specific MAbs using short synthetic peptides has proven successful for a number of antibodies mentioned above. Unfortunately, not all proteins will present the perfect scenario and may pose a difficult challenge in obtaining a specific MAb which recognises the native protein and works in various assays. The production of an MRP1-specific MAb has proven to be one such challenge demanding that a number of the variables discussed above be examined.

Two collaborations were set up to aid the production and characterisation of an MRP1 specific MAb. Our laboratory participated in collaboration with Professor Balazs Sarkadi of The Institute of Enzymology, Biological Research Center, Hungarian Academy of sciences, H-1113 Budapest, Hungary. Professor Sarkadi kindly provided

our laboratory with various fragments of the MRP1 protein expressed in SF9 insect cells and two large MRP1 fusion proteins These fusion proteins were used to immunise mice and screen the resulting hybridomas for an MRP1 specific MAb The SF9 insect membranes expressing various MRP1 fragments were in a limited quantity and were used to characterise the rabbit and mouse MRP1 antibodies produced in our laboratory In return we made available our three MRP1 polyclonals and our MRP1 MAbs to Professor Sarkadi's laboratory Another collaboration was set up between our laboratory and Mr George Scheffer of Professor Rik Schepers' laboratory, Department of Pathology and Oncology, Free University Hospital, 1081 HV Amsterdam Our laboratory received various MRP isoform MAbs as well as the parental ovarian cancer 2008 cell line, MRP1, MRP2 and MRP3 transfected 2008 cell lines from MR George Scheffer Permission for the use of the MRP3 transfected cell line (Kool *et al* 1999a) came from Professor Marcel Kool, Academic Medical Center, Laboratory of Neurozintuigen, Room K2-212, Meibergdreef 9, 1105 AZ Amsterdam These MRP MAbs and MRP transfected cell lines were used to further characterise our MRP1 antibodies and for a small cell line survey

During the course of this thesis many MRP1 antibodies were produced Rabbits, mice and rats were immunised with peptides, fusion proteins, whole cell lysates or cell membranes to generate antibodies for the detection of MRP1 Here efforts to produce MRP1-specific antibodies have proven successful in rabbit (polyclonal antibodies, PAbs) and rat (monoclonal antibodies, MAbs), but has proven more complicated in mouse (monoclonal antibodies, MAbs) However, these complications may point to the existence of another form of MRP1

4.2. Anti-MRP1 rabbit polyclonal antibodies.

Three polyclonal antibodies (see Table 4 2 1), designated 1, 2 and 3 were obtained by immunising New Zealand White Rabbits with short synthetic peptide sequences (see Section 3 6) corresponding to three separate amino acid sequences of human MRP1 The suitability of these synthetic peptides for use in monoclonal antibody production was assessed by polyclonal production

Polyclonal 1 was raised against amino acids 1520-1531, an internal epitope in the C-terminus Polyclonal 2 was raised against amino acids 235-246 and Polyclonal 3 was raised against amino acids 246-260, both internal epitopes in the N-terminus region (see Diagram 3 5 2) All three polyclonals, found to be suitable for Western blot analysis, reacted strongly with a 190kD band in the MRP over-expressing, drug-selected cell line, HL60ADR, and weakly or not at all with the parental HL60S cell line (Refer to Figures 3 6 1 , 3 6 3 and 3 6 5 , Results section) These results strongly suggested that the polyclonals were reacting with MRP1

To confirm the specificity of these polyclonals, further Western blot analysis was performed Polyclonal 1, 2 and 3 detected a 150kD band in human MRP-expressing SF9 insect membranes and a shift of the 190kD band to a 150kD band in tunicamycin-treated HL60ADR cells None of the polyclonals reacted with WT-MDR expressing SF9 insect membranes All three polyclonals also reacted with a 140kD band in SF9 insect membranes expressing DeltaMRP1 (Δ1), containing the MRPr1 rat antibody epitope (amino acids 192 - 360), while only polyclonal 1 detected a 120kD band in SF9 insect membranes expressing DeltaMRP2 (Δ2), which does not contain the MRPr1 epitope (see Figures 3 6 2 , 3 6 4 , 3 6 6 and 3 6 7)

In summary, we have generated three polyclonal antibodies which are MRP1-specific and are suitable for Western blot analysis (see Table 4.1.1.). As expected Polyclonal 3, raised against the C-terminus, behaved exactly as the mouse monoclonal MRPm6 (Bakos E. *et al.* 1996), also raised to the C-terminus, does in trypsinised and chymotrypsinised experiments (Results not shown, carried out by Emese Sinkoe of Professor Sarkadis' laboratory, The Institute of Enzymology, Biological Research Center, Hungarian Academy of sciences, H-1113 Budapest Hungary). As expected Polyclonals 1 and 2 reacted with SF9 insect membranes, expressing the N-terminus of human MRP1 which contains the MRPr1 fusion peptide sequence intact, but did not when this region was deleted (see Figures 3.6.2. and 3.6.4.).

Heat treatment of the MRP expressing whole cells lysates, @ 100°C for 3 minutes, did not affect the ability of the three polyclonals to detect their epitopes (results not shown). Taken together, these results provide strong confirmation of the MRP1 specificity of Polyclonals 1, 2 and 3 and the suitability of peptides 1, 2 and 3 for the production of monoclonal antibodies against MRP1.

Table 4.2.1. Summary of Polyclonal antibodies.

POLYCLONAL	IMMUNOGEN	SPECIFICITY	SUITABLE APPLICATIONS
Polyclonal 1	Peptide 1	MRP1	Western blot analysis and immuno-cytochemical analysis.
Polyclonal 2	Peptide 2	MRP1	Western blot analysis and immuno-cytochemical analysis.
Polyclonal 3	Peptide 3	MRP1	Western blot analysis and immuno-cytochemical analysis.

4 3 Anti-MRP1 mouse monoclonal antibodies

A number of MAbs were obtained by immunising BALB/c mice with short synthetic peptides or fusion proteins taken from the amino acid sequence of human MRP1 or whole cell lysates or cells membranes of MRP1 over-expressing COR-L23R cells (see Section 3 7) The production of these MRP1-specific mouse MAbs using various immunogens resulted in a number of antibodies (see Table 4 3 1) However, the quest for an anti MRP1 MAb involved the changing of many variables in the immunisation and screening protocols

Immunisations using the synthetic peptides 1, 2 and 3 were carried out following confirmation of their suitability by the MRP1 polyclonal results The subsequent fusions were screened by the ELISA screening method using the peptide immunogen All MAbs obtained by this regime identified MAbs which reacted with the linear peptide but did not identify any protein by Western Blotting The resulting anti-peptide MAbs, 58C, 76F and 2B5, indicated that an immune response was elicited against each of the short peptides The fact that they did not recognise the native protein by Western blot analysis suggests that the MAbs were reacting with a linear epitope which was conformationally different in the native protein Other possibilities may be that the epitope is destroyed by Western blotting procedures or blocked by glycosylation sugars Various Western blotting conditions, such as immunoprecipitation, non-denaturing and non-reducing conditions were examined Glycosylation studies in which sugar formation was prevented in MRP1 over-expressing cells were also examined However, the various western blotting conditions and glycosylation studies did not reveal any destroyed or hidden epitopes The most likely case for these MAbs is that immunising with the short synthetic and the ELISA screening system employed produced MAbs to

linear epitopes which do not recognise the native conformational protein

Following these results variations in both screening methods and immunogens were developed A Western blotting screening system was set up to optimise the chances of obtaining MAbs which recognise the native conformational protein and work under Western blotting assay procedures Immunisations with Peptide 1 and Peptide 3 were carried out and screened under this screening system Variations in immunogens included fusion proteins, whole cell lysates and cell membranes containing the native MRP1 protein In an attempt to increase the chances of finding common linear epitopes between the linear peptide and the mature native MRP1 protein, fusion proteins containing 200 to 300 amino acids were used in immunisations Further efforts to obtain a MAb which recognised the native MRP1 protein were made by immunising with the native protein itself and screening against the native protein by Western Blotting COR-L23R whole cell lysates and cell membranes over-expressing the native MRP1 protein were used as immunogens All MAbs obtained by the Western blotting method identified MAbs which reacted with a 190kD band in the MRP1 over-expressing COR-L23R cell line in Western blots However, these MAbs also reacted with a 190kD, sometimes doublet band, in the sensitive parental COR-L23S cell line and various other cell lines Fusion Protein 1 also yielded an antibody, 5E7, which recognised a 190kD band in the MRP1 over-expressing COR-L23R cell line and also reacted with a 190kD, sometimes doublet band, in the sensitive parental COR-L23S cell line

Another immunisation, using a purer source of native MRP1 protein than whole cell lysates, was carried out using COR-L23R plasma membranes Initial screening

identified two hybridomas designated MAb 2A7 and MAb 6C6 which identified a 190kD band on COR-L23R but not COR-L23S cells (see Figure 3 7 18) Both these MAbs appeared to be extremely "sticky" on Western blots and required high dilution in buffers to produce clearer results This high background is possibly due to the fact that neither of these MAbs were sub-cloned at this stage and other membrane protein MAbs were possibly present in the hybridoma supernatants However, despite recognising a 190kD band in the COR-L23R cell line, neither MAb 2A7 nor MAb 6C6 recognised a 190kD band in the HL60ADR MRP1 over-expressing cell line suggesting that they were not reacting with MRP1 (see Figures 3 7 19 and 3 7 20) MAb 2A7 detected two lower bands in the drug resistant HL60ADR cells which decreased to one lower band with higher dilution of MAb 2A7 MAb 2A7 did not detect any protein in the drug sensitive HL60S cell line (see Figure 3 7 19) Preliminary immunocytochemical analysis showed that MAb 2A7 stained both COR-L23R and COR-L23S cells in the plasma membrane region similar to the staining observed in COR-L23R cells by the rat monoclonal MRPr1 (see Figure 3 7 25) However, MAb 2A7 stained the nucleoli of both HL60ADR and HL60S cells (see Figure 3 7 25) This suggests that MAb 2A7 is recognising a plasma membrane protein in COR-L23R and COR-L23S cells which is located in the nucleoli of HL60ADR and HL60S cells Other investigators (Krishnamachary *et al*, 1993) have reported MRP1 expression in the membranes of intracellular vesicles such as the endoplasmic reticulum It may be possible that the epitope recognised by 2A7 may only be exposed on these intracellular membranes

An immunisation using COR-L23R whole cell lysates resulted in the MAb 4F6 MAb 4F6 recogmsed a 190kD band in the drug resistant COR-L23R and HL60ADR cell lines but also their drug sensitive parental cell lines COR-L23S and HL60S (see Figure

3 7 16) However, immunocytochemical analysis of COR-L23R and COR-L23S cells with 4F6 demonstrated strong staining of COR-L23R cells but not the COR-L23S cells (see Figure 3 7 17) Unfortunately the hybridoma secreting MAb 4F6 was unstable and stopped secreting antibody before further characterisation was carried out

The use of the Western blotting screening method to screen the immunisations carried out with Peptide 1 and Peptide 3 resulted in two antibodies, 3A7 (Peptide 3) and 1A2 (Peptide 1) Both of these antibodies recognised a 190kD band in the COR-L23R and COR-L23S cells However, after further characterisation, the hybridoma producing MAb 3A7 ceased antibody secretion MAb 3A7 proved to be unstable despite efforts to re-clone it However, the 1A2 antibody has remained stable and consistently recognises a 190kD band in the COR-L23R, COR-L23S and HL60ADR cells but not the HL60S cells Due to the fact that 1A2 was the most stable mouse MAb produced which worked under many procedures such as Western blotting and immunocytochemistry, further characterisation concentrated on the 1A2 MAb

In summary, none of the mouse MAbs obtained behaved in a manner similar to that of the rat monoclonal MRPr1 antibody (see Table 4 2 1) Anti-peptides MAbs were achieved which detected the linear peptide but not the native MRP1 Changes in immunogens and screening procedures resulted in antibodies which detected a 190kD band by Western blotting and stained cells by immunocytochemical procedures The only mouse MAb which behaved in a similar manner to the MRPr1 antibody by immunocytochemical techniques was the 4F6 antibody (whole cell lysate fusion) which unfortunately was unstable The most stable mouse MAb which worked in western blotting and immunocytochemical techniques was the 1A2 MAb (Peptide 1 Fusion)

However, unlike the MRPr1 MAb, 1A2 recognises a 190kD band in both MRP1 expressing and non-expressing cell lines

Table 4.3.1. Summary of all mouse MAbs obtained

		SUITABLE APPLICATIONS			
MAb	**IMMUNO-GEN**	**PEPTIDE ELISA**	**IMMUNOCYTO-CHEMISTRY**	**WESTERN BLOTTING**	**STABILITY**
58C	Peptide 1	Positive on Peptide 1	Does not recognise native protein	Does not recognise native protein	Stable and frozen
1A2	Peptide 1	Positive on Peptide 1	Not done	Recognises a 190kD band on COR-L23R, COR-L23S and HL60ADR (but not HL60S) cells, various other cell lines and a 150kD band on MRP1 transfected SF9 insect cells	Cloned and stable
76F	Peptide 2	Positive on Peptide 2	Faintly stains both COR-L23R and COR-L23S cells	Does not recognise native protein	Stable and frozen
2B5	Peptide 3	Positive on Peptide 3	Does not recognise native protein	Does not recognise native protein	Stable and frozen
3A7	Peptide 3	Positive on Peptide 3	Cytomplasmic staining on COR-L23R and COR-L23S	Recognises a 190kD band on COR-L23R and COR-L23S cells, various other cell lines and a 150kD band on MRP1 transfected SF9 insect cells	Unstable even when cloned Stopped secreting antibody
5E7 *	Fusion Protein 1	Not done	Not done	Recognises a 190kD band on COR-L23R and COR-L23S cells	Not checked and frozen
2A6	Fusion Protein 2	Recognises Maltose Binding Protein (MBP)	Faint plasma membranous staining on COR-L23R and COR-L23S cells	Does not recognise native protein	Stable and frozen

* (MAb produced by Dr Irene Cleary)

Table 4.3.1. Summary of all mouse MAbs obtained (continued)

		SUITABLE APPLICATIONS			
MAb	**IMMUNO-GEN**	**PEPTIDE ELISA**	**IMMUNOCYTO-CHEMISTRY**	**WESTERN BLOTTING**	**STABILITY**
4F6	Whole cell lysates from the MRP1 over-expressing cell line COR-L23R	Not applicable	Plasma membranous staining on COR-L23R but not COR-L23S cells	Recognises a 190kD band on COR-L23R, COR-L23S, HL60ADR and HL60S cells	Unstable even when cloned Stopped secreting antibody
6C6	Membranes from the MRP1 over-expressing cell line COR-L23R	Not applicable	Plasma membranous staining on COR-L23R and COR-L23S cells and cytoplasmic staining in HL60ADR and HL60S cells	Recognises a 190kD band on COR-L23R, COR-L23S cells but not HL60ADR and HL60S cells	Stable and frozen
2A7	Membranes from the MRP1 over-expressing cell line COR-L23R	Not applicable	Plasma membranous staining on COR-L23R and COR-L23S cells and nucleolar staining in HL60ADR and HL60S cells	Recognises a 190kD band on COR-L23R, COR-L23S cells but not HL60ADR and HL60S cells	Stable and frozen

The possibility that the anti-MRP1 mouse MAbs produced in this study are cross-reacting with comparable regions in other mammalian ABC proteins most closely related to MRP1 can be considered The resulting mouse MAbs which detected protein at 190kD by Western Blotting in MRP1 over-expressing cell lines also detected a 190kD band in sensitive and MRP1 negative cell lines These results suggest cross-reaction of these MAbs with possibly another MRP1 isoform or conformation The amino acid

sequences for the recently described MRP1 homologues, MRP2, MRP3, MRP4 and MRP5 (Kool et al, 1997) are now fully known Peptide 1 (a a 1520 -1531) is situated in the C-terminus of MRP1 (see Diagram 3 5 2) The C-terminus is a highly conserved region of the mammalian ABC transporters thus presenting regions of the highest possible cross-reaction The mouse MAb 1A2 was produced to this region Peptide 1 contains at least 3 to 4 amino acids in alignment for each of MRP2, MRP3, MRP4 and MRP5 when compared to MRP1 (see Table 4 3 2)

Table 4.3.2. Protein alignment of the amino acid sequence of Peptide 1 taken from human MRP1 with comparable regions in several MRP related isoforms Common ammo acids are highlighted

MRP ISOFORM (Human)	**SEQUENCE (Peptide 1) Amino acids 1520 to 1531**
MRP1	L F Y S M A K D A G L V
MRP2	P F Y F M A K E A G I E
MRP3	I F Y G M A R D A G L A
MRP4	L F Y K M V Q Q L G K A
MRP5	R F Y A M F A A A E N K
MRP6	L F Y R L A Q E S G L A

MRP1 shares the highest sequence homology with MRP3 Peptide 1 has one region sharing 4 ammo acids in sequence with MRP3, which is all that is required to form an epitope However, there are also regions of three or less amino acids in common with all the above MRP isoforms Therefore, it is possible that MAb 1A2 may be cross-reacting with other MRP isoforms

Cross-reactivity studies by Western blot analysis with MAb 1A2 and the 2008 Parental, 2008 MRP1, 2008 MRP2 and 2008 MRP3 transfectants showed that 1A2 detected a 190kD band in all these cell lines (see Figure 3 7 2 1) However, characterisation of the MRP isoform antibodies (section 3 1) revealed that neither MRP2 nor MRP3 is expressed in the COR-L23R, COR-L23S or HL60ADR cell lines As MAb 1A2 recognises a 190kD band in all these cell lines it appears to be more likely that 1A2 is cross-reacting with another protein, or possibly with a different conformation of MRP1

The possibility that MAb 1A2 is recognising another protein or conformationally different form of MRP1 is also evident A cell line survey (section 3 10) was carried out on a small number of cell lines (see section 1 6 for information on cell lines used) with the MRPr1, P2A8(6), 1A2, M2III-6, M3II-21 and M5II-54 antibodies As expected, the results showed that the MRP1-specific MRPr1 MAb and the P2A8(6) MAb detected a 190kD band in the MRP1 over-expressing cell lines, COR-L23R, HL60ADR, A549 and RPMI 2650 Melphalan selected The MRPr1 and P2A8(6) MAbs did not detect a 190 kD band in the COR-L23S, HL60S, DLKP, DLKPA, RPMI 2650 parental or RPMI 2650 Taxol-selected MRP2 was detected in the A549 and RPMI 2650 Melphalan selected cell lines with the M2III-6 MAb These results also correlate with an RT-PCR study carried out in our lab (Yizheng Liang, PhD Thesis submitted, 1999) The 1A2 MAb detected a 190kD band in all these cell lines except HL60S, DLKPA and RPMI 2650 Taxol selection Therefore, the antigen recognised by the 1A2 MAb appears to be down-regulated in Pg-P over-expressing cell lines The down-regulation of MRP1 in P-gp over-expressing cell lines is commonly observed (Brock *et al*, 1995) However, a survey of the 1A2 MAb in a wider selection of cell lines is required to confirm this pattern MRP3 was detected with the M3II-21 MAb in the

2008 MRP3 transfected cell line only MRP5 was not detected in any cell line with the M5II-54 MAb See Table 4 2 3 for summary of protein expression in these cell lines

Hipfner *et al*, 1998, reported that the MRPr1 MAb recognised a linear epitope It was found in this study that the MRPr1 epitope was destroyed in heat treatment studies (see Figure 3 2 9) which suggested that MRPr1 may recognise a conformational epitope However, heat treatment studies with the 2008 M1 cell lines did not destroy the MRPr1 epitope (see Figure 3 2 9) This may be due to the fact that the 2008 cells are MRP1 transfectants and the MRP1 may be incorporated into the cell membrane in a slightly different conformation to that of MRP1 expressed intrinsically in the cell Heat treatment studies showed that the epitope recognised by MAb 1A2 was not destroyed in MRP1 drug-selected or –transfected cells suggesting that 1A2 recognises a linear epitope

The cell line survey carried out in section 3 10 of the 1A2, P2A8(6), MRPr1 (MRP1), M2II-6 (MRP2), M3II-21 (MRP3) and M5II-54 (MRP5) antibodies on various cells lines was compared to P-gp expression in Table 4 3 3 This cell line survey showed that the P2A8(6) followed an expression pattern similar to that of the well characterised MRPr1 antibody The 1A2 protein was found to be expressed in all of the examined parental cell lines except the HL60S cell line It was expressed in the MRP1 induced HL60ADR cell line However, it was also noted that the 1A2 protein appeared to be downregulated (compared to the parental cell lines) in P-gp induced cell lines such as the DLKPA and RPMI 2650 cell lines Therefore, these results suggest a link between MRP1 expression, P-gp expression and expression of the protein detected by the 1A2 antibody It may be possible that the P-gp pump downregulates expression of the

protein detected by the 1A2 antibody and this protein is dependent on the MRP1 pathway

Table 4.3.3. Summary of protein expression observed in the cell line study (section 3 10) and compared with P-gp expression

	PROTEIN EXPRESSION LEVELS (as determined by western blotting and/or immunocytochemistry)						
CELL LINE	**Pg-p**	**1A2**	**P2A8(6)**	**MRPr1** MRP1	**M2III-6** MRP2	**M3II-21** MRP3	**M5II-54** MRP5
COR-L23R	— (Twentyman *et al* 1986)	+	+	+	—	—	—
COR-L23S	— (Twentyman *et al* 1986)	+	+/-	+/-	—	—	—
HL60ADR	— (Marsh *et al* 1986)	+	+	+	—	—	—
HL60S	— (Marsh *et al* 1986)	—	—	—	—	—	—
DLKP	— (Law *et al* 1992)	+	—	—	—	—	—
DLKPA	+ (Clynes *et al* 1992)	+/-	—	—	—	—	—
A549	— (Center *et al* 1993)	+	+	+	+	—	—
RPMI 2650 Parental	+/- (Yizheng Liang, PhD Thesis submitted)	+	—	+/-	—	—	—
RPMI 2650 Taxol	+ (Yizheng Liang, PhD Thesis submitted)	—	—	—	—	—	—
RPMI 2650 Melphalan	— (Yizheng Liang, PhD Thesis, submitted)	+	+	+	+	—	—

Table 4.3.3. Continued.

	PROTEIN EXPRESSION LEVELS (as determined by western blotting and/or immunocytochemistry)						
CELL LINE	**Pg-p**	**1A2**	**P2A8(6)**	**MRPr1** MRP1	**M2III-6** MRP2	**M3II-21** MRP3	**M5II-54** MRP5
2008 Parental	mRNA +/- (Kool *et al.*, 1997)	+	—	+/-	—	—	mRNA + (Kool *et al.*, 1997)
2008 MRP1	Not done	+	+	+	—	—	Not done
2008 MRP2	Not done	+	—	+/-	+	—	Not done
2008 MRP3	Not done	+	—	+/-	—	+	Not done

Table 4.3.3. KEY:

-	undetectable levels
+	Over-expression
+/-	Very low basal levels

In summary the antigen recognised by 1A2 is expressed in MRP1 over-expressing drug-selected and MRP1 transfected cell lines The 1A2 antigen is also expressed in sensitive cell lines but appears to be down-regulated in Pg-p over-expressing cell lines It may be possible that MRP1 is present in sensitive cells in a different conformation or active state from that of drug selected MRP1 over-expressing cells A number of results point to this conclusion The Western blotting screening system identified the 1A2 MAb Heat treatment did not destroy the 1A2 MAb epitope The MRPr1 antibody detects MRP1 in COR-L23R and 2008 MRP1 transfected cells Heat treatment destroys the MRPr1 epitope in drug selected MRP1 over-expressing cells COR-L23R cells but not the MRP1 transfected 2008 MRP1 cell line This suggests a difference in conformation between drug selected and transfected MRP1 The conformational difference may be due to another pattern of insertion of the protein in the cell membrane caused by different lipid composition in the separate cell lines It may be possible that the drug-selected MRP1 is heat labile while the transfected MRP1 is more stable because the conformation difference creates a stronger anchorage for the transfected MRP1 into the cell membrane Taken together these results suggest that the conformation of active drug selected MRP1 is unique to resistant cells and another MRP1 conformation which is probably inactive may be present in sensitive cells It is, therefore, possible that the epitope recognised by 1A2 is present in both these conformations

Alternatively, the 1A2 MAb may recognise MRP1 and cross react with all or some of the recently discovered MRP1 homologues Cross reactivity studies showed that the 1A2 MAb detected a 190kD band in the MRP1, MRP2 and MRP3 transfected 2008 cell lines Unfortunately the 1A2 MAb also reacted with a 190kD band in the parental un-transfected 2008 cell line However, there are basal levels of MRP1 expressed in the

parental un-transfected 2008 cell line also Therefore the 1A2 MAb may be cross-reacting with MRP1, MRP2 and MRP3, detecting the basal level of MRP1 in all the cell lines or cross-reacting with another MRP1-related protein expressed in all these cell lines The fact that the parental un-transfected 2008 cell line expresses a basal level of MRP1 is an obstacle in the interpretation of these results The 1A2 MAb recognises a heat stable conformation of the antigen This could be due to a different conformation of the MRP1 protein and may also be reflected in a poorer efflux activity of this same conformation 1A2 detects a 190kD band in the sensitive COR-L23S cell Preliminary pharmacological experiments in this cell line which does not express MRP1, suggests the presence of another MRP related protein (S Tuohey Personal communication) Toxicity assays were carried out and the results compared between the COR-L23R and 2008 MRP1 cell lines to investigate whether there is any difference in the levels of drug resistance (Tuohey, S , PhD Thesis, to be submitted) Lower levels of resistance were found in the transfected cells Recently, Kool *et al* , (1999a), described a limited range of resistance in the transfected 2008 MRP3 cell line

Therefore the antigen recognised by the 1A2 MAb is probably MRP1-related For example, the 1A2 MAb also recognises the MRP1 transfected SF9 insect membranes but not the control SF9 membranes and is inhibited by peptide 1 in peptide inhibition studies Purification by immunoprecipitation, followed by N-terminal sequence analysis may determine the specificity of this MAb for MRP1

4 4 Anti-MRP1 rat monoclonal antibodies

A number of rat MAbs were obtained by immunising female Wistar rats with Peptide 2 and Peptide 3 in combination (see Table 4 4 1) The subsequent fusions were screened and a number of monoclonal antibodies resulted These antibodies were designated P2A8(6), P1D7, P1A6 and P2E4 All MAbs, except P1D7, obtained in this regime recognised a 190KD band in MRP1 over-expressing cell lines but not the sensitive counterparts by Western blotting P1D7 did not react with the COR-L23R cell line by Western blotting but did react with a 190kD band in the MRP1 transfected 2008 cell line Therefore, P1D7 appears to recognise the transfected MRP1 but not the drug-selected MRP1 This difference may be due to a slight difference in the conformation of the transfected and drug-selected MRP1 The transfected MRP1 may be incorporated into the transfected cells membrane in a slightly different conformation to that of the drug selected MRP1 over-expressing cells, possibly due to different lipid conformations in the membranes of these cell lines The P2A8(6) MAb also reacted with MRP1 over-expressing cell lines by immunocytochemical techniques in a similar manner to that of the well characterised rat monoclonal antibody MRPr1 That is P2A8(6) stained all cells in MRP1 over-expressing cell lines However, unlike the rat MAb, MRPr1, the P1D7 antibody stained a subpopulation of cells in MRP1 over-expressing cells These results suggest that this P1D7 MAb may recognise a conformationally or structurally different form of MRP1 All of these MAbs were MRP1 specific and did not cross-react with MRP2 or MRP3 as determined by Western blot analysis and immunocytochemical techniques Peptide inhibition studies, confirmed by Western blot analysis, indicated that the characterised selected rat MAbs reacted with an epitope present on Peptide 3 However, further stocks of selected hybridomas remain frozen Further characterisation of these frozen hybridomas may detect MAbs which recognise an epitope on Peptide 2

An attempt to raise rat MAbs specific for external MRP1 epitopes was unsuccessful Resulting MAbs reacted with both the COR-L23R and COR-L23S cell lines by live cell immunofluoresence Other investigators have reported similar results in similar attempts (Scheffer, G L Unpublished work)

Table 4.4.1. Summary of rat MRP1 monoclonal antibodies

MAb	PEPTIDE SPECIFICITY	SUITABLE APPLICATIONS	MRP1 SPECIFICITY
P2A8(6)	Peptide 3	Western blot analysis and immuno-cytochemistry	Specific for MRP1 in a similar manner to the rat monoclonal antibody, MRPr1, by Western blot and immunocytochemical analysis
PID7	Peptide 3	Western blot analysis and immuno-cytochemistry	Specific for MRP1 in MRP1 transfected cells but not drug selected MRP1 expressing cells by Western blot analysis Recognises a sub-population of cells in MRP1 transfected and drug selected cells by immunocytochemical analysis
P1A6	Peptide 3	Western blot analysis Very sticky in immuno-cytochemistry	Specific for MRP1 in MRP1 tansfected and drug selected cell lines in a similar manner to the rat monoclonal antibody, MRPr1, by Western blot analysis
P2E4	Peptide 3	Western blot analysis and immuno-cytochemistry	Specific for MRP1 in MRP1 tansfected and drug selected cell lines in a similar manner to the rat monoclonal antibody, MRPr1, by Western blot analysis Recognises a sub-population of cells in MRP1 transfected and drug selected cells by immunocytochemical analysis in a similar manner to P1D7

4.5. Species difference:

The failure to produce a drug-induced MRP1 specific mouse monoclonal, which behaves in a manner similar to that of the well characterised MRPr1 rat MAb, may also be due to a species difference. Considering the success of the peptides 1, 2 and 3 for the production of rabbit polyclonals and the success of co-immunising rats with peptides 2 and 3, another possibility may be that the BALB/c mice may not be a suitable host species for immunisation of the MRP1 protein. The epitopes recognised by the MRP1 specific MAbs MRPr1 and MRPm6 (Flens *et al.,* 1994) have recently been published (Hipfner *et al.,* 1998). MAb MRPr1 was mapped to the decapeptide a.a. 238 to 247 of human MRP1. This region is similar to Peptide 2 (a.a. 235 to 246 of human MRP1) which was used in this study to successfully produce a rabbit polyclonal antibody but failed to produce a mouse MAb specific for the mature MRP1 protein. The mouse monoclonal MRPm6 was mapped to the decapeptide a.a. 1511 to 1520. This epitope is adjacent to Peptide 3 (a.a. 1520 to 1531) used in this study to successfully produce a rabbit polyclonal antibody but which failed to produce a mouse MAb specific for the mature MRP1 protein. The murine mrp mRNA encodes a protein of 1528 amino acids that is 88% identical to human MRP (Stride *et al.,* 1996). Rat and rabbit MRP are less homologous to human MRP and may be a more suitable choice of species for immunisation. That is, murine MRP is so closely related to human MRP1 that it may be seen as self-protein resulting in a lack of immunological response. Opposing this theory is the fact that there are two mouse monoclonals to MRP1, MRPm6 (Flens *et al.,* 1994) and QCRL-1 (Hipfner *et al.,* 1994). However, both MRPm6 and QCRL-1 reinforce the species theory. Their epitopes are specific for rare un-conserved regions in murine MRP, which are not as closely homologous to human MRP as most of the regions.

Hipfner et al., 1998, stated that alignment of the MRPr1 and MRPm6 epitope sequences

with the comparable regions in mammalian ABC proteins most closely related to MRP1 indicates, with the exception of murine mrp, the sequences are poorly conserved This suggests that antibodies produced to Peptide 2, which contains the MRPr1 epitope sequence, will not cross-react with any other members of the mammalian ABC transporter family

Murine mrp is 88% identical to human MRP1 Table 4 5 1 shows that the epitopes mapped for the commercial MAbs, MRPm6 and QCRL-1, are rare regions which are not highly conserved in mouse mrp That is, they both have at least two amino acids which are in contrast to corresponding amino acids in mouse MRP These sequences may be more immunogenic in mice than a more highly conserved sequence The sequence used for the production of the commercial MRPr1 antibody is more highly conserved However, the MRPr1 antibody was produced in rat The rat model of immunisation may have a higher chance of eliciting a strong immune response against most regions of human MRP1 because of a lower conservation of amino acid homology between human MRP1 and rat MRP isoforms The same case is true for EBCR, the rabbit homologue of MRP1

Table 4.5.1. AMINO ACID SEQUENCE COMPARISONS OF COMMERCIAL MRP1 ANTIBODY EPITOPES IN VARIOUS SPECIES. Differences in amino acids present in the antibody epitope and corresponding amino acids in various species highlighted in red.

PROTEIN	**MRPr1** (Rat MAb) epitope human MRP1 a.a.238 – 247 Human and murine specific.	**MRPm6** (Mouse MAb) epitope human MRP1 a.a1511-1520 Human specific.	**QCRL-1** (Mouse MAb) epitope human MRP1 a.a.918 – 924 Human specific.
Human MRP1	GSDLWSLNKE	PSDLLQQRGL	SSYSGDI
Murine MRP	SSDLWSLNKE	PSELLQQRGI	SSHSGDT
Rat MOAT	LEDVWDIDEG	PEEOOQIPGP	ENSLRRT
Rabbit EBCR	LEDVWDIDEE	PEELLESAGP	SRSSRSS

Table 4.5.2. Amino acid sequence comparisons Peptide 1, 2 and 3 with corresponding amino acids in various species Differences in amino acids between human MRP1 and various species are highlighted in red

PROTEI	PEPTIDE 1 a a 1520-1531	PEPTIDE 2 a a 235-246	PEPTIDE 3 a a 246-260
Human MRP	LFYSMAKDAGLV	PLEGSDLWSLNK	KEDTSEQVVPVLVKN
Murine MRP	IFYSMAKDAGLV	PLESSDLWSLNK	KEDTSEQVVPVLVNN
Rat MOAT	YFYLMAKEAGIE	PLTLEDVWDIDE	EGFKTRSVTSKFEAAM
Rabbit EBCR	SPEELLESAGPF	PLTLEDVWDIDE	EEFKAKT I VSRFEVH

4 6 Antigen presentation

The route of immunisation may also play a role in the production of antibodies which recognise the native protein (Harlow and Lane, 1988) All three species of animals were immunised by various routes The rabbits were immunised subcutaneously, the rats in the footpad and the mice intraperitoneally Various routes create various environments for presentation of the antigen and may structurally change the conformation of the antigen presented to immune cells Therefore, the antibodies produced from similar antigens in various species may detect various forms of the antigen and may not be suitable for all assays This may explain the production of mouse MAbs specific for the linear MRP1 peptide but unable to recognise the native MRP1 protein

4 7 Production of an external epitope MRP1-specific antibody·

The production of external epitope MRP1-specific antibodies has proven unsuccessful in both rat and mouse MRP1 specific external epitope PAbs (rabbit) were not attempted because immunisation with whole cells lysates or membranes would probably result in polyclonal serum containing many antibodies specific for various components of the cell or cell membrane thus masking the polyclonals specificity for the MRP1 protein alone Future work using external epitope peptides could be valuable, but it is possible that the external epitopes may be masked by glycosylation sugars An obvious solution would be to remove the glycosylation sugars from whole cells prior to immunisation However, the resulting MAbs would probably be unable to recognise the mature, fully glycosylated protein found in the native protein, thus destroying the end objective Hipfner *et al*, 1997 and Kast *et al*, 1997 have determined that MRP1 does have external protein fragments protruding from the membrane However, they do not tell us how well these fragments are available to the immune response It is possible that these

external epitopes do not elicit an immune response Scheffer *et al*, 1997, have tried directing the immune response to the antigen of interest by masking unwanted cell surface epitopes as described by Shen *et al*, 1994, without success They also tried fusion proteins corresponding to external fragments proposed in the membrane model by Hipfner *et al*, 1997 and Kast *et al*, 1997 MAbs reacting with these peptides were easily selected but these MAbs did not react with the membranes of MRP1 expressing cells They suggested that the immune response was directed mainly towards aberrant epitopes due to e g peptide aggregation and not towards the native epitopes

5.0. CONCLUSIONS.

5 0 CONCLUSIONS

The use of various immunogens, species, immunisation protocols and screening procedures can greatly enhance the chances of obtaining a desired antibody Antibodies were produced in rabbits, mice and rats for the detection of MRP1 The rabbit immunisations produced three MRP1 specific polyclonals The mouse monoclonals produced include MAbs which recognise the peptide but not the native protein and the 1A2 MAb which detected MRP1 expressed in un-glycosylated form in SF9 membranes and an MRP1-like antigen in cells, possibly a different conformational form of MRP1 A collaboration between our laboratory and Professor Balazs Sarkadi's laboratory in Budapest, Hungary made available MRP1 fusion proteins and a limited amount of various MRP1 segments expressed in SF9 insect cells The rat immunisations which also included two of the peptides used in the mouse immunisations produced a number of MAbs One rat MAb (P2A8(6), behaved in a similar manner to that of the well characterised MRPr1 MAb and detected MRP1 homogeneously in drug selected or transfected cells by Western blotting and immunocytochemical analysis Another rat MAb (P1D7), detected transfected but not drug selected MRP1 by Western blot analysis and detected a subpopulation of cells in both transfected and drug selected MRP1 cell lines Preliminary characterisation of these novel antibodies suggests that there are various conformations or isoforms of MRP1 opening up the possibility of a more complex mode of multidrug resistance executed by MRP1 It is possible that there are different conformations of MRP1 (perhaps produced by as yet unknown cofactors, e g insertion into membrane lipids) which differ in drug efflux capability and/or substrate specificity Whether there are various degrees of conformational change or a number of active and less active isoforms and whether each conformation or active/less active isoform elicits varying degrees of resistance is still not clear

Immunisation with the same peptides in mice, rats and rabbits produced different results MRP1-specific antibodies were obtained with the rats and rabbits while the mice generally produced MAbs which recognised the linear peptide but not the native protein and the 1A2 MAb which may recognise a conformationally different (possible less active) MRP1 isoform However, immunisations in mice with MRP1 over-expressing membranes produced the 4F6 MAb which detected plasma-membranous staining in MRP1 over-expressing COR-L23R cells but not the COR-L23S parental cells by immunocytochemistry 4F6 also recognised a 190kD band in COR-L23R, COR-L23S, HL60ADR and HL60S cells by western blotting These bands may be due to the fact that 4F6 was un-cloned at this stage Unfortunately 4F6 was unstable and ceased MAb secretion despite sub-cloning Although it seems to be extremely difficult to obtain an MRP1-specific MAb in mice, the 4F6 results do suggest that it is possible Further proof of this is the two commercial mouse MRP1 MAbs which exist, MRPm6 and QCRL-1 The difficulty may be that mouse MRP is 88% identical to human MRP1 and an immune response may therefore be difficult to elicit as many regions of the amino acid sequence may be seen as self-proteins Both of the commercially available mouse MAbs mentioned above are specific for epitope regions which are unusually un-conserved in mouse MRP Both of these MAbs are also human specific and do not cross react with mouse MRP suggesting that these epitopes are different in mouse and human MRP Therefore they may be regions of higher immunogenicity in mice The 4F6 MAb may have been detecting one of these rare un-conserved regions also The success of the rat and rabbit antibodies using the same peptides to produce MRP1 specific antibodies may be attributed to the fact that rat and rabbit MRP1/MRP1 homologues are highly un-conserved when compared to human MRP1, therefore posing

no problems with the majority of the human MRP1 amino acid sequence in eliciting a strong immunological response in either of these species

The recent sequencing of the genes for several new MRP homologues has led to new MRP isoform transfected cell lines and MRP isoform antibodies MRP 1, 2 and 3 transfected 2008 ovarian cell lines and antibodies specific for MRP1, 2, 3, and 5 were made available just before the end of the thesis to our lab in a collaboration set up with Marcel Kool and George Scheffer from Professor Rik Scheper's lab in Amsterdam These cell lines and antibodies were invaluable in determining the specificity of the MAbs produced in the course of this thesis for MRP1, MRP2 or MRP3

6.0 FUTURE WORK.

6 0 FUTURE WORK

Purification by immunoprecipitation of the antigen recognised by the mouse MAb 1A2 followed by N-terminal sequence analysis of the protein would aid confirmation of the specificity of this MAb A full cell line survey with MAb 1A2 should be carried out to further confirm or disprove the link between P-gp over-expression and down-regulation of the antigen recognised by the 1A2 MAb which was observed in the small cell line survey carried out in this thesis already

The success of raising MRP1 specific rat MAbs suggests that the rat may be the most suitable species for eliciting a strong immunological response against the MRP1 protein Therefore, further efforts to raise an external MRP1 epitope MAb using various immunogens (external peptides, cell membranes, fusion proteins) and screening methods (live cell immunofluorescence) should be tried

The epitope recognised by the rat MAb P2A8(6) is unique compared to other commercially available MRP1 antibodies Therefore further characterisation of this novel MRP1 specific MAb would facilitate both clinical and experimental investigations in unravelling the role of MRP1 in multidrug resistance

Interestingly the P1D7 rat MAb distinguishes between transfected and drug-selected MRP1 by Western blot analysis and detects a subpopulation in transfected and drug selected MRP1 cell lines by immunocytochemical analysis suggesting that there may be at least two conformations of the MRP1 molecule A full cell line survey with the P1D7 MAb should be carried out to determine the expression in various cell line types

A number of interesting antibodies have been produced during this thesis and have raised the possibility that there are different MRP1 conformations (possibly less active or have a different substrate specificity) present in cells and therefore possibly tissues An investigation of the expression of these proteins should be carried out in a tissue survey, including human normal and tumour samples

7.0 BIBLIOGRAPHY.

Almquist, K C , Loe, D W , Hipfner, D R , Mackie, J E , Cole, S P and Deeley, R G (1995) Characterisation of the M_r 190,000 Multidrug Resistance Protein (MRP) in Drug-selected and Transfected Human Tumour Cells *Can Res* (55) 102-110

Bader, P , Schilling, F , Schlaud, M , Girgert, R , Handgretinger, R , Klingebiel, T , Treuner, J , Liu, C , Niethammer, D and Beck, J F (1999) Expression analysis of multidrug resistance associated genes in neuroblastomas *Oncol Rep* 6(5) 1143-6

Bakos, E , Hegedus, T , Hollo, Z , Welker, E , Tusnady, G E , Zaman, G J R , Flens, M J , Varadi, A and Sarkadi, B (1996) Membrane Topology and Glycosylation of the Human multidrug Resistance-associated Protein *J Biol Chem* 271(21) 12322-12326

Bakos, E , Evers, R , Szakacs, Tusnady, G E , Welker, E , Szabo, K , de Haas, M , van Deemter, L , Borst, P , Varadi, A and Sarkadi, B (1998) Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain *J Biol Chem* 273(48) 32169-75

Baldini, N , Scotlandi, K , Barbanti-Brodano, G , Manara, M C Maurici D , Bacci, G , Bertoni, F , Picci, P , Sottili, S , Campanacci, M , *et al* (1995) Expression of P-glycoprotein in high-grade osteosarcomas in relation to clinical outcome *N Engl J Med* 333(21) 1380-1385

Barrand, M A , Rhodes, T , Center, M S and Twentyman, P R (1993) Chemosensitisation and drug accumulation effects of cyclosporin A, PSC-833 and verapamil in human MDR large cell lung cancer cells expressing a 190kD membrane protein distinct from P-glycoprotein *Eur J Cancer* 29A(3) 408-415

Beck, J , Bohnet, B , Brugger, D , Bader, P , Dietl, J , Scheper, R J , Kandolf, R , Liu, C , Niethammer, D and Gekeler, V (1998) Multiple gene expression analysis reveals distinct differences between G2 and G3 stage breast cancers, and correlations of PKCN with MDR1, MRP and LRP gene expression *Br J Cancer* 77(1) 87-91

Belinsky, M G , Bain, L J , Balsara, B B , Testa, J R and Kruh, D (1998) Characterisation of MOAT-C and MOAT-D, New Members of the MRP/cMOAT Subfamily of Transporter Proteins *J Natl Can Inst* 90(22) 1735-1741

Bellamy, W T (1990) Multidrug resistance in human tumours *Curr Opin Oncol* 2(6) 1126-1132

Bellamy, W T (1996) P-glycoprotein and multidrug resistance *Ann Rev Pharmacol* Toxocol (26) 161-183

Berger, W , Elbling, L , Hauptmann, E and Micksche, M (1997a) Expression of the multidrug resistance-associated protein (MRP) and chemoresistance of human non-small-cell lung cancer cells *Int J Cancer* (73) 84-93

Berger, W , Hauptmann, E , Elbling, L , Vetterlein, M , Kokoschka, E and Micksche, M (1997b) Possible role of the multidrug resistance-associated protein (MRP) in chemoresistance of human melanoma cells *Int J Cancer* (71) 108-115

Bordow, S B , Haber, M , Madafiglio, J , Cheung, B , Marshall, G M and Norris, M D (1994) Expression of the Multidrug Resistance-associated Protein (MRP) Gene Correlates with Amplification and Overexpression of the N-*myc* Oncogene in Childhood Neuroblastoma *Can Res* (54) 5036-5040

Boring, C C , Squires, T S , and Tong, T (1993) Cancer statistics, 1993 *C A Cancer J Clin* 43(1) 7-26

Borst, P Genetic mechanisms of drug resistance (Review) (1991) *Acta Oncol* (30) 87

Breuninger, L M , Paul, S , Gaughan, K , Miki, T , Chan, A , Aaronson, S A and Kruh, G D (1995) Expression of multidrug resistance associated with increased drug efflux and altered intracellular drug distribution *Cancer Res* 55(22) 5342-5347

Brock, I , Hipfner, D R , Nielsen, B S , Jensen, P B , Deeley, R G , Cole, S P and Shested, M (1995) Sequential coexpression of the multidrug resistance genes MRP and mdr1 and their products in VP-16 (etoposide)-selected H69 small cell lung cancer cells *Cancer Res* 55(3) 459-62

Broxterman, H J , Giaccone, G and Lankelma, J (1995) Multidrug resistance proteins and other drug transport-related resistance to natural product agents *Curr Opin Oncol* 7(6) 532-540

Brugger, D , Herbart, H , Gekeler, V , Seitz, G , Liu, C , Klingebiel, T , Orlikowsky, T , Einsele, H , Dennlinger, C , Bader, P , Niethammer, D and Beck, J F (1999) Functional analysis of P-glycoprotein and multidrug resistance associated protein related multidrug resistance in AML-blasts *Leuk Res* (5) 467-75

Center, M S (1993) Non-P-glycoprotein multidrug resistance in cell lines which are defective in the cellular accumulation of drug *Cytotechnology* (12) 109-125

Chan, H S L , Haddad, G and Thorner, PS , de Boer, G , Lin, Y , Ondrusek, N , Yeger, H and Ling, V (1991) P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma *N Engl J Med* (325) 1608-1614

Chan, H S L , Thorner, P S , Haddad, G and Ling, V (1990) Immunohistochemical detection of P-glycoprotein prognostic correlation in soft tissue sarcoma of childood *J Clin Oncol* (8) 689-704

Chang, X B , Hou, Y X , Jensen, T J and Riordan, J R (1994) Mapping of cystic fibrosis transmembrane conductance regulator membrane topology by glycosylation site insertion *J Biol Chem* 269(28) 18572-18575

Chen, C J , Chin, J , Ueda, K , Clark, D , Pastan, I , Gottesman, M and Rominson, I (1986) Internal duplication and homology with bacterial transport proteins in the mdr-1 (p-glycoprotein) gene from multidrug resistant cells *Cell* (47) 381-389

Chuman, Y , Sumizawa, T , Takebayashi, Y , Njwa, K , Yamada, K , Haraguchi, M , Furukawa, T , Akiyama, S and Aikou, T (1996) Expression of the multidrug-resistance-associated protein (MRP) gene in human colorectal, gastric and non-small-cell lung carcinomas *Int J Can* (66) 274-279

Claassen, E , Zegers, N D , Laman, J D and Boersma, W J A (1993) Use of Synthetic Peptides for the Production of Site (Ammo Acid) Specific Polyclonal and Monoclonal Antibodies *Year Immunol* (7) 150-161

Clynes, M , Redmond, A , Moran, E and Gilvarry, U (1992) Multiple drug resistance in variants of a human non-small-cell lung carcinoma cell line, DLKPA *Cytotechnology* (10) 75-89

Clynes, M , Heenan, M and Hall, K (1993) Human cell lines as models for multidrug resistance in solid tumours *Cytotechnology* (12) 231-256

Cole, S P , Bhardwaj, G , Gerlach, J H , Mackie, J E , Grant, C E , Almquist, K C , Stewart, A J , Kurz, E U , Duncan, A M V and Deeley, R G (1992) Overexpression of a Transporter Gene in a Multidrug-Resistant Human Lung Cancer Cell Line *Science* (258) 1605-1654

Cole, S P , Sparks, K E , Fraser, K , Loe, D W , Grant, C E , Wilson, G M and Deeley, R G (1994) Pharmacological characterisation of multidrug resistant MRP-transfected human tumour cells *Cancer Res* 54(22) 5902-5910

Cole, S P and Deeley, R G (1998) Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP *Bioessays* (2) 931-940

Coop, J M (1993) P-glycoprotein structure and evolutionary homologues In Multidrug Resistance in cancer cellular molecular and clinical approaches Clynes, M (Ed) *Kluwer Academic Press London* 1-32

Dano, K (1973) Active outward transport of daunomycin in resistant Ehrlich ascites tumour cells *Biochem Biophys Acta* (323) 466-483

Davey, R A , Longhurst, T J , Davey, M W , Belov, L , Harvie, R M , Hancox, D and Wheeler, H (1995) Drug resistance mechanisms and MRP expression in response to epirubicin treatment in a human leukaemia cell line *Leuk Res* 19(4) 275-282

De Vita, V T (1989) principles of chemotherapy In Principles and practice of Oncology De Vita, Helman and Rosenberg (Eds) *JP lippincott Company, Philadelphia* 276-300

Dingemans, A M C , van Ark-otte, J , van der Valk, P , Apolinario, R M , Scheper, R J , Postmus, P E and Giaccone, G (1996) Expression of the human major vault protein LRP in human lung cancer samples and normal lung tissue *Ann Oncol* (7) 625-630

Evers, R , Kool, M , van Deemter, L , Janssen, H , Calafat, J , Oomen, L C , Paulusma, C C , Oude Elferink, R P , Baas, F , Schinkel, A H and Borst, P (1998) Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA *J Clin Invest* 101(7) 1310-1319

Filipits, M , Suchomel, R W , Dekan, G , Haider, K , Valdimarsson, G , Depisch, D and Pirker, R (1996a) MRP and MDR1 Gene Expression in Primary Breast Carcinomas *Clin Can Res* (2) 1231-1237

Filipits, M , Suchomel, R W , Zochbauer, S , Malayeri, R and Pirker, R (1996b) Clinical relevance of drug resistance genes in malignant diseases *Leukemia* 10(3) S10-S17

Filipits, M , Suchomel, R W , Zochbauer, S , Brunner, R , Lechner, K and Pirker, R (1997) Multidrug Resistance-associated Protein in Acute Myeloid Leukemia No impact on Treatment Outcome *Clin Can Res* (3) 1419-1425

Filipits, M , Stranzl, T , Pohl, G , Suchomel, R W , Zochbauer, S , Brunner, R , Lechner, K and Pirker, R (1999) MRP expression in acute myeloid leukemia An update *Adv Exp Med Biol* (457) 141-50

Flens, M J , Izquierdo, M A , Scheffer, G L , Fritz, J M , Meijer, C J , Scheper, R J and Zaman, G J (1994) Immunocytochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumour cells by monoclonal antibodies *Cancer Res* 54(17) 4557-45563

Flens, M J , Zaman, G J R , van der Valk, P , Izquierdo, M A , Schroeijers, A B , Scheffer, G L , van der Groep, P , de Haas, M , Meijer, C J L M and Scheper, R J (1996) Tissue Distribution of the Multidrug Resistance Protein *Am J Path* 148(4) 1237-1247

Ford, J M (1995) Modulators of multidrug resistance Preclinical studies *Hematol Oncol Clin North Am* 9(2) 337-361

Fromm, M F , Leake, B , Roden, L D M , Wilkinson, G R and Kim, R B (1999) Human MRP3 transporter identification of the 5'-flanking region, genomic organization and alternative splice variants *Biochimica et Biophysica Acta* (1415) 369-374

Gately, D P and Howell, S B (1993) Cellular accumulation of the anticancer agent cisplatin a review *Br J Cancer* (67) 1171 - 1176

Georges, E , Bradley, G , Gariepy, J and Ling, V (1990) Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies *Proc Natl Acad Sci USA* (87) 152-156

Gerlach JH et al , (1987) P-glycoprotein in human sarcoma evidence for multidrug resistance J Clin Oncol 5 1452 - 1460

Germann, U A , Willingham, M C , Pastan, I and Gottesman, M M (1990) *Biochemistry* (29) 2295-2303

Giaccone, G , van Ark-otte, J , Rubio, G J , Gazdar, A F , Broxterman, H J , Dingemans, A M , Flens, M J , Scheper, R J and Pinedo, H M (1996) MRP is frequently expressed in human lung-cancer cell lines, in non-small-cell lung cancer and in normal lungs *Int J Cancer* 66(6) 760-767

Goldstein, L J , Pastan, I and Gottesman, M M (1992) Multidrug resistance in human cancer *Crit Rev Oncol Hematol* 12(3) 243-253

Gottesman, M M , and Pastan, I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter *Ann Rev Biochem* (62) 385-427

Gottesman, M M , Pastan, I , and Ambudkar, S V (1996) P-glycoprotein and multidrug resistance *Current Opinion in Genetics & Development* (6) 610-617

Grant, C E , Valdimarsson, G , Hipfner, D R , Almquist, K C , Cole, S P and Deeley, R G (1994) Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs *Cancer Res* 54(2) 357-361

Gros, P , Croop, J and Housman, D (1986) Mammalian Multidrug Resistance Gene Complete cDNA Sequence Indicates Strong Homology to Bacterial Transport Proteins *Cell* (47) 371-380

Hamada, H , Hagiwara, K , Nakajima, T and Tsuruo, T (1987) Phosphorylation of the Mr 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumour cells effects of verapamil, trifluoperazine, and phorbol esters *Cancer Res* 47(11) 2860-2865

Harlow, E and Lane, D (1988) Antibodies A Laboratory Manual *Cold Spring Harbour Laboratory* (Pub)

Hart, S M , Ganeshaguru, K , Hoffbrand, A V , Prentice, H G and Mehta, A B (1994) Expression of the multidrug resistance-associated protein (MRP) in acute leukaemia *Leukemia* 8(12) 2163-2168

Higgins, C F (1992) ABC transporters, from microorganisms to man *Ann Rev Cell Biol* (8) 67-113

Hipfner, D R , Gauldie, S D , Deeley, R G and Cole, S P (1994) Detection of the M_r 190,000 Multidrug Resistance Protein, MRP, with Monoclonal Antibodies *Can Res* (54) 5788-5792

Hipfner, D R , Almquist, K C , Stride, B D , Deeley, R G and Cole, S P (1996) Location of a Protease-hypersensitive Region in the Multidrug Resistance Protein (MRP) by Mapping of the Epitope of MRP-specific Monoclonal Antibody QCRL-1 *Can Res* (56) 3307-3314

Hipfner, D.R., Almquist, K.C., Leslie, E.M., Gerlach, J.H., Grant, C.E., Deeley, R.G. and Cole, S.P. (1997) Membrane topology of the multidrug resistance protein (MRP). A study of glycosylation-site mutants reveals an extracytosolic NH2 terminus. *J. Biol. Chem.* 272(38): 23623-236230.

Hipfner, D.R., Gao, M., Scheffer, G., Scheper, R.J., Deeley, R.G. and Cole, S.P. (1998) Epitope mapping of monoclonal antibodies specific for the 190-kDa multidrug resistance protein (MRP). *Br. J. Can.* 78(9): 1134-1140.

Hirohashi, T., Suzuki, H., Ito, K., Ogawa, k., Kume, K., Shimizu, T. and Sugiyama, Y. (1998) Hepatic expression of multidrug resistance-associated protein-like proteins maintained in eisai hyperbilirubinemic rats. *Mol. Pharmacol.* 53(6): 1068-1075.

Hoffman, G. and Mattern, M. (1993) Topoisomerase II in multidrug resistance. *Cytotechnology.* 12(1-3): 137-154.

Hooijberg, J.H., Broxterman, H.J., Kool, M., Assaraf, Y.G., Peters, G.J., Noordhuis, P., Scheper, R.J., Borst, P., Pinedo, H.M. and Jansen, G. (1999) Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Res.* 59(11): 2532-5.

Horio, M., Chin, K.V., Currier, S.J., Goldenberg, S., Williams, C., Pastan, I., Gottesman, M.M. and Handler, J. (1989) Transepithelial transport of drugs by the multidrug transporter in cultured Madin-Darby canine kidney derived cell line epithelia. *J. Biol. Chem.* (264): 14880-14884.

Ihde, D.C. (1992) Chemotherapy of lung cancer. *N. Engl. J. Med.* 327(20): 1434-1441.

Ikeda, K., Oka, M., Yamada, Y., Soda, H., Fukuda, A., Tsukamoto, K., Nogucji, Y., Isomoto, H., Takeshima, F., Murase, K., Kambira, S., Tomonaga, M. and Kohno, S. (1999) Adult T-cell leukemia cells over-express the multidrug-resistance-protein (MRP) and lung-resistance-protein (LRP) genes. *Int. J. Cancer.* 82(4): 599-604.

Ishikawa, T. (1992) The ATP-dependent glutathione S-conjugate export pump. *Trends Biochem. Sci.* (17): 463-468.

Ishikawa, T., and Ali-Osman, F. (1993) Glutathione-associated cis-diamminedichloroplatinum (II) metabolism, and ATP-dependent efflux from leukemia cells. Molecular characterisation of glutathione-platinum complex and its biological significance. *J. Biol. Chem.* (268): 20116-20125.

Ishikawa, T., Wright, C.D. and Ishizuka, H. (1994) GS-X pump is functionally overexpressed in cis-diamminedichloroplatinum (II)-resistant human leukemia HL-60 cells and down-regulated by cell differentiation. *J. Biol. Chem.* 269(46): 29085-29093.

Ishikawa, T., Bao, J.J., Yamane, Y., Akimaru, K., Frindrich, K., Wright, C.D. and Kuo, M.T. (1996) Coordinated induction of MRP/GS-X pump and gamma-glutamycysteine synthase by heavy metals in human leukemia cells. *J. Biol. Chem.* 271(25): 14981-14988.

Ito, K , Fujimori, M , Nakata, S , Hama, Y , Shingu, K , Kobayashi, S , Tsuchiya, S , Kohno, K , Kuwano, Ml and Amano, J (1998) Clinical Significance of the Increased Multidrug Resistance-Associated Protein (MRP) Gene Expression in Patients With Primary Breast Cancer *Oncol Res* (10) 99-109

Izquierdo, M A , van der Zee, A G J , Vermorken, J B , *et al* (1995) Drug resistance associated marker LRP for prediction of response to chemotherapy and prognosis in advanced ovarian carcinoma *J Natl Can Inst* 87(16) 1230-1237

Izquierdo, M A , Neefjes, J J , Mathari, A E , Flens, M J , Scheffer, G L and Scheper, R J (1996a) Overexpression of the ABC transporter TAP in multidrug-resistant human cancer cell lines *Br J Cancer* 74(12) 1961-1967

Izquierdo, M A , Shoemaker, R H , Flens, M J , Scheffer, G L , Wu, L , Prather, T R and Scheper, R J (1996b) Overlapping phenotyes of multidrug resistance among panels of human cancer-cell lines *Int J Cancer* (65), 1-8

Jansen, P L and Oude Elferink, R P (1993) Hereditary conjugated hyperbilirubinemia in Wistar rats a model for the study of ATP-dependent hepatocanalicular organic anion transport *Adv Vet Sci Comp Med* (37) 175-195

Jedlitschky, G , Leir, I , Buchloz, U , Baroun, K , Center, M and Keppler, D (1994) ATP-dependent transport of glutathione S-conjugates by multidrug resistant small-cell lung cancer cells *Can Res* (54) 4833-4836

Jedlitschky, G , Leier, I , Bucholz, U , Hummel-Eisenbeiss, J , Burchell, B and Keppler, D (1997) ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2 *Biochem J* 327(1) 305-310

Juliano, R L and Ling, V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants *Biochem Biophys Acta* (455) 152-162

Kast, C and Gros, P (1997) Topology mapping of the amino-terminal half of multidrug resistance-associated protein by epitope insertion and immunofluorescence *J Biol Chem* 272(42) 26479-87

Keppler, D , Muller, M , klunemann, C , Guhlmann, A , Krauss, K , Muller, J , Berger, U , Leier, I and Mayatepek, E (1992) Transport and in vivo elimination of cysteinyl leukotrienes *Adv Enzyme Regul* (32) 107-116

Keppler, D , Konig, J and Buchler, M (1997) The canalicular multidrug resistance protein, cMRP/MRP2, a novel conjugate export pump expressed in the apical membrane of hepatocytes *Advan Enzyme Regul* (37) 321-333

Kiuchi, Y , Suzuki, H , Hirohashi, T , Tyson, C A and Sugiyama, Y (1998) cDNA cloning and inducible expression of human multidrug resistance associated protein 3 (MRP3) *FEBS Letters* (433) 149-152

Kohler, G and Milstein, C (1975) Continuos cultures of fused cells secreting antibody of predefined specificity *Nature* 256(5517) 495-497

Koike, K , Kawabe, T , Tanaka, T , Toh, S , Uchiumi, T , Wada, M , Akiyama, S , Ono, M and Kuwano, M (1997) A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells *Cancer Res* 57(24) 5475-5479

Konig, J , Rost, D , Cui, Y and Keppler, D (1999) Characterisation of the human multidrug resistance protein isoform MRP3 localised to the basolateral hepatocyte membrane Hepatology (4) 1156-63

Kool, M , de Haas, M , Scheffer, G L , Scheper, R J , van Eijk, M J T , Juijn, J A , Baas, F and Borst, P (1997) Analysis of Expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, Homologues of the Multidrug Resistance-associated Protein Gene (MRP1), in Human Cancer Cell Lines *Can Res* (57) 3537-3547

Kool, M , van der Linden, M , de Haas, M , Scheffer, G L , De Vree, M L , Smith, A J , Jansen, G , Peters, G J , Ponne, N , Scheper, R J , Oude Elferink, R P J , Baas, F and Borst, P (1999a) MRP3, an organic anion transporter able to transport anti-cancer drugs *Proc Natl Acad Sci USA* (96) 6914-6919

Kool, M , van der Linden, M , de Haas, Baas, F and Borst, P (1999b) Expression of Human *MRP6*, a Homologue of the Multidrug Resistance Protein Gene *MRP1*, in Tissues and Cancer Cells *Can Res* (59) 175-182

Krishnamachary, N and Center, M S (1993) the MRP Gene Associated with a Non-P-glycoprotein Multidrug Resistance Encodes a 190-kDa Membrane Bound Glycoprotein *Can Res* (53) 3658-3661

Kruh, G D , Chan, A ,Myers, K , Gaughan, K , Miki, T and Aaranson, S A (1994) Expression Complementary DNA Library Transfer Establishes *mrp* as a Multidrug Resistance Gene *Can Res* (54) 1649-1652

Kruh, G D , Gaughan, K T , Godwin, A and Chan, A (1995) Expression Pattern of MRP in Human Tissues and Adult Solid Tumour Cell Lines *Journal of the National Cancer Institute* 87(16) 1256-1258

Kuichi, Y , Suzuki, H , Hirohashi, T , Tyson, C A and Sugiyama, Y (1998) *FEBS Lett* (433) 149-152

Kuss, B J , Deeley, R G , Cole, S P , William, C L , Kopecky, K J , Wolman, S R , Eyre, H J and Callen, D F (1996) The biological significance of the multidrug resistance gene MRP in inversion 16 *leukemias Leuk Lymphoma* 20(5-6) 357-364

Lacave, R , Coulet, F , Ricci, S , Touboul, E , Flahault, A , Rateau, J G , Cesari, D , Lefranc, J P and Bernaudin, J F (1998) Comparative evaluation by semiquantitative reverse transcriptive polymerase chain reaction of MDR-1, MRP and GSTπ gene expression in breast carcinomas *Br J Cancer* (77) 694-702

Lai, S.L., Goldstein, L.J., Pastan, M.M., Tsai, C.M., Johnson, B.E., Mulshine, J.L., Ihde, D.C., Kayser, K. and Gazdar. (1989) MDR-1 gene expression in lung cancer. *J. Natl. Cancer Inst.* (81): 1144-1150.

Larkin, A.M., Moran, E., Kennedy, S. and Clynes, M. (1999a) Investigations of multidrug resistance (MDR) protein levels in pre- and post-treatment samples from breast cancer patients. *Br. J. Cancer.* (78) S1 (Abstract).

Larkin, A.M., Moran, E., Alexander, D., Doherty, J., Connolly, L., Kennedy, S. and Clynes, M. (1999b) A new monoclonal antibody that specifically recognises the mdr-3 gene product. *Int. J. Can.* 80(2): 265-271.

Law, E., Gilvarry, U., Lynch, V., Gregory, B., Grant, G. and Clynes, M. (1992) Cytogenetic comparison of two poorly differentiated human lung squamous cell carcinoma lines. *Cancer Genet. Cytogenet.* (59): 111-118.

Lee, J.S., Scala S., Matsumoto Y., Dickstein B., Robey R., Zhan Z., Altenberg G., and Bates SE. (1997) Reduced drug accumulation and multidrug resistance in human breast cancer cells without associated P-glycoprotein or MRP expression. *J. Cell. Biochem.* (65): 513 - 526.

Lee, K., Belinsky, M.G., Bell, D.W., Testa, J.R. and Kruh, G. (1998) Isolation of MOAT-B, a Widely Expressed Multidrug Resistance-associated Protein/Canalicular Multispecific Organic Anion Transporter-related Transporter. *Can. Res.* (58): 2741-2747.

Legrand, O., Perrot, J.Y., Tang, R., Simonin, G., Gurbuxani, S., Zittoun, R., Marie, J.P. (1996) Expression of the multidrug resistance-associated protein (MRP) mRNA and protein in normal peripheral blood and bone marrow haemopoietic cells. *Br. J. Haematol.* 94(1): 23-33.

Leier, I., Jedlitschky, G., Bucholz, U., Cole, S.P., Deeley, R.G. and Keppler, D. (1994) the MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J. Biol. Chem.* 269(45): 27807-10.

Leveille-Webster, C. and Arias, I. (1995) The biology of the P-glycoproteins. *J. Membrane Biol.* (143): 89-102.

Linn, S.C., Pinedo, H.M., van Arke-Otte, J., van der Valk, P., Hoekman, K., Honkool, A.H., Vermrken, J.B. and Giaccone, G. (1997) Expression of drug resistance proteins in breast cancer, in relation to chemotherapy. *Int. J. Cancer.* (71): 787-795.

Loe D.W., Deeley R.G., and Cole S.P.C. (1996a) Biology of the multidrug resistance associated protein, MRP. *Europ. J. Cancer.* (32A): 945-957.

Loe D.W., Almquist, K.C., Deeley R.G., and Cole S.P.C. (1996b) Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. *J. Biol. Chem.* 271(16): 9683-9.

Longhurst, T J , O'Neill, G M , Harvie, R M and Davey, R A (1996) The anthracycline resistance-associated (*ara*) gene, a novel gene associated with multidrug resistance in a human leukaemia cell line *Br J Can* (74) 1331-1335

Lutzky, J , Astor, M B , Taub, R N , Baker, M A , Bhalla, K , Gervasoni, J E , Rosado, M , Stewart, V , Krishna, S and Hindenburg, A A (1989) Role of glutathione and dependent enzymes in anthracycline-resistant HL60/AR cells *Cancer Res* 49(15) 4120-4125

Marie, J P (1995) P-glycoprotein in adult hematologic malignancies *Hematol Oncol Clin North Am* (2) 239-249

Masuda, S , Saito, H and Inui, K I (1997) Interactions of nonsteroidal anti-inflammatory drugs with rat renal organic anion transporter, OAT-K1 *J Pharmacol Exp Ther* 283(3) 1039-1042

Marsh, W , Sicheri, D and Center, M S (1986) Isolation and characterisation of Adriamycin-resistant HL-60 cells which are not defective in the initial intracellular accumulation of drug Cancer Res (46) 4053-4057

McGrath, T , Latoud, C , Arnold, S T , Safa, A R , Felsted, L and Center, M S (1993) Mechanisms of multidrug resistance in HL60 cells Analysis of resistance associated membrane proteins and levels of *mdr* gene expression *Biochem Pharmacol* 38(20) 3611-3619

Mechetner, E , Cummiskey, R , Kyshtoobayeva, A , Garcia, R , Mera, R , Salwen, H , Cohn, S , Fruehauf, J and Levine, E (1997) MRP expression as a predictive marker in multidrug resistant breast cancer (Abstract) *Proc Am Assoc Ca Res* (38) 389

Michieli, M , Damiani, D , Ermacora, A , Masolini, P , Raspadori, D , Visani, G , Scheper, R J and Baccarani, M (1999) P-glycoprotein, lung resistance-related protein and multidrug resistance associated protein in de novo acute non-lymphocytic leukaemias biological and clinical implications *Br J Haematol* 104(2) 328-35

Moore, G E and Sandberg, A A (1964) Studies of human tumour cell line with a diploid karyotype *Cancer* (17) 170-175

Moore, S (1980) William H Stein *J Biol Chem* 255(20) 9517-9518

Moran, E , Larkin, A , Doherty, G , Kelehan, P , Kennedy, S and Clynes, M (1997) A new mdr-1 encoded P-170 specific monoclonal antibody (6/1C) on paraffin wax embedded tissue without pretreatment of sections *J Clin Pathol* 50(6) 465-71

Moscow, J and Dixon, H (1993) Glutathione related enzymes, glutathione and multidrug resistance *Cytotechnology* (12) 155-170

Muller, M , Meijer, C , Zaman, G J , Borst, P , Scheper, R J , Mulder, N H , de Vries, E G and Jansen,P L (1994) Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport *Proc Natl Acad Sci USA* 91(26) 13033-7

Naredi, P , Heath, D D , Enns, R E and Howell, S B (1994) Cross resistance between cisplatin and antimony in a human ovarian carcinoma cell line *Cancer Res* (54) 6464-6468

Nooter, K , Westerman, A M , Flens, M J , Zaman, G J R , Scheper, R J , van Wingerden, K E , Burger, H , Oostrum, R , Boersma, T , Sonneveld, P , Gratama, J W , Kok, T , Eggermont, A M M , Bosman, F T and Stoter, G (1995) Expression of the Multidrug Resistance-associated Protein (MRP) Gene in Human Cancers *Clin Can Res* (11) 1301-1310

Nooter, K , Bosman, F T , Burger, H , van Wingerden , K E , Flens, M J , Scheper, R J , Oostrum, R G , Boersma, A W M , van der Gast, A and Stoter, G (1996) Expression of the multidrug resistance-associated protein (MRP) gene in primary non-small-cell lung cancer *Annals of Oncology* (7) 75-81

Nooter, K , de la Riviere, G B , Look, M P , van Wingerden, K E , Henzen-Logmans, S C , Scheper, R J , Flens, M J , Klijn, J G M, Stoter, G and Foekens, J A (1997a) The prognostic significance of expression of the multidrug resistance-associated protein (MRP) in primary breast cancer *Br J Can* 76(4) 486-493

Nooter, K , de la Riviere, G B , Klijn, J , Stoter, G and Foekens, J (1997b) Multidrug resistance protein in recurrent breast cancer *Lancet* 349(9069) 1885-6

Norris, M D , Bordow, S B , Marshall, G M , Haber, P S , Cohn, S and Haber, M (1996) Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma *New Engl J Med* 334(4) 231-238

Norris, M D , Bordow, S , Haber, P S , Marshall, G M , Kavallaris, M , Madafiglio, J , Cohn, S L , Salwen, H , Schmidt, M L , Hipfner, D R , Cole, S P C , Deeley, R G and Haber, M, (1997) Evidence that the *MYCN* Oncogene Regulates MRP Gene Expression in Neuroblastoma *Eur J Can* 33(12) 1911-1916

Ota, E , Abe, Y , Oshika, Y , Ozeki, Y , Iwasaki, M , Inoue, H , Yamazaki, H , Ueyama, Y , Takagi, K , Ogata, T , Tamaoki, N and Nakamua, M (1995) Expression of the multidrug resistance-associated protein (MRP) gene in non-small-cell lung cancer *Br J Can* (72) 550-554

Oude Elferink, R P , Meijer, D K , Kuipers, F , Jansen, P L , Groen, A K and Groothuis, G M (1995) Hepatobiliary secretion of organic compounds, molecular mechanisms of membrane transport *Biochim Biophys Acta* 1241(2) 215-268

Paulusma, C C and Oude Elferink, R P (1997) The canalicular multispecific organic anion transporter and conjugated hyperbilirubinemia in rat and man *J Mol Med* 75(6) 420-428

Ross, D D , Doyle, L A , Schiffer, C A , Lee, E J , Grant, C E , Cole, S P , Deeley, R G , Yang, W and Tong, Y (1996) Expression of multidrug resistance-associated protein (MRP) mRNA in blast cells from acute myeloid leukemia (AML) patients *Leukemia* 10(1) 48-55

Schachner, M *et al* , (1977) Brain surface antigens detected by anti-corpus callosum antiserum *Brain Research* 127 87 - 97

Schaub, T P , Kartenbeck, J , Konig, J , Vogel, O , Witzgall, R , Kriz, W and Keppler, D (1997) Expression of the conjugate export pump encoded by the mrp2 gene in the apical membrane of kidney proximal tubules *J Am Soc Nephrol* (8) 1213-1221

Scheper, R J , Broxterman, H J , Scheffer, G L , et al (1993) Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein mediated multidrug resistance *Can Res* (53) 1475-1479

Schneider, E , Cowan, K H , Bader,H , Toomey, S , Schwartz, G N , Karp, J E , Burke, P J and Kaufmann, S H (1995) Increased Expression of the Multidrug Resistance-Associated Protein Gene in Relapsed Acute Leukemia *Blood* 85(1) 186-193

Schummer, B , Siegsmund, M , Steidler, A , Toktomambetova, L , Kohrmann, K U and Alken, P (1999) Expression of the gene for the multidrug resistance-associated protein in human prostate tissue *Urol Res* 27(3) 164-8

Shen, R , Su, Z Z , Olsson, C A , Goldstein, N I and Fisher, P B (1994) Surface-epitope masking a strategy for the development of monoclonal antibodies specific for molecules expressed on the cell surface *J Natl Cancer Inst* 86(2) 91-98

Stride, B D , Valdmarsson, G , Gerlach, J H , Wilson, G M , Cole, S P and Deeley, R G (1996) Structure and expression of the messenger RNA encoding the murine multidrug resistance protein, an ATP-binding cassette transporter *Mol Pharmacol* 49(6) 962-971

Sugawara, I , Arai, T , Yamashita, T , Yoshida, A , Masunaga, A and Itoyama, S (1994) Expression of multidrug resistance-associated protein (MRP) in anaplastic carcinoma of the thyroid *Cancer Lett* 82(2) 185-185

Sugawara, I , Akiyama, S , Scheper, R J and Itoyama, S (1997) Lung resistance protein (LRP) expression in human normal tissues in comparison with that of MDR1 and MRP *Cancer lett* (112) 23-31

Sugawara, I , Kataoka, I , Morishita, Y , Hamada, H , Tsuruo, T , Itoyama, S and Mori, S (1988) Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16 *Cancer Res* 48(7) 1926-9

Sugiyama, Y., Kato, Y. and Chu, X. (1998) *Cancer Chemother. Pharmacol.* Suppl.42: 44-49.

Taniguchi, K., Wada, M., Kohno, K., Nakamura, T., Kawabe, T., Kawakami, M., Kagotani, K., Okumura, K., Akiyama, S. and Kuwano, M. (1996) A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. *Cancer Res.* 56(18): 4124-9.

Tusnady, G.E., Bakos, E., Varadi, A. and Sarkadi, B. (1997) Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters. *FEBS Letters.* 402(1): 1-3.

Twentyman, P.R., Fox, N.E., Wright, K.A. and Bleehen, N.M. (1986) Derivation and preliminary characterisation of adriamycin resistant lines of human cancer cells. *Br. J. Cancer.* (53): 529-537.

Twentyman, P.R. and Versantvoort, C.H. (1996) Experimental modulation of MRP (multidrug resistance-associated protein)-mediated resistance. *Eur. J. Cancer.* 32A(6): 1002-9.

Uchiumi, T., Hinoshita, E., Haga, S., Nakmura, T., Tanaka, T., Toh, S., Furukawa, M., Kawabe, T., Wada, M., Kagotani, K., Okumura, K., Kohno, K., Akiyam, S. and Kuwano, M. (1998) Isolation of a novel human canalicular multispecific organic anion transporter, cMOAT2/MRP3, and its expression in cisplatin-resistant cancer cells with decreased ATP-dependent drug transport. *Biochem. Biophys. Res. Commun.* 252(1): 103-10.

van Der Valk, P., van Kalken, C.K., Ketelaars, H., Broxterman, H.J., Scheffer, G., Kuiper, C.M., Tsuruo, T., Lankelma, J., Meijer, C.J., Pinedo, H.M., et al. (1990) Distribution of multidrug resistance associated P-glycoprotein in normal and neoplastic human tissues. Analysis with 3 monoclonal antibodies recognising different epitopes of the P-glycoprotein molecule. *Ann. Oncol.* (1): 54-64.

Versantvoort, C.H.M., Broxterman, H.J., Pinedo, H.M., de Vries, E.G., Feller, N, Kuiper. C.M. and Lankelma, J. (1992) Energy-dependent processes involved in reduced drug accumulation in multidrug-resistant human lung cancer cell lines without P-glycoprotein expression. *Cancer Res.* 52(1): 17-23.

Versantvoort, C.H., Broxterman, H.J., Lankelma, J., Feller, N., Pinedo, H.M. (1994) Competitive inhibition by genistein and ATP dependence of daunorubicin transport in intact MRP overexpressing human small cell lung cancer cells. *Biochem. Pharmacol.* 48(6): 1129-36.

Versantvoort, C.H.M., Broxterman, H.J., Bagrij, T., Scheper, R.J. and Twentyman, P.R. (1995) Regulation by glutathione of transport in multidrug resistant human lung tumour cell lines overexpressing multidrug resistance associated protein. *Br. J. Can.* (72): 82-89.

Walker, J E , Saraste, M , Runswick, M J and Gray, N J (1982) EMBO J (1) 945-951

Wijnholds, J , Evers, R , van Leusden, M R , Mol, C A , Zaman, G J , Mayer, U , Beijnen, J H , van der Valk, M , Krimpenfort, P and Borst, P (1997) Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein *Nature Medicine* (11) 1275-1279

Wright, S R , Boag, A H , Valdmarsson, G , Hipfner, D R , Campling, B G , Cole, S P and Deeley, R G (1998) Immunohistochemical detection of multidrug resistance protein in human lung cancer and normal lung *Clin Can Res* 4(9) 2279-89

Young, L C , Campling, B G , Voskoglou-Nomiskos, T , Cole, S P , Deeley, R G and Gerlach, J H (1999) Expression of Multidrug Resistance Protein-related Genes in Lung Cancer Correlation with Drug Response *Clin Can Res* (5) 673-680

Zaman, G J R , Versantvoort, C H M , Smit, J J M , Eijdems, E W H M , de Haas, M , Smith, A J , Broxterman, H J , Mulder, N H , de Vries, E G E , Baas, F and Borst, P (1993) *Can Res* (53) 1747-1750

Zaman, G J R , Flens, M J , van Leusden, M R , de Haas, M , Mulder, H S , Lankelma, J , Pinedo, H M , Scheper, R J , Baas, F , Broxterman, H J and Borst, P (1994) The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump *Proc Natl Acad Sci USA* (91) 8822-8826

Zaman, G J R , Lankelma, J , van Tellingen, O , Beijnen, J , Dekker, H , Paulusma, C , Oude Elferink, R P , Baas, F and Borst, P (1995) Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein *Proc Natl Acad Sci USA* (92) 7690-7694

Zaman, G J R , Cnubben, N H , van Bladeren, P J , Evers, R and Borst, P (1996) Transport of the glutathione conjugate of ethacrynic acid by the human multidrug resistance protein MRP *FEBS Lett* 391(1-2) 126-30

Zhang, J T and Ling, V (1991) Study of membrane orientation and glycosylated extracellular loops of mouse P-glycoprotein by in vitro translation *J Biol Chem* 266(27) 18224-32

Zhang, J T , Duthie, M and Ling, V (1993) Membrane topology of the N-terminal half of the hamster P-glycoprotein molecule *J Biol Chem* 268(20) 15101-10

Zhu, Q Sun, H and Center, M S (1997) Functional Analysis of the Nucleotide Binding Domains of the Multidrug Resistance Protein (MRP) *Oncol Res* (9) 229-236

8.0. APPENDICES.

Appendix 1

ABBREVIATIONS

MRP1	Multidrug resistance-associated protein 1 (protein or gene)
P-gp	P-glycoprotein or P-170 (MDR1 gene protein product)
MDR1	Multidrug resistance protein 1 gene (P-gp encoding gene)
ABC/HRP	Streptavidin/biotin-horseradish peroxidase conjugate
Adr	Adriamycin
ATCC	American Tissue Culture Collection
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
DAB	Diaminobenzidine
dH_2O	Deionised water/glass distilled water
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethyl sulfoximide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin (Adriamycin)
ECL	Enhanced chemiluminescence
ECACC	European Collection of Animal Cell Culture
EDTA	Ethylene diamino tetra-acetic acid
ELISA	Enzyme-linked immunoabsorbent assay
FCS	Foetal calf serum
FITC	Fluorescein-isocyanate

HAT	Hypoxanthine, aminopterin, thymidine
HCL	Hydrochloric acid
HEPES	4-(2-hydroxethyl-)-piperazine ethane sulphonic acid
HT	Hypoxanthine, thymidine
IMS	Industrial methylated spirits
kD	KiloDalton
LRP	Lung resistance related protein
MAb/s	Monoclonal antibody/monoclonal antibodies
MDR	Multidrug resistance
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
Mw	Molecular weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCTCC	The National Cell and Tissue Culture Centre
ND	Not done
NEAA	Non-essential amino acids
NSCLC	Non-small cell lung carcinoma
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol

PMSF	Phenylmethyl sulfonyl fluoride
PNAG	Phenyl-N-acetyl-α-D-galactosaminide
PNPP	P-nitrophenyl phosphate
PVDF	Polyvinyldifluoride (blotting membrane)
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TBS	Tris buffered saline
TEMED	N,N,N,N,-tetramethyl-ethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
TV	Trypsin/versene
UV	Ultra violet
v/v	Volume to volume ratio
w/v	Weight to volume ratio

Appendix 2

LIST OF SUPPLIERS

- Amersham International plc Bucks HP7 9NA, UK
- Antec International Ltd Sudbury, Suffolk CO16 6XD, UK
- BDH Laboratory Supplies, Poole, BDH15 1TD, England
- Beckman Instruments (UK) Ltd High Wycombe, Bucks HP12 4YH, UK
- Becton Dickinson UK Ltd, Between Towns Road, Crowley, Oxford OX4 3LY, UK
- Bio Rad laboratories Ltd Herts HP2 7TD, UK
- Bioresearch Ireland, Forbairt, Glasnevin, Dublin 9, Ireland
- Biosyn Ltd , 10 Malone Road, Belfast, BT9 5BN, Northern Ireland
- Boehringer Mannheim, Roche Diagnostics Ltd Bell Lane, Lewes, East Sussex BN7 1LG, UK
- Clintech, Clacton-on-Sea, Essex, UK
- Costar Cambridge MA, USA
- Dako, 16 Manor Courtyard, Hughenden Avenue, High Wycombe, Bucks HP13 5RE, UK
- Gelman, United Kingdom Pall Gelman Sciences, Brackmills Business Park, Caswell Road, Northampton NN4 7EZ, UK
- Gibco, Life Technologies Ltd, 3 Fountain Drive, Inchinnan Buisness Park, Paisley PA4 9RF, UK
- Greiner Labortechnik Gmbh, Maybachstabe 2, D-72636 Frickenhaussen
- Hoefer Hoefer Scientific Instruments, San Fransisco, USA
- Immune Systems Ltd , PO Box 120, Paignton, TQ4 7XD, UK
- Merck, Magna Park, Little-worth, Leicestershire, UK
- NUNC A/S Roskilde, Denmark
- Chemical Products, R Borghgraef s a Belgium
- Nuaire, 2100 Fernbrook Lane, Plymouth, Minnesota 55447, USA
- Oxoid Ltd, Basingstoke, Hampshire, UK

- Pharmingen, BD Diagnostic Systems, Pottery Road, Kill O'The Grange, Dun Laoghaire, Co Dublin, Ireland
- Pierce, Post Office Box 117, Rockford, Illinois 61105 USA
- Sigma Diagnostics, St Louis, MO 63178 USA
- Sigma, Sigma-Aldrich Company LTD Fancy Road, Poole, Dorset, BH12 4QH, UK
- Sterlin Ltd, Middlesex TW148QS, UK
- Vector Laboratories Ltd , 16 Wulfric Square, Bretton, Peterborough PE3 8RF, UK
- Vector Laboratories, Inc Burlingame, CA 94010 USA