Transcriptional Regulation of Gene Expression in Lung Carcinoma Cells

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By

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Discussion, Conclusions & Future work, Bibliography, Appendices

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Table of Contents Page

Volume 1:

Section 4.0

Discussion

4.1 General Introduction

It has been shown previously that BrdU induces differentiation in the poorly differentiated lung carcinoma cell line, DLKP, and in the lung adenocarcinoma cell line, A549 (McBride *et al.*, 1999; P. Meleady, PhD. Thesis, 1997; F. O' Sullivan, PhD. Thesis, 1999; D. Walsh, PhD. Thesis, 1999). Associated with this alteration in differentiation status of the cell lines was an alteration in expression levels of various proteins. Protein expression of the cytokeratins K8 and K18, the eukaryotic initiation factors eIF-4E and 2α and the transcription factors Yin-Yang 1 (YY1) and c-Myc1 was increased in both cell lines when induced to differentiate by BrdU. BrdU-induced differentiation of the DLKP cell line also resulted in increased protein expression of α_2 and β_1 integrins, the cytokeratin K19 and the cell-adhesion proteins, Ep-CAM, α-actinin and α-catenin. The mRNA expression levels of these genes, with the exception of the α_2 integrin, remained unaffected following BrdU treatment. As a result, regulation of expression of these genes (with the exception α_2 integrin) appeared to be at the translational level in these cell lines.

BrdU (a thymidine analogue) integrates into the DNA of the target cells prior to inducing the differentiation of those cells (Ashman and Davidson, 1980). As such, it was expected that BrdU would exert its effects primarily via a transcriptional mechanism. The speculation that the poorly differentiated DLKP cell line (Law *et al.*, 1992) may represent a stem cell-like population of the lung provided a unique opportunity to study the possible transcriptional mechanisms regulating early lung development and differentiation *in vitro*.

4.2 BrdU-induced differentiation in epithelial lung cell lines

Studies were carried out on DLKP and the diffuse, glandular adenocarcinoma lung cell line, A549 (Smith, 1977), both exposed to the differentiating agent, BrdU. It was decided to examine whether BrdU was capable of inducing effects in these cell lines which were exerted at the transcriptional level. To this end, RT-PCR and DNA microarray analyses were employed to identify genes whose expression was capable of being transcriptionally-regulated by BrdU.

While RT-PCR analysis remained the primary method of examination, due to its reliability and reproducibility, the DNA microarray method (which only became available during the later stages of the thesis) was included to highlight gene expression changes in groups of genes which would not necessarily have been considered for analysis. It was felt that the RT-PCR results, which were repeated threefold, were more reliable than those obtained for the DNA microarray analysis. However, the DNA microarray results are also valuable, as they may highlight future areas of research of BrdU-regulated genes.

4.2.1 RT-PCR analysis reveals that BrdU induces gene expression changes predominantly in the DLKP cell line

RT-PCR analysis of the DLKP cell line revealed low levels of expression of most of the twenty-five genes studied. Immunocytochemical analysis of DLKP had already determined that the cell line lacked several expression markers for differentiation, including the major keratins (Daly *et al.*, 1998).

RT-PCR analysis of the BrdU-exposed DLKP cell line revealed increased expression of a number of genes not previously examined in differentiation studies. In general, expression changes were felt to be of significance if they exceeded a two-fold increase or decrease. Significant increases in expression of the MRP1, MRP3, COX-2, eIF-2α, BAXα, and MRIT genes were observed. Less significant increases in expression were also observed for the MRP2, BCRP, α-catenin and E-cadherin genes. The anti-apoptotic

Survivin gene was the only gene surveyed by RT-PCR which showed decreased expression in DLKP following BrdU treatment.

The RT-PCR results profile obtained for the BrdU-treated A549 cells was distinctly different. The A549 cell line appears much more differentiated than DLKP and in the normal state expressed many of the genes surveyed here in the RT-PCR analysis, and at a much higher level of expression. Following exposure to BrdU, only two genes out of the twenty-five studied, MRP1 and BCRP, showed increased expression in this cell line. Three genes, MRP2, MRP4 and mdr-1, decreased in expression in A549 following BrdU treatment. Only the MRP4 and mdr-1 expression decreases were felt to be of significance.

It is important to note that the expression changes observed here have not been analysed in terms of mRNA stability in the differentiating cells. It is therefore possible that these alterations in mRNA may be due to posttrancriptional mechanisms. Paine *et al.* (1992) has previously demonstrated using nuclear run-ons that alterations in mRNA expression in differentiating rat anaplastic carcinomas was due to posttranscriptional (although pretranslational) downregulation. In the absence of such tests being carried out here to confirm or deny this hypothesis, this possibility cannot be discounted.

4.2.1.1 BrdU induces expression of MDR-related genes in DLKP and A549

The MRP1, MRP2, MRP3 and BCRP genes have been associated with the multidrug-resistance phenotype in various cell systems (Section 1.8). RT-PCR analysis demonstrated that all four of these genes were observed to be significantly upregulated in expression at the mRNA level in DLKP cells following exposure to the differentiating agent BrdU. The most significant increases were observed for the MRP1 and MRP3 genes. In the A549 cell line, increased expression of MRP1 and BCRP was observed following BrdU treatment, although the increases were less dramatic than those obtained in the DLKP cell line. In addition, A549 registered significant decreases in expression of the MDR-related MRP4 and mdr-1 genes as well as a slight decrease in MRP2 gene expression. Apart from mdr-1, none of these MDR-related genes had previously been associated with altered expression levels in differentiating cells.

4.2.1.1.1 Previous reports of MDR-related gene expression changes associated with differentiation

The majority of studies carried out on the effect of differentiation on the multidrug-resistance phenotype and genotype have focussed on the activity and expression of mdr-1/Pgp. Increased expression of Pgp in humans has been correlated with the differentiation status of murine oesteoblastic sarcomas (Takeshita *et al.*, 2000), and oral and maxillofacial tumours (Xie *et al.*, 2000). Increased mdr-1 gene expression has also been correlated with increased differentiation status in human gastric and colorectal cancers (Motoo *et al.*, 1998), in the colon carcinoma cell line LS180 (Herzog *et al.*, 1993) and in non-small cell lung carcinoma (NSCLC) (Bosch *et al.*, 1997). While the prognostic implications of mdr-1 gene and protein expression have already been reviewed for a host of clinical settings (see Section 1.8.1), Tokunaga *et al.* (2001) have correlated the increased Pgp phenotype in well-differentiated colorectal cancer with resistance to cancer chemotherapy and poor clinical prognosis. Expression of the mdr-1 gene has also been correlated with expression of other differentiation-specific markers. Gamelin *et al.* (1999) observed that mdr-1 gene expression correlated with cytokeratin-8 (K8) protein expression levels in renal cell carcinoma. As already outlined, cytokeratin-8 has previously been identified in this laboratory as a possible differentiation marker in BrdU-differentiated DLKP cells (McBride *et al.*, 1999). Pgp has also been proposed as a differentiation marker in chronic myeloid leukaemia (CML) (Turkina *et al.*, 1999).

The effect of the process of differentiation on the expression of other MDR-related gene and protein markers has not been extensively studied. Studies attempting to correlate differentiation and expression of MRP1 have met with mixed results. Sugawara *et al.* (1995) observed higher levels of MRP1 protein expression in well-differentiated adenocarcinoma of the lung than for poorly differentiated tumours. However, further studies on differentiated tumours (Nooter *et al.*, 1998) and cell lines (Schippers *et al.*, 1997) have revealed no significant correlation of MRP1 expression and differentiation status.

4.2.1.1.2 Increased expression of MDR genes does not necessarily confer MDR phenotype

DLKP cells induced to differentiate by BrdU were examined at each previously selected time frame of the differentiation process (day 2, day 5, etc.) using *in vitro* drug toxicity assays. The cells were exposed to adriamycin, cisplatin, taxol and VP16 in an attempt to correlate the increased MDR gene expression profile of the cells with any change in their multidrug resistance profile. It was observed from these assays that the cells did not display any significantly altered drug resistance phenotype to the normal, undifferentiated DLKP (Section 3.1.2). It is important to note here that the increases observed in mRNA expression in BrdU-treated DLKP may not necessarily have been translated into protein expression in those cells.

In a number of previous studies, increased differentiation has resulted in an increase in the MDR phenotype (Ho *et al.*, 1994; Su *et al.*, 1994). Thus far, the increase in the MDR phenotype has largely only been correlated with expression of mdr-1/Pgp, although the role of some other MDR-specific markers have also been tentatively examined (Scheper *et al.*, 1993; Kitazono *et al.*, 2001). However, several studies have also noted induction of MDR gene expression does not tally with the induction of an MDR phenotype. Mickley *et al.* (1989) correlated mdr-1 gene expression with the degree of induced differentiation in SW-620 and HCT-15 cells, but observed that in neither case was the MDR phenotype induced. Exposure of breast, ovarian and colon carcinoma cell lines to the differentiation-inducing aromatic fatty acids phenylacetate and phenylbutryate also resulted in increased mdr-1 gene expression (Shack *et al.*, 1996) without the induction of the multidrug resistance phenotype.

In this study, it also was noted that, after seven days exposure to BrdU, a marked decrease in normal cell growth was observed. This facilitated the elimination of the cells at a far lower drug concentration than that previously determined by toxicity analysis. This result was observed to be identical over the four structurally and functionally different drugs studied. This indicates the possibility that one or several genes or proteins responsible for essential cell function in these cells are adversely affected by exposure to the agent. This effect is not due simply to the inherent toxicity or anti-tumour effects of BrdU (see Section 1.4.3.6) as cell viability returned to near normal patterns of growth after fifteen days exposure to BrdU. This indicates that this is a specifically targetted effect of BrdU which disrupts normal cell growth and division and

peaks at seven days of exposure. This was an interesting observation and one which has obvious implications for the future analysis of BrdU effects in cell lines using DNA microarray analysis (see Section 4.2.3).

4.2.1.1.3 Why are MDR-specific markers increased in expression during differentiation?

The functional significance, if any, for the increase in expression of MDR markers in differentiation is currently unclear. It is possible that expression of MDR-related genes, such as mdr-1, may fulfill some as yet unidentified role in the developing cell. Mylona *et al.* (1996) observed expression of the mdr-1 gene at all stages of development and differentiation in human placenta and increased expression of Pgp was also observed in differentiating megakaryocytes (Sato *et al.*, 1995). These two studies indicate that mdr-1 expression may have some function in the normal process of development of a non-carcinoma cell, and that its upregulation during differentiation may not necessarily be connected with multidrug resistance. In fact, there is evidence that the availability of transcriptionally active forms of MDR-related genes may actually be a requirement for successful differentiation. Shannon and Iacopetta (2001) observed that methylation of the mdr-1 gene correlated with a poor histological differentiation status in colorectal carcinoma.

4.2.1.1.3.1 Pgp and MRP1 as possible transporters of differentiating-inducing agents

Pgp has been postulated as a possible efflux pump for the well-characterised differentiating agent, Retinoic acid (RA) in RA-resistant human myeloid leukaemic HL60 and APL cell lines (Matsushita *et al.*, 1998). It has also been suggested that Pgp may also be capable of transporting polyamines, low molecular weight organic cations that are essential for cell growth and differentiation (Aziz *et al.*, 1996).

Scala *et al.* (1995) noted increased differentiation in human MCF-7 breast cancer cells accompanied by decreased Pgp activity induced by the Pgp antagonist, 8-Cl-cAMP. The NSAID indomethacin, a known MRP1 inhibitor (Duffy *et al.*, 1998) has been observed

to induce differentiation in adipocyte precursors (Ye and Serrero, 1998) and in fibroblasts (Williams and Polakis, 1977), although it was observed to inhibit differentiation in mouse leukaemia cells (Honma *et al.*, 1979).

However, in studies carried out in mouse lymphoma cells (Molnar *et al.*, 1998) and human colon carcinomas (Herzog *et al.*, 1993), reversal of Pgp activity by the use of Pgp antagonists has not been correlated with increased differentiation. Additionally, work carried out in this laboratory has indicated that BrdU may be an "irreversible" inducer of differentiation of DLKP cells (D. Walsh, PhD. Thesis, 1999) (Section 1.4.3.7). In this case, the proposed model of induced transcription in response to perceived toxic threat posed by BrdU does not seem viable.

4.2.1.2 BrdU induces expression of eIf-2α in DLKP cells

A significant increase in gene expression of the eukaryotic translation initiation factor eIF-2α in DLKP cells was observed following exposure to BrdU. No significant effect on expression of the gene was observed in the BrdU exposed A549 cell line. RT-PCR analysis did not reveal any significant effect on expression of the eIF-4E gene in either cell line following BrdU exposure.

Preliminary western blotting carried out in this laboratory on the DLKP cell line (Walsh, PhD. Thesis, 1999) had identified increased protein expression of eIF-2α following BrdU treatment. At the time, RT-PCR analysis on eIF-2α mRNA levels was not carried out, and it was assumed that the eIf-2α protein was translationally upregulated in DLKP by the action of the c-Myc 1 protein.

However, it was also postulated that the c-*myc* gene may transcriptionally increase levels of eIf-2α. An interesting observation is that the 3'UTR of differentiation-related mRNAs, such as tropomyosin, activate PKR (Protein Kinase RNA-activated) activity (Davis and Watson, 1996). Active PKR phosphorylates eIF2α leading to a decrease in translational efficiency (Rastinejad *et al.*, 1993) and an induction of differentiation (Rastinejad and Blau, 1993). Phosphorylation of eIf-2α has previously been observed in

PC12 cells induced to differentiate with nerve growth factor (Munoz *et al.*, 2001). In this scenario, the transcription factor c-*myc* would provide PKR with its substrate by transcriptionally increasing the levels of eIf-2α mRNA.

It is not clear whether or not the increase in eIF-2α is due to the action of c-*myc*. However, no significant change in c-*myc* gene expression was observed here in either A549 or DLKP cells following BrdU treatment using RT-PCR. DNA microarray analysis did however, indicate a significant decrease in c-*myc* gene expression in DLKP after seven days exposure to BrdU (Section 4.2.2). BrdU has previously been reported to induce differentiation of the leukaemic cell line, HL60 (Yen and Forbes, 1990) and melanoma cells (Valyi-Nagy *et al.*, 1993) associated with decreased levels of the c-*myc* mRNA transcript. It is therefore possible that the gene is transcriptionally regulating differentiation in DLKP through transcriptional expression of eIf-2α.

It would therefore be of interest to examine further the possible influence of increased eIf-2α protein expression, either in the active or inactive phosphorylated form, during differentiation. eIf-2α protein levels and activity are thought to regulate global translation (Kimball *et al.*, 1998), and may therefore play a role in regulating the overall protein synthesis and growth rates during differentiation. eIf-2α has also previously been implicated in translational regulation in differentiating myeloid leukaemia HL60 cells (Konno *et al.*, 1986) and has been implicated in the regulation of haemoglobin synthesis in differentiating erythroid cells (Crosby *et al.*, 2000). Also, the induction of eIf-2α protein expression by both the differentiating agent thiazolidinedione (Palakurthi *et al.*, 2001) and interferon, a product of cell growth (Petryshyn et al., 1996) indicates a regulatory mechanism for this protein in cellular differentiation. It would appear from research carried out here that expression of eIf-2α during BrdU-induced differentiation may be regulated at both transcriptional and translational levels in the DLKP cell system.

4.2.1.3 BrdU increases expression of BAXα and MRIT and reduces Survivin expression in DLKP

A total of eight apoptotic gene markers were examined for expression in BrdU-differentiated DLKP and A549 cells. Significant increases in expression of the pro-apoptotic BAXα and MRIT genes were observed in DLKP following exposure to BrdU. No change in expression of the pro-apoptotic BAP and Bcl-x_S genes was observed in these cells. Decreased gene and protein expression of anti-apoptotic Survivin were also observed in the BrdU-treated DLKP. No change in expression of the anti-apoptotic Bcl-2α, Bcl-x_L and BAG genes was observed in DLKP. Exposure to the differentiating agent was not observed to have an effect on the expression of any of the apoptotic genes studied in the A549 cell line.

The role of apoptotic genes in the process of cellular differentiation remains to be elucidated. In general terms, explanations for apoptotic gene and protein expression in this process may be inferred from an overview of tumour progression. The growth of a tumour is a general term indicating an alteration in size of a cell mass and is the end result of several interrelated influences, such as proliferation, differentiation and cell death, as well as cell contacts and blood supply. In patients, changes in cell number represent a balance between proliferation and death so alteration of any of these parameters can influence the size of a tumour mass. Thus, in a normal tissue, cell number remains constant because of a balance between proliferation, death and differentiation. In abnormal situations, increased cell number can result from either blocked death and/or differentiation or increased proliferation with no changes in the other two properties. Each of these routes is used in carcinogenesis. It is apparent, therefore, that apoptosis may play a role in regulating cell number in differentiating cell populations. If this were the case, the expression of apoptotic-specific markers in cells undergoing differentiation would be expected.

4.2.1.3.1 Cell types undergoing apoptosis and differentiation concomitantly

A number of studies have been carried out correlating the causes and effect of apoptotic behaviour in cells with their differentative capacity. Apoptosis has been induced in human leukaemia cell lines (Benito *et al.*, 1996; Elstner *et al.*, 1997) and endocrine cells (Eerola *et al.*, 1999) undergoing differentiation. Maruoka *et al.* (1997) observed apoptosis in terminal differentiation of keratinocytes and concluded that terminal differentiation was a pathway to apoptosis in this cell system. A putative model for this synergistic combination of apoptosis and differentiation in tumours was provided recently in ameloblastomas by Sandra *et al.* (2001). Ameloblastomas are malignant jaw tumours which stem from ameloblasts, cells which form tooth enamel. The authors proposed an anti-apoptotic proliferating site in the outer peripheral layer of the tumour, and an inner pro-apoptotic differentiating layer at the centre. This model was devised by the specific locational identification of various pro- and anti-apoptotic proteins in several tissue sections of ameloblastomas.

Apoptosis has also previously been induced by several agents known to induce differentiation. Dual induction of both differentiation and apoptosis by the differentiating agent vitamin D3 has been observed in acute promyelocytic leukaemia (Elstner *et al.*, 1997). Apoptosis has also been induced in colorectal carcinomas by this agent (Diaz *et al.*, 2000), and by Retinoic acid (RA) in acute promyelocytic leukaemia (Gianni *et al.*, 2000) and cultured keratinocytes (Islam *et al.*, 2000). Further studies have indicated the effect of the DIF-1 differentiating inducing factor from Dicytostelium discoideum to induce apoptosis in human insulin-secreting INS-1 cells (Fujimaki *et al.*, 2001).

These studies indicate that DLKP and A549 induced to differentiate by BrdU may also spontaneously undergo apoptosis. In this scenario, the expression of pro-apoptotic markers might be expected to increase in differentiating cells, possibly accompanied by a decrease in anti-apoptotic markers. In the DLKP cell line, this is what was observed at the mRNA level. It is therefore relevant to examine the role these markers may play in the induced differentiation of lung cell lines.

4.2.1.3.2 The effect of differentiation on the expression of apoptotic genes and proteins

Little is known of the expression of MRIT in cellular differentiation. No comparative studies have been carried out on expression of the MRIT gene or protein, although MRIT is known to interact with Bcl-X_L in mediating cell death. Expression of Bcl-x_L protein has been observed to be upregulated following differentiation of promyelocytic leukaemia cells (Chatterjee *et al.*, 1997), liver carcinoma cells (Wakabayashi *et al.*, 2000), neuronal cells (Guillemain *et al.*, 2000), and primary macrophages (Sevilla *et al.*, 2001).

The expression of the anti-apoptotic Survivin protein has also been correlated with differentiation in human and mouse fetal tissue (Adida *et al.*, 1998). However, studies relating expression of Survivin gene or protein with regard to differentiation are limited.

As stated previously, the BAXα gene codes for the 21kDa BAX protein product. Studies examining the expression of the BAXα gene in differentiating cells have been limited. However, expression of BAX protein has previously been correlated with differential regulation in tumours of the central and peripheral nervous system (Krajewski *et al.*, 1997). The significance of BAX protein expression in the process of cellular differentiation has been outlined in a study by Feuerhake *et al.* (2000). The group observed different staining patterns of BAX and Bcl-2 proteins in specific glandular epithelial cells which appeared to reflect particular cell differentiation states in human mammary gland epithelium. The different patterns also suggested that there are also different grades of susceptibility towards apoptotic stimuli in individual glandular epithelial cells. Additional studies utilising the differentiating agent Retinoic Acid (RA) have identified a possible role for the BAX protein during differentiation. Sermadiras *et al.* (1997) observed increased expression of BAX protein in cultured human melanocytes, but not keratinocytes, which were induced to differentiate. A further study by Sano *et al.* (2001) found increased BAX protein expression in human embryonal carcinoma cells following differentiation and apoptosis. However, studies comparing induced differentiation and apoptotic proteins in HL60 myeloid leukaemia cells (Manfredini *et al.*, 1998) and neuronal cells (Guillemain *et al.*, 2000) found no significant correlation with expression of the BAX protein.

It is becoming apparent that to carry out a comprehensive analysis of pro- and anti-apoptotic genes in any cell system requires analysis of all known apoptotic genes in order to ascertain the extent of possible interactions between them. To date, little is understood of the roles these apoptotic genes play at the transcriptional level of differentiation.

4.2.1.4 BrdU-induced expression of genes involved in tumour progression

A significant increase in COX-2 mRNA expression was observed in DLKP cells induced to differentiate following exposure to BrdU. No significant effect on expression of the gene was observed in the BrdU-exposed A549 cell line. The expression of the COX-1 gene was not significantly affected in either cell line following BrdU-induced differentiation.

COX-2 protein expression has previously been implicated in the differentiation of human keratinocytes (Leong *et al.*, 1996), endometrial stromal cells (Han *et al.*, 1996) and osteoblast-like cells (Koide *et al.*, 1999). Expression of COX-2 protein has also been associated with abnormal differentiation of mouse epidermis, which has been found to be characteristic of epithelial tumours, including squamous cell carcinomas of the skin (Neufang *et al.*, 2001). Investigations into the effects of COX-2 inhibitors on differentiation have met with varying results; Nakanishi *et al.* (2001) found that inhibitors of cyclooxygenase-2 were able to suppress differentiation of human leukaemia cell lines, while a COX-2 selective NSAID was observed to enhance the differentative properties of sodium butyrate on colorectal carcinomas (Crew *et al.*, 2000).

It is currently unknown why expression of COX-2 should be upregulated during differentiation, or indeed what effect the gene/protein may have in the process. However, a study by Takiguchi *et al.* (1999) revealed that Prostaglandin E_2, exerts a biphasic effect on differentiation in osteoblast cells. Additionally, COX-2 expression can be increased dramatically in fibroblasts, vascular smooth muscle or endothelial cells to growth factors, hypoxia, phorbol esters or cytokines and in monocytes/macrophages by lipopolysaccharides (LPS) (Bolten, 1998; Vane *et al.*, 1996). In light of these studies, the

inducible response of COX-2 gene expression in response to BrdU is not wholly surprising, if not fully understood.

4.2.1.5 BrdU induces mRNA expression of E-cadherin and α-catenin in DLKP

RT-PCR analysis was carried out to examine expression of genes involved in cell adhesion in BrdU-exposed DLKP and A549 cells. Three genes were chosen for analysis; E-cadherin, α-catenin and β-catenin. Expression of E-cadherin and α-catenin was increased in the BrdU-treated DLKP cells. It must be noted that these increases, while significant, were lower than those obtained for any of the other genes studied. No change was observed in β-catenin expression in BrdU-treated DLKP. The slight increase in α-catenin (two-fold) was duplicated in the DNA microarray analysis (Section 4.2.2). No changes in expression of any of the three genes studied was observed in the BrdU-exposed A549 cells.

Previous work in this laboratory (F. O'Sullivan, PhD. Thesis, 1999) had identified the induction of the Ca^{2+} dependent cell-cell adhesion molecule in BrdU-treated DLKP cells. Litvinov *et al.* (1997) had proposed a model in which Ep-CAM-mediated disruption of adherens junctions was due to a redistribution of E-cadherin on the cell surface, rather than a downregulation. This redistribution of E-cadherin, and hence disruption of its function, by Ep-CAM was via alterations in the focal adhesion proteins (specifically α-catenin and α-actinin) involved in binding E-cadherin to the cytoskeleton. O'Sullivan (PhD. Thesis, 1999) subsequently observed significant downregulation of α-catenin and α-actinin protein in BrdU-treated A549 and DLKP cell lines. The effect of BrdU on E-cadherin expression was not examined in this study.

Cadherins are the "glue" by which adjacent epithelial cells are attached to each other; they are important in determining the pattern of cells in a tissue and function as Ca^{2+}-dependent homophilic cell-cell binding proteins (Nose *et al.*, 1990). They are believed to modulate differentiation by co-signalling with other cell adhesion molecules (Section 1.7.4.1). It is obvious therefore, that a change in expression of any of these

genes/proteins may have a knock-on effect on expression of some or all of the other genes.

While disruption to E-cadherin binding is often seen as a prelude to metastatic events in cellular physiology, its downregulation is also considered a normal event during development and differentiation (Christofori and Semb, 1999). It is possible, however, that decreased expression of these proteins may lead to a decrease in cell adhesion with an attendant increase in invasiveness phenotype. Down-regulation of the E-cadherin/catenin complex has been implicated in oesophageal cancer (Kadowaki *et al.*, 1994), gastric cancer (Streit *et al.*, 1996, Shun *et al.*, 2001) and colon cancer (Vermeulen *et al.*, 1995; Ghadimi *et al.*, 1999). At present, little is known about cadherin expression in SCLC (Shimoyama *et al.*, 1989), although decreased α-catenin expression has been correlated with high cell proliferation and levels of metastasis in NSCLC (Kimura *et al.*, 2000; Pirinen *et al.*, 2001).

It is apparent, therefore, that low levels of expression of the cadherin/catenin proteins may be associated with a poorly differentiated phenotype (Kimura *et al.*, 2000; Shun *et al.*, 2001). Increased gene expression of both E-cadherin and α-catenin has been previously observed in differentiating pancreatic ductal carcinoma (Yonemasu *et al.*, 2001), while increased protein expression has been observed in well-differentiated hepatic bile duct carcinomas (Mikami *et al.*, 2001). Induction of expression of E-cadherin and α-catenin protein has also been observed in response to the differentiating agent 1,25 dihydroxyvitamin D3 in human keratinocytes (Gniadecki *et al.*, 1997).

It is apparent from these studies that increased in E-cadherin and α-catenin gene expression associated with increased differentiation is not an isolated phenomenon. Indeed, the altered expression of these two genes may provide further evidence of a differentiated phenotype in lung carcinomas. Evidence has also been presented here that may correlate increased expression of these genes with a reduced level of cellular invasiveness. However, further work would be necessary to confirm this phenotype.

4.2.2 DNA microarray analysis reveals BrdU-induced gene expression changes after seven days exposure in the DLKP cell line

Using DNA microarray analysis (Section 3.5), a total of 597 genes were assayed for changes in expression in the DLKP cell line following seven days exposure to BrdU. This preliminary experiment was carried out both to examine the power and versatility of this method in examining the effects of various agents, including BrdU, on different cell systems. The seven-day BrdU DLKP sample was chosen for analysis for two reasons;

1. Most of the gene expression changes already detected were in the DLKP, not the A549, cell line.
2. Most of the genes which had increased expression in DLKP following BrdU exposure had already been significantly increased by this time point of the experiment (Section 3.1.1).
3. At seven days BrdU exposure, a specific decrease in cell viability was observed which facilitated the death of the cells at a significantly lowered concentration of drug relative to normal cells (Sections 3.1.2 and 4.2.1.1.2).

Following DNA microarray analysis, the expression of eighty-one genes was altered in the DLKP cells following BrdU exposure. However, many of these increases or decreases were not felt to be of significance (less than two-fold). As a result, a total of only thirty-eight genes were considered significantly affected in the cell line. Genes which were detected increased using this assay are summarised in table 3.5.2, genes displaying decreased expression are listed in table 3.5.3. Only the more significant expression changes will be discussed in detail here.

Sixteen genes (Table 3.5.2) were substantially increased in expression following BrdU treatment in DLKP. The largest increase was in the transcription factor ETR103, whose expression increased eleven-fold after seven days exposure to BrdU. ETR103 expression has previously been detected to be increased in HeLa cells exposed to the gene-inducing agent (see Section 1.5.4.5) benzo[a]pyrene diol epoxide (BPDE) (Yu *et al.*, 2000). Other highly significant increases were observed for p55CDC and Ini1 (both eight-fold) and for the Nuclease-sensitive element DNA-binding protein (NSEP) and EB1 protein (both seven-fold). p55CDC is a regulatory cell cycle protein (Weinstein, 1997) which is transcriptionally upregulated in HeLa and NIH3T3 cells following taxol exposure (Makino et al., 2001). Ini1 has been postulated as a potential tumour suppressor gene (Biegel *et al.*, 1999). The EB1 family of proteins have recently been

implicated in microtubule assembly in mammalian cells (Bu and Su, 2001), while NSEP-1 has been postulated as a transcription factor which may recognise DNA with unusual secondary structure (Kolluri *et al.*, 1992).

There were also a number of gene expression increases which were deemed interesting although small in size. Expression of the glutathione-S-transferase gene (GSTP1) was also increased two-fold in this sample. The MRP1 protein has previously been demonstrated to transport glutathione (GSH) conjugates of drugs (Leier *et al.,* 1994; Jedlitschly *et al.,* 1994), while expression of the gene was significantly increased in BrdU-treated DLKP (Sections 3.1.1 and 4.2.1.1). Whether or not the increased expression of both genes are connected in this system remains to be elucidated. Additionally, expression of the α-catenin gene was increased two-fold after seven days exposure to BrdU. RT-PCR analysis had also detected a slightly smaller increase in expression of this gene following BrdU treatment in DLKP (Section 3.1.1). Less significant increases in expression were observed for the DNA binding proteins ID-1 and GNAS, the heat shock proteins HSPA1 and HSP27, ribosomal protein S19, the fibronectin receptor β subunit (FNRB), mitogen-activated protein kinase p38, the DNA-damage-inducible GADD153 gene and the hepatoma-derived growth factor (HDGF).

A total of twenty genes were substantially decreased in expression in the BrdU-treated DLKP. Half of these downregulated genes, including the two largest decreases, coded for transcription factors. The largest decreases were in cAMP-response element binding protein (CREB2) and the c-*myc* oncogene (both seven-fold) after seven days exposure to BrdU. The c-*myc* oncogene has already been discussed (Section 1.7.4). CREB2 has previously been identified as essential for differentiation of murine lens fibres (Tanaka *et al.*, 1998). Other significant decreases were the 60S ribosomal protein L6 (RPL6) (five-fold) and Transcription initiation factor IID (four-fold). Little is known of human RPL6, as the gene has only recently been mapped and sequenced (Kenmochi, *et al.*, 2000). TFIID is the only general transcription factor (GTF) capable of binding core promoter DNA both independently and specifically. The other GTFs bind DNA weakly on their own and it is the network of cooperative protein-protein interactions with TFIID and each other that allows them to form stable, specific DNA interactions (Carey and Smale, 2000). The functional implications of downregulation of transcription

factors during differentiation are currently unclear. Decreased expression of the CNBP, HIP116, GABP-β2, oct-2, STST6, COUP, Activator 1 and CACCC-box binding transcription factors was also observed. Additionally, human SPL6 ribosomal protein, SOD1 protein, N-ras, DNA-dependant protein kinase, VEGF, GPI, the EGF receptor and acyl-CoA-binding protein (ACBP) were also decreased in DLKP cells following seven days BrdU treatment. A two-fold decrease of the glutathione-S-transferase gene M1 polymorphism (GSTM1) was also observed. This result stands in marked contrast to the increase in expression of the GSTP1 polymorphism mentioned earlier.

Also, DNA microarray analysis indicated that the housekeeping genes Ubiquitin and Cytoplasmic β-actin were increased three- and two-fold respectively following BrdU exposure in DLKP cells. BrdU has also previously been shown to upregulate expression of the actin gene in B16 melanoma cells (Gomez *et al.*, 1995). The expression of the other housekeeping genes was not affected, indicating that the amount of RNA sample examined was not significantly greater than for the control sample. This result would indicate that BrdU upregulated expression of these genes specifically.

This has important implications for the RT-PCR analysis of RNA samples from BrdU-treated cells. Expression of β-actin was used as the internal control for all RT-PCRs outlined in this thesis. If BrdU were to upregulate β-actin expression in DLKP or A549 cells, this would have the affect of decreasing the impact of gene expression changes in the examined cells. However, as can be seen from Figs. 3.1.1 – 3.1.13, expression of the β-actin gene as detected by RT-PCR was not increased significantly in the BrdU-treated cells. Additionally, it may be accepted that the significant results detailed herein remain significant, as a lowered expression profile for β-actin in these cells would have the effect of accentuating rather than decreasing the expression changes. It would be preferable, however, that all future RT-PCRs on BrdU-treated RNA samples utilise another, or additional internal control, such as GAPDH.

Very little overlap was observed between the genes examined by RT-PCR and those examined by DNA microarray. Gene overlap only occurred in five cases; the apoptotic genes BAX, Bcl-x and Bcl-2, α-catenin and c-*myc*. As already mentioned, the RT-PCR expression changes detected for α-catenin closely resemble those obtained by DNA

microarray. Expression levels of the Bcl-x and Bcl-2 genes remained unaffected in the seventh-day day sample, whether these levels were determined by DNA microarray or RT-PCR. However, a number of discrepancies have arisen with comparison of the two techniques. Significantly, c-*myc* gene expression was decreased seven-fold following BrdU treatment. As mentioned previously (Section 4.2.1.2), BrdU-induced differentiation has been associated with decreased levels of the c-*myc* mRNA transcript (Yen and Forbes, 1990; Valyi-Nagy *et al.*, 1993). c-*myc* mRNA levels were also decreased following adriamycin-induced differentiation in MCF-7 cells (Fornari *et al.*, 1994). RT-PCR analysis, however, failed to detect any significant change in c-*myc* mRNA expression levels. In addition, expression of the BAX gene remained unaffected by BrdU exposure in DLKP cells when examined by DNA microarray analysis, but is increased six-fold when analysed by RT-PCR (Section 3.1.1.9).

These comparisons show that out of five overlapping genes assayed for expression between the two techniques, three express the same amount no matter which technique is used, while two, c-*myc* and BAX, are different. Additionally, the unexpected increased levels of β-actin expression observed using DNA microarray analysis was not replicated in the RT-PCR technique. This poses the obvious question; which of the techniques used give is more accurate? Due to the fact that the RT-PCR results were repeated at least twice before confirmation of a significant increase or decrease was made, the results obtained via the RT-PCR technique must be considered the more accurate. However, this result, especially when coupled with the effects of BrdU on the housekeeping gene expression levels detailed above, would tend to cast a dubious light on the expression levels detected by DNA microarray analysis. As a result, this method would warrant additional repeats to confirm the results already obtained.

However, this method has demonstrated the ability of DNA microarrays to examine expression levels of a larger number of genes quickly and, when compared to RT-PCR, relatively effortlessly. Use of this method has already identified a number of genes significantly affected by BrdU in DLKP cells, which may form the basis of future studies into BrdU-related effects in this cell system.

4.2.3 Gene expression in differentiated DLKP: The identification of new transcriptional differentiation-specific markers?

The bulk of the changes in gene expression observed here in response to BrdU exposure were observed in the DLKP cell line. As DLKP is a poorly differentiated cell line of the lung (Law *et al.*, 1992) and BrdU had induced differentiation in this cell line, it is possible that these gene expression changes mimic those which would be observed during normal cellular differentiation in the lung. A number of protein differentiation-specific markers for lung cell lines have already been identified, including fibroblast growth factor (Park *et al.*, 1998) and cytokines of the IL-6 group (McCormick and Freshney, 2000). Work carried out in this laboratory has also identified the K8, K18 and K19 cytokeratins as differentiation-specific markers in lung cell lines in response to BrdU (McBride *et al.*, 1999; Meleady and Clynes, 2001). Previous studies have also identified the ribosomal protein L35A (Siavoshian *et al.*, 1999) and the cytochrome P450 gene CYP26 (Kim *et al.*, 2000) as differentiation-specific markers at the transcriptional level in lung cell lines. However, little is known of gene induction in response to differentiation in lung. It is possible, therefore, that these genes may constitute novel differentiation-specific markers in lung epithelial cell lines.

4.3 Exposure to chemotherapeutic drugs induces gene expression but no change in drug resistance in DLKP cells

Expression changes in various genes studied here were divided up into those cells which were transiently-exposed to drug (Section 3.2) and the drug- selected cell lines (Section 3.3). Cisplatin significantly induced gene expression of MRP1, MRP2 and BCRP in the transiently-exposed DLKP, while selection in cisplatin resulted in increased MRP4 and MRP5 gene expression. Short-term exposure to VP16 significantly increased gene expression of MRP1, MRP2 and α-catenin, while selection in VP16 resulted in increased MRP1 and MRP5 gene expression. Taxol significantly induced gene expression of MRP2, α-catenin and E-cadherin in DLKP. This drug was not used as a selection agent in DLKP.

DLKP cells were exposed to a variety of chemotherapeutic drugs in order to ascertain the gene induction profiles for the treated cells. The benefits of this study are threefold:

1. An examination of genes induced by the drugs in DLKP would yield an accurate gene induction profile for each chemotherapeutic agent. It would then be possible to compare expression levels for each drug in order to build up an accurate picture of the types of genes induced, and hence, a picture of the expected phenotype of the treated cells.
2. If the profile of gene induction for any given drug was observed to be similar to that obtained for BrdU-treated DLKP, it could be inferred from this result that part of the anti-tumour chemotherapeutic effect of that drug might involve inducing a differentiated phenotype in the cancer cells.
3. A comparison between transiently-exposed (Section 3.2) and drug-selected (Section 3.3) cells could also be made, in order to identify differences in expression incurred by the different treatment methods.

The results from the cisplatin- and VP16-selected DLKP will be discussed here together with the short-term, or transient exposures, while the results for the other drug selections will also be discussed separately.

DLKP cells were exposed to three chemotherapeutic drugs routinely used in treating lung cancer, namely: cisplatin, taxol and VP16. The modes of action of these drugs have previously been described (Section 1.5). The DLKP cells were exposed to the drugs over the same time frame as that examined for the BrdU-treated cells. DLKP cells were also selected in ten chemotherapeutic agents and their gene expression profiles elucidated.

4.3.1 Morphology: An indication that differentiation is induced by exposure to chemotherapeutic drugs in DLKP?

The morphological effects of BrdU-induced differentiation have already studied in this laboratory (P. Meleady, PhD. Thesis, 1997; D. Walsh, PhD. Thesis, 1999; F. O'Sullivan, PhD. Thesis, 1999). The principal alterations in morphology of A549 and DLKP cells exposed to BrdU include cell flattening and enlargement, accompanied by a decreased growth rate.

When the poorly differentiated DLKP cell line was exposed to chemotherapeutic drugs (Section 3.2.1), induction of a differentiated phenotype was evidenced by significant changes in morphology. The cells became enlarged and assumed a flattened, elongated shape. These changes in cell size and shape were also accompanied by a decreased growth rate of the cells.

Exposure to chemotherapeutic drugs other than those examined here has previously been shown to induce differentiation in human and animal cell lines (see Section 1.5.3). Taxol has been reported to induce differentiation in human choriocarcinoma cells (Marth *et al.*, 1995), leukaemia cells (Olah *et al.*, 1996) and in colon adenocarcinoma cells (Cohen *et al.*, 1999). Cisplatin induced a differentiated phenotype in oesteosarcoma cells (Tsuchiya *et al.*, 1993). The DNA topoisomerase II poison VP16 also elicited a differentative effect in U937 promonocytic cells (Perez *et al.*, 1994; Perez *et al.*, 1997), as well as in human HL60 cells (Yung, 1994).

It was therefore apparent that in the DLKP cell system, exposure to the chemotherapeutic drugs cisplatin, taxol and VP16 could induce a differentiated

phenotype in those cells, similar to that induced by BrdU. It was therefore considered to be of interest to compare gene expression profiles of cells induced to differentiate by these separate agents.

4.3.2 Induced differentiation in DLKP results in gene induction of specific genes

Previous instances of gene induction following exposure to cisplatin, taxol and VP16 has already been reviewed (see Section 1.5.3.1). Here, the implications of the induced genes in DLKP shall be discussed.

4.3.2.1 Gene induction response to cisplatin in DLKP

Cisplatin was observed to significantly induce gene expression of MRP1, MRP2 and BCRP in the transiently-exposed DLKP. Selection of DLKP cell lines in cisplatin resulted in increased MRP4 and MRP5 gene expression.

The method of action of cisplatin has been outlined in Section 1.5.2.2. MRP1 gene induction in response to exposure to cisplatin has previously been observed in human lung cancer specimens (Oguri *et al.*, 1998) and in human leukaemic HL60 cells (Ishikawa *et al.*, 1996). Expression levels in the HL60 cells could be induced to rise within 30hrs of exposure to the drug. MRP2 gene induction has also previously been observed in human colorectal carcinomas (Hinoshita *et al.*, 2000) in response to cisplatin exposure. There is no evidence in the literature of increased BCRP expression following exposure to cisplatin.

MRP1 is thought to cause multidrug resistance by decreasing the intracellular concentration of cytotoxic drugs, and although most MRP1-expressing cell lines show a defect in accumulation (Gaj *et al.,* 1998; Zaman *et al.,* 1993; Krishnamachary *et al.,* 1993; Zaman *et al.,* 1994), others do not (Gaj *et al.,* 1998). Also, despite the expression of MRP1/2 in cisplatin-resistant cell lines (Oguri *et al.*, 1998), there is no evidence that any of the MRP homologues or BCRP actively transport cisplatin. Decreased intracellular accumulation of cisplatin and carboplatin has been associated with

resistance to these chemotherapeutic drugs (Shen *et al.*, 2000). The mechanism(s) by which cisplatin enters the cell, and by which decreased accumulation occurs in resistant cells have yet to be determined. It has generally been believed that cisplatin enters cells largely through passive diffusion, however, evidence provided by Shen *et al.* (2000), suggest the involvement of a novel active transport process in the uptake of cisplatin and carboplatin in to the human liver carcinoma cell line, BEL-7404. This uptake was significantly reduced in the cisplatin–resistant derivative 7404-CP20. Shen *et al.* (2000), demonstrated decreased MRP1 and MRP2 protein expression in this cisplatin resistant cell line making it highly unlikely that MRP1 or cMOAT are involved in reducing cisplatin or carboplatin influx or efflux in this particular cell line.

Selection in cisplatin appears to have induced a rather different expression profile to that observed for the short-term exposures. MRP4 expression increased two-fold in cisplatin-selected DLKP. MRP4 expression has previously been associated with high-level resistance to a number of anti-human immunodeficiency virus drugs (reviewed in Borst *et al.*, 2000). However, this is the first instance in the literature linking MRP4 overexpression and cisplatin resistance. In contrast, MRP5 gene overexpression has been previously observed in lung cell lines exposed to cisplatin (Oguri *et al.*, 2000). The authors observed that MRP5 expression was not rapidly induced by cisplatin over 24 hrs. This result may explain the lack of MRP5 gene induction in the transiently-exposed samples.

4.3.2.2 Gene induction response to taxol (Paclitaxel) in DLKP

Taxol significantly induced gene expression of MRP2, α-catenin and E-cadherin in the DLKP cell line. Taxol was not used as a selection agent in DLKP.

The mode of action of this drug has been outlined in Section 1.5.2.3. To date, many of the studies examining the effect of taxol on gene induction have focussed on mdr-1. Dumontet *et al.* (1996) found that taxol exposure resulted in activation of expression of the mdr-1 gene in the human MES-SA sarcoma cell line. Induction of expression of mdr-1 mRNA was also observed in a number of human ovarian cell lines by Yamamoto

et al. (2000), following exposure of the cell lines to taxol. Work in this laboratory (Liang *et al.*, 2001) revealed an increase in MRP1, MRP2 and MRP3 protein expression in human RPMI cells selected in taxol. Previous studies had also revealed increased expression of MRP1 protein in taxol-selected epitheloid sarcoma cell lines (Reinecke *et al.*, 2000) and in colorectal carcinoma (Uchiyama-Kokubu *et al.*, 2001). However, the authors concluded that this increased expression was not deemed sufficient to confer the observed resistance levels to the drug. Additional studies involving the characterisation of MRP1-overexpressing cell lines with a number of chemotherapeutic drugs have demonstrated that these cell lines do not show increased resistance to taxol (Zaman *et al.*, 1994; Breuninger *et al.*, 1995; Doyle *et al.*, 1995; Binaschi *et al.*, 1995; Huang *et al.*, 1997). There is no mention in the literature of MRP2 gene induction in response to taxol exposure.

Taxol exposure decreased expression of β-catenin protein in human NSCLC cell lines without affecting α-catenin protein expression (Ling *et al.*, 2001). The drug also decreased cytosolic E-cadherin in nasopharngeal carcinoma cells (Lou *et al.*, 2000). Docetaxel ("taxotere"), a taxol analogue, was also found to enhance the expression of E-cadherin in human colon cancer cell lines (Eckert *et al.*, 1997).

4.3.2.3 Gene induction response to VP16 in DLKP

VP16 significantly induced gene expression of MRP1, MRP2 and α-catenin in the poorly differentiated DLKP cell line. Selection in VP16 resulted in increased MRP1 and MRP5 gene expression.

The method of action of VP16 has been outlined in Section 1.5.2.5. Increased MRP1 expression has previously been observed in VP16-exposed A549 cells (Trussardi *et al.*, 1998). VP16-resistant lung (Doyle *et al.*, 1995) and bladder (Hasegawa *et al.*, 1995) cell lines also overexpress MRP1. Additionally, Cui *et al.* (1999) reported a five-fold resistance to the drug in canine cells overexpressing MRP2 protein, although Kawabe *et al.* (1999) and Chen *et al.* (1999) both concluded that MRP2 gene overexpression did

not confer resistance to VP16. No correlations between α-catenin or MRP5 gene or protein expression and VP16 treatment have been found in the literature.

4.3.3 Induction of gene expression does not affect the drug resistance profile of DLKP

The resistance profile of the drug exposed DLKP cells to adriamycin, cisplatin, taxol and VP16 was examined using *in vitro* toxicity assays. No change in the drug resistance profile of the DLKP was observed following exposure of the cells to the chemotherapeutic agents cisplatin, taxol and VP16 (Section 3.2.2).

This was observed despite the induction of gene expression in genes thought to be associated with mediating multidrug resistance and apoptosis in the DLKP cells. This phenomenon has also been observed in previous studies (Licht *et al.*, 1991). It would appear from this result that the induction of expression of these genes may not translate into active, functioning proteins. The functional implications of the induction of these genes remain to be elucidated. However, as described in Section 4.4, the induction of these genes may be related less to their functionality and more to the type of transcriptional machinery they utilise.

A significant fact to note is that short-term exposure to, and long-term selection in, VP16 results in increased expression of the MRP1 gene in DLKP. However, the latter treatment results in increased resistance to the drug, while the former situation does not. It is apparent, therefore, that additional elements must be functioning in the drug-selected cell line which may combine to confer the resistant phenotype.

4.3.4 Examination of drug-selected cell lines and their correlation with transiently-exposed cells

As has been shown in the previous sections, selection of a cell line in a particular agent tends to confer a dissimilar gene expression response to that of transient exposure. Transient exposure to cisplatin induced expression of MRP1, MRP2 and BCRP, while

selection in the drug significantly increased expression of MRP4 and MRP5 only. Short-term exposure to VP16 significantly increased gene expression of MRP1, MRP2 and α-catenin, while selection in VP16 resulted in increased MRP1 and MRP5 expression. Comparisons between selection in Taxol and transient exposure to the drug could not be made as Taxol was not used as a selection agent in DLKP.

These results indicate that not only do separate drugs elicit differing gene expression profiles in the same cell system, but that these profiles are also subject to the method of exposure to that drug. This result has implications for the treatment of cancer in the clinical setting as it indicates that the profiles of genes expressed in tumours may vary over, as well as a result of the chemotherapeutic regimen employed. However, further work would be necessary to confirm this hypothesis.

4.3.5 Examination of drug-selected cell lines not correlated with transiently-exposed cells

The DLKP cell line was also selected out in a number of chemotherapeutic agents not examined under transient exposure. A total of eight extra drugs were used; these drugs and the gene expression changes which were elicited are summarised in Table 3.3.1.

All of the observed changes were increases in gene expression, as observed for the transiently exposed DLKP. The most significant expression increases were for mdr-1 expression in the taxotere-selected line (35-fold) and BCRP in the mitoxantrone-selected DLKP. Increased Pgp protein expression has previously been associated with resistance to taxotere in pancreatic carcinomas (Liu *et al.*, 2001), while the drug has also been demonstrated to interact with Pgp (Shirakawa *et al.*, 1999). BCRP expression has previously been observed in cell lines following exposure to mitoxantrone (Maliepaard *et al.*, 1999; Ross *et al.*, 1999), and indeed the BCRP gene is sometimes referred to as the Mitoxantrone resistance gene (MXR).

4.4 BrdU- and drug-mediated induction of gene expression

It has been demonstrated in this study that the thymidine analogue BrdU was capable of inducing differentiation and gene expression in the poorly differentiated DLKP cell line and the adenocarcinoma A549 cell line. This induced differentiation was accompanied by significant increases and decreases in mRNA expression of a number of genes (Section 3.1.1). The particular gene expression profile observed for each cell line differed markedly, indicating that gene induction profiles may be dependant on the type of cell system under scrutiny, as well as on the differentiation status of the cell line.

Further work demonstrated that a number of chemotherapeutic drugs were also capable of inducing a similar differentiated phenotype in the DLKP cell line. Short-term exposures (up to two weeks) to cisplatin, taxol and VP16 induced differentiation which was indicated both by morphological changes and the induction of different gene expression profiles (Section 3.2.1). The profiles of genes induced by the drugs differed from those induced by BrdU. In addition, the induced gene expression profiles differed depending on which type of drug was used and also on whether the exposures were short-term or if the cells were selected in drug over a longer period.

Increased expression of these genes in the various systems described did not confer the expected phenotype in the cell systems studied. For instance, significant gene expression increases in DLKP cells of MRPs 1, 2, 3 and BCRP, genes thought to be associated with conferring multidrug resistance, following exposure to BrdU did not increase the drug resistance of those cells (Section 3.1.2). Similar results were observed for the drug-treated DLKP (Section 3.2.2). In marked contrast, DLKP cells which were selected for resistance to cisplatin and VP16 displayed different gene expression profiles to those which were exposed to the drugs in the short-term (Section 3.3). Additionally, the existence of certain similarities in gene induction between drug-exposed and drug-selected cells (i.e.) were not sufficient to significantly alter the phenotype of the drug-exposed cells. For example, the VP16 drug-exposed DLKP cells expressed higher levels of MRP1 but did not show an increase in VP16 resistance. In contrast, the DLKP cells selected for resistance to the drug also revealed elevated expression levels of MRP1 (Section 4.3.3).

It was clear, therefore, that the type of genes expressed in drug- and BrdU-treated cells depended on a number of criteria which were not apparently linked to a functional occupation in those cells.

4.4.1 BrdU-upregulated genes in DLKP share common potential transcription factors

Transfac™ sequence analysis of the 5' promoter sequences of nine of the ten genes upregulated by BrdU identified one hundred and forty-seven potential transcription factor recognition sites when all the possible recognition motifs were collated. The 5' promoter sequence of the BAXα gene, although upregulated in BrdU-exposed DLKP cells, was not examined in this study as BLAST™ searches failed to identify the correct sequence data. Analysis of these factors by hand, however, detected that only seven of these factors were shared between all nine genes. It was therefore possible that BrdU was upregulating expression of the genes via activation of one (or all) of the factors.

In order to attempt to explain the different gene induction profiles observed for the different inducing agents, a transcriptional model of BrdU- and drug-induced differentiation was proposed. In this model, the up- or down-regulation in mRNA expression of each of the different genes could be explained by the utilisation of different transcription factors by the different agents studied. It would be presumed from this model that the genes induced to increase by a particular agent, for example BrdU, would share a common transcription factor which would be utilised to mediate upregulation of the mRNA.

The gene induction effect of BrdU was chosen as the first set of results to analyse, as the greatest number of gene expression changes were observed in this cell line in response to differentiation induced by this agent. To simplify the analysis of the BrdU effect in DLKP, it was decided to examine only those genes which were upregulated in the cell line. In this manner, the action of BrdU could be broadly said to have a similar effect: the increase in expression of the examined genes. In this way, it could be hypothesised that any putative transcription factors discovered could be expected to affect expression of their prescribed genes in a positive regulatory fashion.

A total of one hundred and forty-seven potential transcription factors were identified which could bind to the promoter sequences of nine of the ten genes whose expression was upregulated in DLKP by BrdU (MRP1, MRP2, MRP3, BCRP, MRIT, COX-2, eIF-2α, α-catenin and E-cadherin). The reduction in transcription factor number from one hundred and forty seven potential factors displayed *by* all nine genes to just seven factors potentially expressed *in* all nine also greatly simplified matters.

4.4.1.1 Classification of the common transcription factors

Despite the fact that the 147 factors were drawn roughly equally from all four Superclasses, the seven common potential factors were drawn largely from one Superclass; the Zinc-coordinating DNA-binding domains. Transcription factors are divided into four distinct domain Superclasses; based on different motifs used to bind DNA; Basic Domains, Zinc-coordinating DNA-binding domains, helix-turn-helix and β-scaffold factors with minor groove contacts. These Superclasses are also sub-divided into Classes, Families, and Subfamilies, in descending order.

The Zinc-coordinating DNA binding domain Superclass includes the zinc finger motif Class of factors. These were originally recognised in Factor TFIIA, which is required for RNA Pol III to transcribe 5S rRNA genes (Lewin, 1997). Zinc fingers take their name from their structure in which a small group of conserved amino acids binds a zinc ion, and forms a relatively independent domain in the protein. They are a common motif in DNA-binding proteins, and are usually organised as a single series of tandem repeats; for instance the Sp1 general transcription factor has a DNA-binding domain that consists of three zinc-fingers (Desjarlais and Berg, 1992). Two Classes of factors are of relevance within this Superclass; these are the diverse Cys4 zinc fingers and the Cys2His2 zinc finger domains. The diverse Cys4 zinc fingers contain the GATA Family of transcription factors, of which three (out of the seven) are represented as being potential common factors between the ten BrdU-upregulated genes. Ik-2 (or Ikaros) and MZF1 are from the second Class, the Cys2His2 zinc finger domains. The remaining two common transcription factors are CdxA, from the helix-turn-helix Superclass of transcription factors, and AML-1a, which is one of the β-scaffold factors. This

classification of the seven transcription factors obtained from BrdU-induced expression was carried out with the aid of the *Transfac*™ Transcription factor classification system (http://transfac.gbf.de/TRANSFAC/cl/cl.htm).

From this perspective, it is apparent that the Zinc-finger superfamily of transcription factors may play a disproportionate role in the BrdU-mediated increase in gene expression in human lung epithelial cells. An examination of the other transcription factors contained within this Superclass tends to fortify this theory.
Of the seven common potential factors, five (>70%) of these factors are contained within two Classes of this Superclass of transcription factors. Other members of this Superclass include the Retinoic acid receptor factors, which have already been postulated as being important in RA-mediated differentiation (Brand *et al.*, 1990) and the Vitamin D receptor, which may be of importance in Vitamin D_3-mediated differentiation (Lazarova *et al.*, 2001). Other potentially relevant transcription factors in this Superclass include the Sp1 transcription factor, which has been postulated as a potential transcriptional regulator of MRP1 (Zhu and Center, 1994) and YY1, which has been suggested in this laboratory to be a potential transcriptional regulator involved in BrdU-mediated differentiation of DLKP cells (D. Walsh, PhD. Thesis 1999). Both Sp1 and YY1 are members of the same Class as Ik-2 and MZF1, but they belong to the Ubiquitous Factor Family, whereas Ik-2 and MZF1 are from the Cell Cycle Regulator Family.

Additional transcription factors have previously been described as associated with differentiation in human cells. These factors include the AP-1 (Activating Protein-1) transcription factor, the *c-fos* and *c-jun* proto-oncogenes of AP-1, c-myc and the Yin-Yang 1 (YY1) transcription factor. Pankov *et al.* (1994) discovered that RA-induced differentiation of EC (Embryonal Carcinoma) and ES (Embryonal Stem) cells resulted in increased AP-1 expression, implying a possible role for AP-1 in the positive regulation of differentiation. Expression of *c-fos* and c-*jun* may also be induced throughout the cell cycle in both quiescent and differentiating cells (Curran, 1988). As described previously, downregulation of the c-*myc* transcription factor expression has been demonstrated during differentiation of human leukaemic and melanoma cell lines (Yen and Forbes, 1990; Valyi-Nagy *et al.,* 1993). Also, treatment of myoblasts with

BrdU results in inhibition of myogenesis, associated with an increase in expression of YY1 (Lee *et al.,* 1992).

4.4.1.2 Identification of transcriptional elements already associated with genes induced by BrdU and chemotherapeutic drugs

4.4.1.2.1 Transcriptional elements associated with BrdU-affected genes

Several previous studies have identified transcription factors which may be involved in transcriptionally regulating the expression of genes upregulated here in DLKP by BrdU.

Zhu and Center (1994, 1996) identified the Sp1 transcription factor as the major transcription factor for MRP1 in a number of human cell lines. The human Y-box binding protein YB-1 has previously been linked with the MDR phenotype in cells (Ohga *et al.*, 1996) and was recently associated with expression of MRP1 in human colon carcinoma cells (Stein *et al.*, 2001). Additional studies have identified the AP-1 binding site in the MRP1 promoter as important for gene expression (Kurz *et al.*, 2001). It has also been proposed that transcriptional expression of the MRP1 gene may be suppressed by wild-type p53 tumour suppressor gene expression (Wang and Beck, 1998). Subsequent studies have shown MRP1 protein expression to be associated with aberrant p53 expression in colorectal carcinoma (Fukushima *et al.*, 1999) and prostate cancer (Sullivan *et al.*, 2000).

Expression of the MRP2 gene has been associated with the expression of retinoic acid receptors (Denson *et al.*, 2000) and with the transcription factor C/EBPβ (Tanaka *et al.*, 1999) in human hepatic cells. The retinoic acid receptor family has already been mentioned, C/EBPβ belongs to the Basic Domain Superclass of transcription factors.

The alpha-1 fetoprotein transcription factor (FTF) is the only transcription factor currently associated with regulation of MRP3 (Inokuchi *et al.*, 2001). This study

correlated expression of the factor in response to stimulation by bile salts in the enterohepatic circulation system.

Expression of the pro-apoptotic BAX and MRIT genes have also been associated with transcriptional control. MRIT has previously associated with expression of the NF-kappaB transcription factor (Chaudhary *et al.*, 1999; Chaudhary *et al.*, 2000). Expression of BAX has been demonstrated to be linked with expression of the E2F-1 transcription factor in gastric carcinoma (Atienza *et al.*, 2000) and cortical neurons (Giovanni *et al.*, 2000). It has also been postulated that transcriptional expression of this gene may, like MRP1, be under p53 control (Fortin *et al.*, 2001).

Expression of a number of transcription factors has also been associated with transcriptional regulation of the eukaryotic initiation factor gene eIF-2α. These include NF-kappaB (Srivastava *et al.*, 1998) and the Nuclear Respiratory binding Factor 1 (NRF-1) together with p3A2 and ewg (Efiok *et al.*, 1994), poly-A binding protein (PABP) (Hensold *et al.*, 1996), CCAAT/enhancer-binding protein β (Raught *et al.*, 1996) and α-pal (Jacob *et al.*, 1989; Bassey *et al.*, 1994; Efiok and Safer, 2000).

Expression of COX-2 has been associated with the transcription factors NF-kappaB (Inoue and Tanabe *et al.*, 1998), C/EBPβ (Yuan *et al.*, 2000) and AP-1 (Guo *et al.*, 2001).

The cell-cell adhesion protein E-cadherin has also been associated with expression of transcription factors. E-cadherin expression has recently been correlated with expression of transcription factors AP-2 (Baldi *et al.*, 2001), the vitamin D receptors (Palmer *et al.*, 2001) and the lymphoid enhancer-binding factor-1 (LEF-1) (Kobeliak *et al.*, 2001). α-catenin expression has also been correlated with expression of the TNF-alpha gene (Lapteva *et al.*, 2001).

4.4.2 Expression of GATA-2 and GATA-3 is upregulated in BrdU-treated DLKP but not in A549 cells

RT-PCR analysis using primers designed for the seven transcription factors revealed increased expression of the GATA-2 and GATA-3 genes in BrdU-treated DLKP cells. Neither GATA-2 nor GATA-3 gene expression was significantly altered in the BrdU-treated A549 cells. Furthermore, expression of the other transcription factors could not be assayed in these samples as the PCR optimisations had failed for these factors. It is therefore possible that a number of the other factors may also be utilised by BrdU in mediating the gene expression increases observed. Further work, incorporating the design of more successful primers for these (and other) factors is necessary.

The increase in GATA-2 and GATA-3 gene expression following BrdU treatment raises the distinct possibility that one (or both) of these factors is involved in mediating BrdU-induced upregulation of gene expression in the DLKP cell line.

4.4.2.1 GATA-2, but not GATA-3, is upregulated in VP16-treated DLKP cells

Expression of GATA-2 was observed to be significantly increased in the drug-exposed DLKP cells. However, expression of the gene was increased only in response to VP16 exposure in DLKP, whereas exposure to cisplatin and taxol had no effect on expression of the gene. Also, expression of GATA-3 was unaltered in any significant fashion following exposure to cisplatin, taxol and VP16 in DLKP.

It appears from this result that cisplatin and taxol may not utilise expression of the GATA-2 or GATA-3 transcription factors in mediating gene expression. Some other, alternative factor(s) may be important in mediating gene upregulation from these agents. It is clear from the lists of potential factors compiled for these drugs that there are an additional number of potential factors which may be utilised in mediating transcription of the selected genes. The cisplatin induced genes share twenty-three common potential factors, the taxol genes eighteen and the VP16-induced genes have a total of twenty-seven factors potentially in common (Section 3.4.5). Further work on these factors

would be necessary to determine a potential transcription factor involved in mediating gene upregulation in response to these agents.

4.4.2.2 VP16: A similar mode of action to BrdU?

The observation that in DLKP, expression of the GATA-2 gene is increased following exposure to both VP16 and BrdU indicates that gene upregulation mediated by the drug may mimic that mediated by the differentiating agent, BrdU.

This result also demonstrates a distinct difference between BrdU and VP16 in mediating gene expression; BrdU may utilise both GATA factors whereas VP16 appears to only utilise GATA-2. The reasons for this are currently unclear although it would appear that BrdU-mediated gene expression may recruit a wider number of factors than that utilised by VP16.

Little is known of the role the GATA-2 and GATA-3 factors may play in lung cell differentiation. However, it is hoped that this finding may lead to further research elucidating the pathways these factors may take in mediating gene expression during lung cell differentiation.

4.4.2.3 *In vitro* examination of the effects of BrdU and chemotherapy on the promoter regions of the MRP1 gene

Previous studies (Wang and Beck, 1998; Fukushima *et al.*, 1999; Turzanski *et al.*, 1999) identified a correlation with expression of MRP1 and aberrant p53 expression. The indication that this gene was transcriptionally controlled presented an opportunity to examine the role of the gene *in vitro.* As has been shown here, MRP1 gene expression has also been induced in the DLKP and A549 cell lines following differentiation with BrdU and in DLKP cells exposed to cisplatin and VP16. It was considered interesting to examine *in vitro* the effects of these agents on the relative activity of various regions of the MRP1 gene promoter. It was hoped that exposure of truncated regions of the MRP1

promoter to these agents would result in the identification of the location of action of these agents on the 5' UTR of the gene.

4.4.2.3.1 Sp1 may be an important promoter of MRP1 in untreated DLKP cells

Previous work by Zhu and Center (1994) in human leukaemic HL60 cells identified the major promoter region of the MRP1 gene as residing in bases –91 to +103 relative to the transcriptional start site of the gene. This area of the promoter is highly GC-rich and does not contain a TATA box for directing site-specific transcriptional initiation. This is similar to that found for the promoter region of the mdr-1 gene (Ueda *et al.*, 1987). In the absence of a TATA box, alternate recognition sequences must be important in the recognition process. The authors observed that this area of the promoter contains recognition sequences exclusively for the Sp1 transcription factor (Zhu and Center, 1994). It would be expected from this that these multiple Sp1 sites would participate in Sp-1 factor binding and the modulation of transcription activity. Additional evidence for this model of MRP1 transcription comes from further study by Zhu and Center (1996), in which site-directed mutagenesis of the Sp1 sequences resulted in decreased reporter gene activity in a number of plasmid-transfected cell lines.

In the present study, an identical fragment of the MRP1 promoter (bases –91 to +103) was observed to contain the area of most concentrated activity in the DLKP cell line. This result concurs with the findings of Zhu and Center (1994, 1996) that transcriptional MRP1 gene activity is controlled mainly from this area of the gene promoter and most likely involves the binding of the Sp1 transcription factor.

4.4.2.3.2 Exposure to BrdU alters the transcriptional activity of MRP1 promoter fragments

BrdU exposure of DLKP cells transfected with reporter plasmids attached to the MRP1 promoter had the effect of shifting the transcriptional activity of the gene upstream, to the –411 to –91 base fragment. Luciferase activity based on this area of the promoter was increased four-fold on addition of BrdU to that of untreated cells. *Transfac*™

analysis of this sequence revealed a potential binding site for GATA-2, as well as for the other common transcription factors, AML-1a and CdxA.

Transcriptional activity on either side of this fragment of the promoter was decreased, the –91 to +103 base fragment yielding less than a third of normal activity. This may indicate the presence of a BrdU-repressive element in this section of the promoter. Activity remains low further upstream, in the –660 to –411 fragment, which also may indicate the presence of a BrdU-repressive element. It must be noted that basal luciferase activity for the fragment containing the full 2kb of MRP1 promoter sequence was not affected by BrdU exposure in DLKP. This result suggests that the major promoter for BrdU-mediated upregulation of the MRP1 gene may lie outside of, (possibly upstream of) this 2kb region of the promoter.

4.4.2.3.3 Exposure to VP16, but not cisplatin or taxol, alters the transcriptional activity of the MRP1 promoter fragments

Exposure to cisplatin and taxol was not observed to alter MRP1 promoter activity in DLKP. However, in both cases, luciferase activity was significantly lowered in the –91 to +103 base fragment, the area of the promoter which would normally be the most active at a basal level in DLKP cells. This result may indicate the presence of cisplatin- and taxol-repressor elements in this area of the MRP1 promoter. The result obtained for the cisplatin-treated cells was surprising, as cisplatin increased MRP1 gene expression in DLKP. However, it is possible that, as for BrdU, a cisplatin-responsive element may be located further upstream than the 2kb of promoter sequence analysed here. In general, the levels of promoter activity in the presence of both drugs were slightly less than for normal or BrdU-exposed cells.

Exposure to VP16, on the other hand, induced a similar response in MRP1 promoter activity as that obtained for BrdU. Luciferase production shifted upstream, to the –411 to –91 base fragment, increasing four-fold. Transcriptional activity was again decreased on either side of this fragment, the –91 to +103 base fragment yielding less than half that of normal activity. However, in the –660 to –411 fragment, luciferase is double that of normal untreated cells, which indicates a partial response to the agent. Again, it must

be noted that basal luciferase activity for the fragment containing the full 2kb of MRP1 promoter sequence was not affected by VP16 exposure in DLKP. This result indicates that the major promoter for VP16-mediated upregulation of the MRP1 gene lies outside this 2kb region of the promoter.

4.4.2.4 Role of the zinc finger binding proteins GATA-2 and GATA-3 in cellular transcription

The GATA family of transcription factors bind DNA at a GATA-consensus motif ((T/A)GATA(A/G)) through a highly conserved C-terminal C_4 zinc finger binding domain (Yamamoto *et al.*, 1990). The family comprises six vertebrate members, two of which, GATA-1 and GATA-2, are expressed in various hematopoietic lineages (Tsai *et al.*, 1989; Leonard *et al.*, 1993). GATA-3 expression is abundant in the developing central nervous system, adrenal gland and kidney (Oosterwegel *et al.*, 1992; Nardelli *et al.*, 1999; Van Esch and Devriendt, 2001). Vital and non-redundant roles for the GATA family of transcription factors have already been demonstrated for normal embryonic development (Tsai *et al.*, 1994; Pandolfi *et al.*, 1995).

GATA-2 was one of the original proteins to define the GATA factors as a related family (Yamamoto *et al.*, 1990). It was originally cloned from a chicken embryo erythroid cDNA library but later shown to be expressed in a broad spectrum of different tissues and cell types (Yamamoto *et al.*, 1990). Early attention focussed on the role of GATA-2 in haematopoiesis. Numerous studies demonstrated that expression of GATA-2 is not only closely associated with the proliferation of hematopoietic progenitor cells, but also with lineage specification (Leonard *et al.*, 1993). Murine embryos bearing an inactivating targetted deletion of *gata2* in the germline die at around 10 days post coitus (dpc) from pan-haematopoietic failure (Tsai *et al.*, 1994). Little is known about the requirements for GATA-2 in developmental processes of cells outside the haematopoietic system because of the early embryonic lethality exhibited by the *gata2* germline mutants. When this developmental block was overcome by complementing the germline mutation with a *gata2* YAC transgene, a second critical role for GATA-2 in urogenital development was revealed (Zhou *et al.*, 1998). More recently, Zhou *et al.* (2000) determined that GATA-2 is expressed in the ventral spinal cord in newly

generated V2 interneurons. This finding is in accordance with their earlier suggestion that expression of GATA-2 may be involved in the determination of specific central nervous system (CNS) cell lineages (Zhou *et al.*, 1998). It would thus appear likely that GATA-2 acts very early in stem cell growth and/or differentiation (Jordan and Zant, 1998). Expression of GATA-2 and Sp1 have been shown to be necessary for basal transcription in bovine endothelial cells (Zhang *et al.*, 1995).

GATA-3 was originally identified as a protein that binds to the T cell receptor (TCR) gene enhancer (Ho *et al.*, 1991). Within the hematopoietic system GATA-3 expression is confined to T lymphocytes and NK cells (Ho *et al.*, 1991). GATA-3 is expressed in early steps of mouse hematopoietic development, in the intraembryonic regions known to give rise to hematopoietic precursors (Manaia *et al.*, 2000). Antisense GATA-3 oligonucleotides inhibited T cell development from fetal liver precursors in fetal thymic organ cultures (FTOC), indicating the critical importance of GATA-3 for early T cell development (Hattori *et al.*, 1996). Taken together, these findings demonstrate an essential role for GATA-3 in T cell commitment (Staal *et al.*, 2001). GATA-3-deficient mice, produced by gene targetting, die at 10-11.5 dpc and exhibit severe defects in hematopoiesis, abdominal haemorrhaging, retardation of the lower jaw, abnormal morphology of the central nervous system (CNS) (Pandolfi et al., 1995) and a block of T-lymphocyte differentiation (Ting et al., 1996; Hendriks et al., 1999). A role of GATA-3 in the expression of a Th2 cytokine gene was first established by Siegel *et al.* (1995). Alternatively, GATA-3 might be involved in the induction of cellular proliferation after the successful completion of TCR rearrangments. This hypothesis would be supported by findings that GATA-3 is markedly upregulated in differentiating Th2 cells (Zhang *et al.*, 1997). Altogether, these findings suggest that GATA-3 is involved in multiple, even unrelated, functions during development.

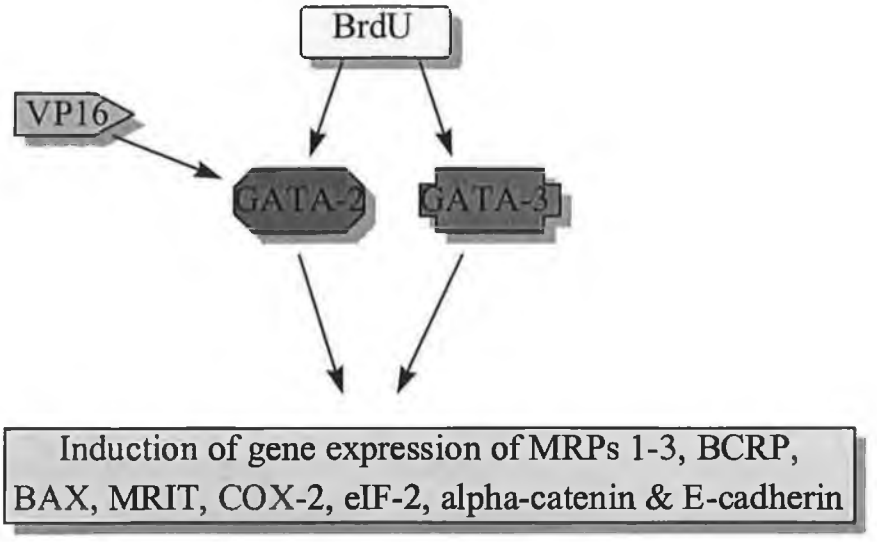
4.4.2.5 A role for the GATA-2 and GATA-3 transcription factors in mediating gene induction in response to BrdU and chemotherapy

The results outlined here imply that gene induction is an entirely subjective process to the agents of induction. Differing expression profiles have been observed in the DLKP cells line following not only exposure to BrdU and the three chemotherapeutic agents

cisplatin, taxol and VP16, but also depending on the length of exposure to that agent. Expression levels of the different genes increased by BrdU vary according to the length of time the differentiating agent has been applied to the cells. This phenomenon is also observed for the chemotherapeutic drugs. As well, selection in a chemotherapeutic drug frequently elicits a different expression profile than does transient exposure to that drug (Section 4.3.4).

This evidence, together with the finding that induction of expression of specific genes may not affect the phenotype of the cell pose an interesting question. What are the reasons for induction of such specific genes in response to these elements?

A transcriptional model of gene induction in response to BrdU and chemotherapy in the DLKP cell line is presented here. The first step of the induction pathway involves the induction of GATA-2 and GATA-3 transcription factor gene expression by BrdU. The method by which BrdU achieves increased expression of these factors is currently unclear, although its interaction with the actin cytoskeleton of the cells may be important (Section 4.2.2.1.6).



Following activation of GATA-2 and GATA-3 expression, the transcription factors interact with their constituent cofactors and the DNA of the DLKP cells to upregulate expression of the various genes expressed following BrdU treatment. Unfortunately, not enough is known of the co-factors recruited by these transcription factors in mediating transcription, nor of the role played by GATA-2 and GATA-3 in lung development and

differentiation. GATA-2 may also be a target for the gene induction effect of the DNA topo II poison VP16, although it would appear that GATA-3 is not recruited by the drug. This result may suggest that the VP16-induced genes; namely MRP1, MRP2 and α-catenin, may be upregulated via the action of GATA-2 solely. This may help explain the differences observed in gene induction profiles between BrdU and VP16, despite a potentially similar method of gene activation (Section 4.3.2.4).

4.5 Analysis of the clinical study results

The clinical study was set up to investigate if a reliable method of analysis could be obtained using basic laboratory collection and analytical procedures. It was found that accurate and reliable results could be obtained from human tissue samples. The study also demonstrated the versatility of the results obtainable from such a study; RNA isolated from these tumours may be used to examine the expression of any gene for which PCR primers are available.

As outlined previously, only the Lung Primary tumour tissue samples constitute a study group of sufficient size from which conclusions may be drawn with any accuracy. The dearth of primary Breast and Oesophageal samples available for analysis render their analysis statistically unviable. The gene expression results for the lung primary samples will therefore be the only results discussed here.

4.5.1 Expression of MDR-related genes in Lung Primary carcinomas

One of the major problems in the cure of advanced NSCLC is the insensitivity to cytotoxic drug treatment of both primary tumours and the metastases. *In-vitro* studies have revealed different mechanisms of cytotoxic drug resistance in lung cancer cells (Clynes, 1993), however there is conflicting evidence as to whether these mechanisms play a significant enough role in the multiple-drug resistance profile of some tumours.

4.5.1.1 Expression of MDR-related genes in cancer

The clinical relevance of MRP1 overexpression is uncertain but studies have indicated that MRP1 is commonly found in cells derived from solid tumours (Kruh *et al.*, 1995; Izquierdo *et al.*, 1996). High levels of MRP1 protein have been found in some cell lines derived from tumours that characteristically respond poorly to chemotherapy, e.g. lung cancer and melanoma, and patients with tumours expressing high levels of MRP1 have significantly decreased survival times following chemotherapy (Ota *et al.*, 1995). Two separate studies by Nooter *et al.* (1995; 1996), identified high expression levels of the

MRP1 protein in chronic lymphocytic leukaemia (CLL) and prolymphocytic leukaemia (PLL), medium expression in oesophagus squamous cell carcinoma (ESCC), acute myeloid leukaemia (AML) and non-small cell lung cancer (NSCLC) and low expression in Wilm's tumour, melanoma, acute lymphocytic leukaemia (ALL), chronic myelotic leukaemia (CML), hairy cell leukaemia (HCL), multiple myeloma (MM), soft tissue sarcoma (STS) and non-Hodgkin's lymphoma (NHL). Low levels of expression were also observed in cancers of the breast, bladder, colon, ovary, testis, head and neck, prostate and kidney. Expression of MRP1 protein in head and neck squamous cell carcinomas has also been observed (Tsuzuki *et al.*, 1998). MRP1 mRNA expression has been observed in high-grade transitional bladder cell carcinomas (Clifford *et al.*, 1996).

Filipits *et al.* (1996) found expression of MRP1 mRNA in all of 134 primary breast carcinoma specimens by RT-PCR. mdr-1 mRNA was also observed in 60% of the samples. Nooter *et al.* (1997a) showed MRP1 is frequently overexpressed in primary breast cancer and could be of prognostic significance in patients with small tumours. Further studies on recurrent breast cancer (Nooter *et al.*, 1997b) which focussed on patients who were treated with chemotherapy as first-line systemic therapy for recurrence, and primary breast cancer patients (Ito *et al.*, 1998), concluded that MRP1 is an important predictor of poor prognosis. Dexter *et al.* (1998) found very low levels of MRP1 and mdr-1 expression in primary breast carcinomas, and while mdr-1 appeared to correlate well with age and histological parameters, MRP1 expression was independent of all other clinical parameters.

Decreased MRP2 expression has been correlated with cholestatic liver disease (Oswald *et al.*, 2001; Zollner *et al.*, 2001), while increased expression has been suggested to be of significance in renal cell carcinomas (Schuab *et al.*, 1999) and colorectal carcinomas (Hinoshita *et al.*, 2000). Mutations in this gene has also been associated with Dubin-Johnson syndrome (Toh *et al.*, 1999; Tsujii *et al.*, 1999; Mor-Cohen *et al.*, 2001).

4.5.1.2 MRP4 and MRP5 are overexpressed in Primary Lung tumour tissue

RT-PCR analysis examined the expression of eight genes which may be associated with multidrug-resistance in cancer cells (MRPs 1 – 6, mdr-1, BCRP) and an additional gene,

known not to be involved in MDR, mdr-3. The study found that expression of MRP4 and MRP5 may be linked with tumour progression in lung cancer. The expression of both genes was observed in the majority of unpaired tumour samples, as well as in a significantly higher number of tumour samples relative to their normal controls. Expression of the other genes could not be correlated with tumour progression in this sample study group. However, low levels of expression of the MRP1 and MRP2 genes was observed over most of the tissues analysed. The gene expression levels of MRP3, MRP6, BCRP, mdr-1 and mdr-3 were not felt to be significant.

Currently, no evidence exists for the association of either MRP4 or MRP5 in mediating tumour progression in any cell system. MRP4 has been reported to be expressed in a wide range of tissues (Lee *et al.,* 1998), and may display a basic structure sufficient for GS-X pump activity (Bakos *et al.,* 1998). Kool *et al.* (1997), screened a large number of human cell lines derived from various tissues and their resistant sublines selected with a number of chemotherapeutic agents. They reported that MRP4 was expressed only at low or very low levels in the cells lines they analysed and no overexpression of MRP4 was detected in resistant sublines. Schuetz *et al.* (1999), discovered that the overexpression and amplification of the MRP4 gene could be correlated with the efflux of nucleoside-based antiviral drugs from mammalian cells. Still less is known about MRP5 (Borst *et al.,* 1995). Kool *et al.* (1997), reported that MRP5 was expressed in a number of cell lines analysed, but was not highly overexpressed in any resistant sublines. Wijnholds *et al.* (1999), reported a possible connection between MRP5 and resistance to thio-purines although this remains to be substantiated by drug accumulation and vesicular transport studies (Borst *et al.,* 1999). However, recently Carter *et al.* (2001) reported high levels of expression of MRP4 and MRP5 mRNA in patients with chronic myelogenous leukaemia (CML), so it is possible that these genes may play a prognostic role in expression of human cancers.

4.5.2 Expression of COX genes in Primary Lung carcinomas

The expression of both COX-1 and COX-2 was examined in the Primary Lung Tumour sample set. Expression of the COX-1 gene was not felt to be of significance in any of the samples, although this may also be due to the poor quality of the PCRs obtained for

this gene. COX-2, on the other hand, was significantly upregulated in tumour tissue relative to the normal counterparts. COX-2 was overexpressed in 75% of the tumour samples that expressed the gene relative to the normal controls.

COX-2 overexpression has previously been associated with carcinogenesis in NSCLC (Ochiai *et al.*, 1999) and in primary lung adenocarcinomas (Achiwa *et al.*, 1999). Expression has also been correlated with poor patient prognosis in NSCLC (Khuri *et al.*, 2001), while the COX-2 inhibitor, nimesulide, has been demonstrated to enhance cytotoxicity of various chemotherapeutic drugs in lung cancer cell lines (Hida *et al.*, 2000). Expression of the gene has also been observed in gastric gliomas (Shono *et al.*, 2001), bladder carcinoma (Komhoff et al., 2000; Shirama *et al.*, 2001), colon carcinoma (Chen *et al.*, 2001), colorectal carcinoma (Hull *et al.*, 2000; Masunaga *et al.*, 2000), pancreatic carcinoma (Merati *et al.*, 2001), cervical cancer (Ryu *et al.*, 2000), prostate (Yosimura *et al.*, 2000; Uotila *et al.*, 2001) and hepatic cancer (Kondo *et al.*, 1999; Rahman *et al.*, 2001). Successful inhibition of COX-2 usually results in potentiation of an attendant chemotherapeutic effect (Reddy *et al.*, 2000; Chen *et al.*, 2001; Kokawa *et al.*, 2001).

4.5.3 Expression of apoptotic genes in Primary Lung carcinomas

RT-PCR analysis examined the expression of eight genes which are involved in apoptosis. Four genes, BAP, BAX, MRIT and Bcl-x_S are pro-apoptotic genes, while four anti-apoptotic genes, Bcl-x_L, Bcl-2, BAG and Survivin were also included. The study found that the altered expression levels of the MRIT, Bcl-x_L and Bcl-2 genes were significant in the lung tissue samples. The gene expression levels of BAP, BAX, Bcl-x_S, BAG and Survivin were not felt to be of significance.

MRIT is pro-apoptotic, and is observed to interact with Bcl-x_L in mediating apoptosis (Han *et al.*, 1997). As such, it would be expected that expression of the gene would be higher in normal tissue relative to tumour tissue. The gene was observed overexpressed in the majority (80%) of normal samples relative to the tumour equivalent in the Lung Primary sample group. This study is the first to suggest a possible link between expression of this gene and cancer development in a clinical setting.

Expression of the anti-apoptotic Bcl-x_L (Boise *et al.*, 1993) and Bcl-2 (Hunter and Parslow, 1996) genes was also observed to be significantly correlated with tumour survival in the Lung Primary sample study. Bcl-x_L was overexpressed in >60% of tumours in tumour/normal sample pairs that expressed the gene, as well as in over 80% of the unpaired tumour samples. No clinical correlations have yet been made for expression of Bcl-x_L in lung cancer, although the gene has been suggested to be a prognostic marker for carcinomas of the pancreas (Friess *et al.*, 1998), ovary (Liu *et al.*, 1998), head and neck (Pena *et al.*, 1999) and breast (Moore *et al.*, 2000), as well as in B-cell lymphoma (Bairey *et al.*, 1999), neuroepithelial tumours (Prayson *et al.*, 2000), neoplastic edometrium (Marone *et al.*, 2000), ameloblastoma (Sandra *et al.*, 2001) and colorectal cancer (Biroccio *et al.*, 2001). Recent studies have also shown that Bcl-x_L may mediate tumour resistance to chemotherapy (*Nita et al.*, 2000) and radiotherapy (Aebersold *et al.*, 2001).

Bcl-2 was overexpressed in exactly 60% of tumours from the paired samples, as well as in all ten unpaired tumour samples. Bcl-2 has previously been postulated as a prognostic marker in NSCLC (Ohsaki *et al.*, 1996; Higashiyama *et al.*, 1997) and SCLC (Kaiser *et al.*, 1996). Expression of the gene has also been correlated with progression of several other cancers, including eosphageal cell carcinoma (Takayama *et al.*, 2001), pancreatic cancer (Campnai *et al.*, 2001), colorectal carcinoma (Elkablawy *et al.*, 2001), bladder carcinoma (Wolf *et al.*, 2001) and prostate cancer (Van Brussel *et al.*, 2001).

4.5.4 Expression of translation initiation genes in Primary Lung carcinomas

High levels of gene expression of eukaryotic initiation factor 2 (eIF-2), as characterised by expression of the eIF-2α subunit was observed in the majority of paired tumour samples (70%) and all unpaired tumour sample. From this result, it would appear that there may be a correlation between tumour proliferation and expression of a gene which is known to be essential for protein translation (Colhurst *et al.,* 1987; Altman and Trachsel, 1993). No correlations were observed between expression of eIF4E in the tumour samples.

Relatively few clinical studies have focussed on the role of eIF-2 in human tumour tissue specimens. Lobo *et al.* (2000) reported that eIF-2α protein expression was implicated in gastrointestinal cancer. However, there is no evidence in the literature of eIF2α gene expression. This finding may indicate a possible role for translation regulation in lung cancer progression.

4.5.6 Relevance of clinical data to gene expression results

Unfortunately, the lack of reliable clinical data available on most of the tumour samples supplied rendered accurate prognosis based on gene expression profiles redundant. However, a number of interesting correlations were drawn from the limited information available. The most interesting of these was that MRP1 gene expression was predominantly observed in tumours from male patients. This is the first case in the literature indicating that expression of this gene in the clinical setting may be sex-linked. Another interesting observation was that the median age of male patients was eight years older than female patients.

Gene expression results obtained from the clinical study demonstrated the high level of expression of the MRP1 and MRP2 genes in most of the tissues analysed. The potential clinical relevance of the following genes; MRP4, MRP5, COX-2, MRIT, Bcl-x_L, Bcl-2α and eIF-2α, was also demonstrated.

4.6 Use of gene therapy to downregulate expression of the MRP1 gene

The Combination *in vitro* toxicity assay and IOV LtC_4 transport studies outlined in Section 3.7 demonstrated the expression of a functioning MRP1 protein in the DLKP-SQ cell line. It was also shown here that MRP1 could be inhibited by the use of known MRP1 NSAID activity antagonists (Duffy *et al.*, 1998). It was therefore considered to attempt to downregulate expression of MRP1 expression using novel gene therapy mechanisms.

There is hope that selective anticancer drugs, with fewer cytotoxic side effects than conventional cancer chemotherapy, will be developed. This optimism is based on the identification of new cancer-associated molecular sites, which could allow the selective targetting of cancer cells, while sparing normal cells. Gene expression may also be altered at the transcriptional stage by use of oligonucleotides that cause the formation of triple helixes without stable integration of genetic material into the genome. An alternative strategy is to use single-stranded oligonucleotides to modify gene expression at the translational step.

4.6.1 Use of Ribozyme Technology

4.6.1.1 Introduction to ribozymes and their usage

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

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

The MRP1 ribozyme used in this study was a hammerhead ribozyme. As a result, only the hammerhead group of ribozymes will be discussed in detail.

4.6.1.2 Introduction to Hammerhead ribozymes

The name "hammerhead" derives from the predicted shape of the ribozymes secondary structure and describes the smallest of the six classes (Kashani-Sabet and Scanlon, 1995). Naturally occurring hammerhead ribozymes were found within RNA viruses and they act in *cis* during viral replication by the rolling circle mechanism (Bratty *et al.,* 1993). Through genetic engineering, the hammerhead was manipulated to enable it to cleave its target in *trans* and act in a truly catalytic manner (Uhlenbeck, 1987). Using *in vitro* mutagenesis studies of the plus strand of satellite RNA of tobacco ringspot virus (sTobRV0), the consensus sequences required to maintain catalytic cleavage by the ribozyme were defined (Haseloff and Gerlach, 1987). It is this information that allows the design of ribozymes to target any gene of interest once the sequence is known.

In terms of secondary structure, the *trans*-acting hammerhead ribozyme developed by Haseloff and Gerlach (1987), is composed of the catalytic core (or hammerhead domain) region and three hybridising helices or stems (Fig. 3.9.2). Stems I and III hybridise to the flanking sequences of the cleavage site and act as an antisense, and the stem loop II is usually composed of eight complementary ribonucleotides in the loop structure (Kashani-Sabet and Scanlon, 1995). In terms of the substrate, the mutational analysis revealed the requirement of XUN sequences, with X being any nucleotide and N being A, C or U (Haseloff and Gerlach, 1988; Ruffner *et al.*, 1990). It is the

likelihood of finding an appropriate target within a given gene sequence that makes hammerhead ribozymes such a potentially useful tool.

The basic reaction scheme of a ribozyme cleaving its target is as follows (Ohkawa *et al.*, 1995): First the substrate (together with Mg^{2+} ions) binds to the ribozyme via the formation of base pairs with stems I and III. Then, a specific phosphodiester bond in the bound substrate is cleaved by the action of the Mg^{2+} ions (the ribozyme functions as a metalloenzyme as it requires the presence of magnesium. Recent research has also identified zinc-dependant ribozymes (Li *et al.*, 2000)). This cleavage generates products with 2', 3'-cyclic phosphate and 5'-hydroxyl groups. Finally the cleaved fragments dissociate from the ribozyme and the liberated ribozyme is now available for a new series of catalytic events. With respect to the ribozyme itself, several requirements must be met for the development of an effective catalytic RNA. Several groups have probed the actual requirements within the catalytic core, which are almost exclusively composed of RNA (Perreault *et al.*, 1990, 1991; Yang *et al.*, 1992). An all DNA ribozyme was shown to be devoid of catalytic activity (Perreault *et al.*, 1990). Further studies using DNA at specific sites established that the minimum ribonucleotide requirement is that at bases A^{15}, A^{9}, G^{5} and G^{8} (Perreault *et al.*, 1990, 1991; Yang *et al.*, 1992).

It has been suggested that 12 bases may represent the optimal length of flanking sequence (Bertrand *et al.*, 1994). In addition, substrate sequences flanking the cleavage site rich in A or U were favoured over GC rich sequences to enhance discrimination (Herschlag, 1991).

Transportation of the ribozyme into the cells has been achieved by using either exogenous delivery (using naked ribozymes complexed with cationic liposomes) or vector-based systems to promote ribozyme expression (endogenous delivery). In the case of exogenous delivery, the susceptibility of RNA oligonucleotides (including ribozymes) to ribonuclease attack intracellularly or in the serum, required the search for modifications to enhance ribozyme stability while maintaining cleavage capability. To this end, a number of chemical modifications of the nucleotides have been made. (Paolella *et al.*, 1992). In contrast to exogenous delivery, many studies utilise the cellular machinery to express the ribozyme. Here, the ribozyme gene is cloned into an

available vector (expression plasmid or retroviral vector) and delivered to the cells by transfection of the plasmid or by retroviral infection. Other delivery systems, such as cationic liposomes, adenoviruses and adeno-associated viruses (AAV), are being studied in order to optimize ribozyme activity (Kashani-Sabet and Scanlon, 1995). The choice of delivery system can depend heavily on the disease type and type of promoter used.

Once the ribozyme has been successfully introduced intracellularly, demonstration of ribozyme activity is required. Expression of the ribozyme itself can be detected by reverse-transcriptase-polymerase chain reaction (RT-PCR) or Northern blot analysis. Proof of efficacy of the ribozyme is reliant on a demonstration of inhibition of the target gene expression (at RNA and/or protein level) and any downstream phenotypic effects such as decreased tumor growth, viral replication or drug resistance. As the ribozyme has the potential to act as antisense, it is desirable to demonstrate that the ribozyme retains the ability to cleave. Kashani-Sabet *et al.* (1992) have designed an *in vitro* assay system for assessing ribozyme efficacy at cleaving the target sequence. Researchers have also shown that cellular extracts of ribozyme expressing cells cleave target RNA *in vitro* (Chang *et al.*, 1990; Scanlon *et al.*, 1991; Kashani-Sabet *et al.*, 1992), suggesting that the ribozyme expressed intracellularly retains the ability to cleave its target. However, detection of the cleavage products has been elusive in many studies, due, most likely, to the rapid degradation of short RNAs. Some studies have detected such products by PCR analysis (Kashani-Sabet *et al.*, 1992; Cantor *et al.*, 1993). Detection of the cleaved fragment of mdr-1 RNA was reported by Northern analysis in human ovarian carcinoma cells (Scanlon *et al.*, 1994).

4.6.1.3 Use of ribozymes in the study of MDR

Several groups have demonstrated ribozyme-mediated modulation of MDR in human cancer cells. Scanlon *et al.* (1994) reported the reversal of the MDR phenotype in human ovarian carcinoma cells using both an mdr-1 ribozyme and a *fos* ribozyme. The mdr-1 ribozyme has also been shown to be effective in a number of other MDR cell types. Holm *et al.* (1994) subsequently reported the reversal of daunorubicin resistance

in resistant human pancreatic carcinoma cells. Previous work in this laboratory identified a similar effect for the ribozyme in two lung cell lines; transfected DLKP-A2B and SKMES-1ADR were found to be more sensitive to adriamycin, vincristine and VP-16 (Daly *et al.*, 1996). The anti-mdr-1 ribozyme does not result in complete reversal of resistance to the level of the sensitive parent. Possibly the level of mdr-1 mRNA remaining in the mdr-1 ribozyme transfectants is capable of mediating drug resistance and/or other mechanism of resistance may be present in these lines.

Ribozyme studies can also be used in functional assays of multi-drug resistance. Eijdems and co-workers report the down-regulation of mdr-1 mRNA using an mdr-1 ribozyme targeted at codon 196 (Kobayashi *et al.*, 1994; Eijdems *et al.*, 1995) in a human non-small cell lung cancer cell line SW-1573 selected in a low concentration of doxorubicin. In a clone having reduced mdr-1 mRNA level there was no detectable change in sensitivity to drug which suggests that mdr-1 does not contribute to drug-resistance in these cells. These authors have concluded that resistance in this cell line is associated with the presence of an altered form of MRP1. Hatanaka *et al.* (2001) recently used a hammerhead anti-MRP1 ribozyme to inactivate MRP1 function in a multidrug resistant cancer cell line, KB8-5. Expression of the ribozyme was β-actin promoter-driven, and was targetted to bases 1162-1176 of the MRP1 coding sequence, an area which has previously been shown to be essential for protein activity (Kast *et al.*, 1997). This is also the same ribozyme which was used to downregulate MRP1 expression in DLKP-SQ cells in this study. The group observed decreased MRP1 mRNA expression in the transfected cells but no significant decrease in the multidrug resistance profile. Further work in that laboratory (Nagata *et al.*, 2001) recorded reversal of the multidrug resistance phenotype in human colon cancer cells using a ribozyme to gamma-Glutamylcysteine (gamma-GCS). Gamma-GCS is a key enzyme in glutathione (GSH) synthesis (reviewed in Griffith and Mulcahy, 1999), which may be utilised by MRP1 in mediating multidrug resistance (Leier *et al.*, 1994; Jedlitschly *et al.*, 1994).

4.6.1.4 The MRP1 ribozyme was not capable of downregulating MRP1 protein or mRNA in DLKP-SQ cells

Expression of the pHβ plasmid containing the MRP1 ribozyme was detected in transfected cells DLKP-SQ clones using pHβ expression primers listed in Appendix A (Table 7.1A) (Ng *et al.*, 1985). These primers bind to the β-actin promoter sequence and at the polylinker site of the plasmid, yielding an RT-PCR product of 118bp. Expression of this PCR product would thus imply expression of the ribozyme, as this section is located just before the cloned MRP1 ribozyme sequence. RT-PCR was not carried out on the ribozyme RNA sequence due to both the small size of the fragment (42 bases) and the fact that most of the sequence would be inaccessible for primer binding.

However, expression of the MRP1 ribozyme in the low-MRP1-expressing DLKP-SQ cells did not result in downregulation of MRP1 mRNA. A decrease in MRP1 protein expression in a small number of ribozyme–expressing clones was observed, although in the majority of clones expression of the protein was not affected. This decrease in protein expression was not accompanied by any change in the drug-resistance profiles of the transfected cells, as measured by vincristine resistance. As a result, it must be concluded that the MRP1 ribozyme, while achieving decreased MRP1 protein expression in a small number of clones, did not succeed in modulating the drug resistance profile of the DLKP-SQ cells.

An *in vitro* cleavage assay carried out on the MRP1 ribozyme as described by Kashani-Sabet *et al.* (1992) demonstrated that the purified ribozyme was capable of cleaving the target sequence *in vitro*. Cell extract isolated from clones also demonstrated cleavage of the target, suggesting that the ribozyme expressed intracellularly retains the ability to cleave its target, and that this process was time dependant, significant cleavage only being observed over 24hrs. Such *in vitro* selection of ribozymes is now currently being utilised for a range of target genes to identify catalytic ribozymes capable of efficient cleavage (reviewed in Pan, 1997). Once a designed ribozyme has been determined as functional, exogenous or endogenous delivery of the ribozyme to the target cells and its subsequent assessment *in vivo* will be carried out. Ribozymes directed against the

multidrug-associated genes MRP2 (Materna *et al.*, 2001) and BCRP (Kowalski *et al.*, 2001) have been designed using this method. However, as outlined here, previous studies have indicated that the efficacy of ribozyme-mediated cleavage *in vitro* may not always translate into similar efficiency *in vivo* (Kato *et al.*, 2001).

4.6.1.5 Was the MRP1 ribozyme capable of targetting α-catenin expression?

In order to investigate if the chosen MRP1 was complementary to other human genes, a *BLAST*™ search (http://www.ncbi.nlm.nih.gov:80/BLAST) examining "short nearly exact matches" was carried out on the ribozyme target sequence. The results are summarised on Table 3.9.1. As can be seen, the MRP1 ribozyme target sequence shared roughly 85% sequence similarity with the human α-catenin coding sequence, among others. It was therefore possible that a catalytically active ribozyme would downregulate expression of this gene as well as MRP1 in the DLKP-SQ cells.

However, it was certain that this would not affect detection of changes in MRP1 gene and protein expression. An identical *BLAST*™ search was carried out on the MRP1 RT-PCR primers, which demonstrated that these primers shared no sequence homology at all with α-catenin. Similarly, the detection of MRP1 protein would not be affected as the size of MRP1 (190kDa) was significantly different to that of α-catenin (102 kDa) (Ozawa *et al.*, 1989). As well, there is no indication that the MRP1 R1 antibody, specific for MRP1, should detect α-catenin protein expression. Additionally, the functional assays carried out in Section 3.7 (combination assay, IOV assay) indicate the expression of a functioning MRP1 protein in the DLKP-SQ cell line, and the ability to impair that function through known MRP1 inhibitory mechanisms (NSAIDs).

It was, therefore, feasible to proceed with the attempted specific downregulation of the MRP1 gene using the ribozyme. Unfortunately, in this study, the ribozyme was not observed to significantly affect expression of either the MRP1 gene or protein or affect drug resistance, despite being demonstrated to be catalytically active *in vivo*. As a result, it was deemed unnecessary to examine the expression levels of the α-catenin gene in the transfected cells.

In conclusion, while attempts to modulate MDR with the use of mdr-1 ribozymes have met with some success, efficient downregulation of the MRP1 mRNA using this method remains elusive. It is hoped that further research in this area, combined with the knowledge gained from MRP1 antisense research (Section 4.7) may yield more appropriate targets.

4.6.2 Use of Antisense Technology

4.6.2.1 Introduction to antisense and their usage

Antisense oligonucleotides are unmodified or chemically modified single-stranded DNA molecules. They are typically 13-25 nucleotides long and are specifically designed to hybridise to corresponding RNA by Watson-Crick binding. This corresponding RNA strand is termed the 'sense' strand. Affinity between the antisense oligonucleotide and target polynucleotide increases as the length of the antisense molecule increases, due to the increased hydrogen bonding between bases (Crooke and Bennett, 1996). Theoretically, at least, this allows for the design of drugs to attack any unwanted or mutated form of a gene, and leave the normal copy of the gene untouched, even if the two forms differ by only a single base pair or nucleotide (Crooke and Bennett, 1996).

Antisense molecules can consist of relatively short synthetic oligonucleotides introduced into cellular systems by various means (Scanlon *et al.*, 1995; Crooke and Bennett, 1996). Alternatively, antisense molecules can consist of a whole gene, or a specific fragment of a gene, cloned into an expression vector in a reverse orientation and transfected into a cell, where it is expressed as antisense RNA either endogenously or upon stimulation (Scanlon *et al.*, 1995; Branch, 1996; Tolume *et al.*, 1996; Zhang, 1996; Sczakiel, 1997).

There are a large number of antisense clinical trials in humans and animals worldwide, including the target genes Bcl-2, Survivin, Bcl-x_L, c-myc, RAS and BCR-ABL (reviewed in Tamm *et al.*, 2001).

4.6.2.2 Mechanisms of antisense action

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

One of the most important mechanisms of action of DNA based-antisense targeted to RNA is the activation of ribonuclease H (RNase H) (Crooke and Bennett, 1996; Bouffard *et al.*, 1996; Giles *et al.*, 1995; Branch, 1996). RNase H is an ubiquitous enzyme that selectively cleaves the RNA component of RNA-DNA duplexes. Other mechanisms of inhibition of translation include interference with secondary structures, such as stem loops, (Ecker *et al.*, 1992; Thierry *et al.*, 1993; Crooke and Bennett, 1996), inhibition of 5' capping (Alahari *et al.*, 1996; Stewart *et al.*, 1996; Crooke and Bennett, 1996) and interference with 3' polyadenylation (Chiang *et al.*, 1991; Alahari *et al.*, 1996; Stewart *et al.*, 1996). Oligonucleotides conjugated to alkylating and photoactivable alkylating species have been synthesised. These can then inhibit the target DNA by covalently modifying them, rendering them non-functional (Crooke and Bennett, 1996). Activation of mRNA breakdown is not universal, however; Probst and Skatella (1996) found elevation of specific mRNAs by antisense, but not by sense treatments.

Numerous chemical modifications have been made to the oligonucleotide backbones and sugar bases to render them more nuclease resistant and give them greater affinity to their targets. The earliest modifications involved substituting the non-bridging oxygen atoms in the internucleotide bonds with either a methyl or a sulphur group to give methylphosphonate and phosphorothioate oligodeoxynucleotides respectively (Marcus-Sekura *et al.,* 1987; Matsukura *et al.,* 1987). This made the oligonucleotides more resistant to nuclease degradation, which was a problem for natural phosphodiester oligonucleotides (Wickstrom, 1986), thus extending the half-life of the oligonucleotides and improving their efficacy. Phosphorothioates retain the ability to activate RNaseH (Gao *et al., 1992*). As a result, phosphorothioate oligos remain the most widely used base analogue, and are currently being tested in a number of clinical trials (reviewed in Tamm *et al.*, 2001).

The Second Generation™ chimera oligonucleotides (see Section 2.4.4.1) used in this study were commercially available oligonucleotides which had previously been used successfully in studies on the intracellular adhesion molecule 1 (Chiang *et al.*, 1991) and against Ha-ras (Harvey-Ras) mRNA (Monia *et al.*, 1992). The structure of these patented oligonucleotides was a combination of phosphorothioate and methylphosphonate. They were designed to incorporate the benefits of both oligo types; increased resistance to nuclease degradation conferred by a phosphorothioate backbone, while also remaining non-toxic to the target cells (methylphosphonate). The oligos were also capable of recruiting RNaseH for cleavage.

The ubiquitous enzyme RNaseH can cause unspecific side effects. It can cleave DNA-RNA duplexes which are as short as 4 bp *in vitro* and 10bp *in vivo* (Nakamura *et al.*, 1991). As a result, it is probably not possible to obtain cleavage of an intended RNA target without causing at least partial degradation of many nontargeted RNAs. It is therefore prudent to screen potential antisense oligonucleotides against gene databases to identify and select those expected to knock out the fewest essential genes.

It is clear that there is a requirement, when designing antisense experiments, for strict and rigorous control measures, to allow the clear and unambiguous demonstration of an antisense effect. There are now a number of generally accepted guidelines for the design and evaluation of antisense experiments (Wagner, 1995; Branch, 1996).

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

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

3. The choice of control sequences is a critical element in the design of any antisense experiment. There are four types of control oligos that should be considered; Sense, Scrambled, Mismatch and Mismatch Target. Ideally, the control should differ from the antisense sequence no more than is necessary to prevent specific hybridisation. There is no scientifically correct number of controls to employ in a certain experiment. The more control oligos that are used, however, the more likely that the observed end point has resulted from a true antisense mechanism.

4.6.2.3 Use of antisense to modulate MDR

Jaroszewski *et al.* (1990), designed five different phosphorothioate oligos which were used to down regulate mdr-1 in the human adriamycin-resistant breast cancer cell line MCF-7 ADR. Of the five oligos tested the most effective was the one targeted at nucleotide +18 to +32 in relation to the first base in the mRNA sequence which caused a 4-fold increase in Adriamycin toxicity. Rivoltini *et al.* (1990) used a 12 bp phosphodiester oligo targeted to bases -6 to +6 of mdr-1 mRNA to reduce Pgp expression levels in human colorectal adenocarcinoma cells LoVo/Dx. This was accompanied by a 100-fold reduction in the cell line's resistance to adriamycin. Clynes

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

Alahari *et al.* (1996) conducted an extensive study, analysing 32 different phosphorothioate oligonucleotides spanning almost every region of the mdr-1 mRNA including the 5' Untranslated, AUG codon, Coding (splice junction), open reading frame (ORF), and stop codon, 3' untranslated and 5' Cap. The cells used were mouse NIH3T3 fibroblasts, which had been transfected with an expression plasmid containing the human mdr-1 cDNA (pSK1 MDR-1). One oligonucleotide which stood out from the others was one overlapping the AUG start codon (*AS 5995*), as it caused a substantial reduction in the mdr-1 message levels as measured by Northern blots. This oligonucleotide was thus chosen to optimise the RNaseH *in vitro* cleavage assay in this study. The other sequences tested were largely ineffective, including two other oligonucleotides that also overlapped the AUG codon. Maximum specific reduction on mdr-1 mRNA was observed after 24h treatment of the cells with *AS 5995* (Alahari *et al.* 1996) but reduction occurred only with the use of serum-free media and cationic liposomes. Multiple treatments with the AS 5995 did not cause any greater specific reduction in the mdr-1 messenger levels than a single treatment, whereas greater cytotoxicity was observed. The reduction in the mRNA expression was found to be readily reversible after the 24h exposure to *AS 5995*, with normal levels returning after 24 h if the cells were returned to complete culture medium.

The first report of the use of antisense oligonucleotides for the reduction of MRP1 expression came from Stewart *et al.* (1996). This group identified two oligonucleotides, (ISIS 7597 and 7598) from sixteen designed oligonucleotides (15 phosphorothioate and one 2'-O-methyl derivative) which were effective at downregulating MRP1 protein and mRNA in HeLa cells. Subsequent experimentation was discontinued on ISIS 7598 because of its complementarity to a region highly conserved among the ABC transporter superfamily which could potentially affect the expression of other proteins, making it less specific. A 2'-modified ISIS 7597 oligonucleotide was again observed to decrease MRP1 mRNA and protein levels in the HeLa cell line in a subsequent study (Canitrot *et al.*, 1996). The authors also demonstrated the role of RNaseH in the reduction of MRP1

mRNA levels. By using two DNA probes for Northern blots corresponding to regions in the 5' and 3' coding ends of the mRNA, they were able to detect the oligonucleotide induced cleavage fragments of MRP1 mRNA in whole cells.

Stewart *et al.* (1996), also proposed that variations in secondary structure at different sites within the MRP1 mRNA may have been the cause of differences in efficacy of the various oligonucleotides tested. The AUG start site, as seen above, has been targeted in many studies because of the proposed accessibility of this sequence. However, this group found that oligos complementary to the coding region to be the most effective. These results indicate the importance of evaluating the activity of a number of oligonucleotides complementary to different regions of a given mRNA target rather than testing oligonucleotides directed against a single site.

Further studies on the ability of antisense to modulate the activity of MRP1 have involved the endogenous delivery of a full-length MRP1 antisense sequence to doxorubicin-selected, multidrug-resistant GAOK cells (Gao *et al.*, 1997). The authors observed almost complete inhibition of expression of the MRP1 protein in the transfected cells. Additional successful studies have included the transfection of MRP1 and Bcl-2 oligonucleotides into A549 cell lines (Wang *et al.*, 2000) and the use of hairpin loop antisense (Niewiarowski *et al.*, manuscript submitted).

4.6.2.4 Selected MRP1 antisense do not affect MRP1 gene expression or drug resistance in DLKP-SQ cells

The oligonucleotide identified by Stewart *et al.* (1996) and Canitrot *et al.* (1996), *ISIS 7597*, was chosen to be used to downregulate expression of the MRP1 gene in the DLKP-SQ cell line. Unfortunately, several transfections of the antisense including a sense control oligonucleotide into the DLKP-SQ cell line failed to induce any significant decrease in expression of either the MRP1 mRNA or protein or effect on the resistance to the MRP1 substrate chemotherapeutic drug, vincristine. Subsequent discussion on the subject of this oligonucleotide with external colleagues revealed that the molecule was not as efficient at downregulating the MRP1 mRNA as had been previously assumed (Dr. Finbarr Cotter, Personal communication).

Several additional antisense were designed to target different regions of the MRP1 gene and mRNA coding strand as outlined in Section 3.10.2. The reasons chosen for each target area of the coding strand are as outlined in Section 3.10.2.1.

Unfortunately, MRP1 gene expression was in the main increased following transfection with many of the selected antisense. In addition, the drug resistance profiles of the transfected cells were not altered in an MRP1-specific fashion.

However, a number of interesting results were observed from the antisense experiment. The two oligonucleotides which were observed to be the most toxic to the cells (particularly at 1.5μm concentrations) were *MA*s 14 and 19. *MA*14 was targetted against the Walker A motif in the NBD1 region of the MRP1 gene, which is one of the shared homology regions between the various MRP genes (MRPs 1 – 6). It is possible therefore, that the toxicity of this oligonucleotide may be related to the fact that it is interfering with the functioning of this common, and therefore essential, sequence. The possibility that this interference may have led to the downregulation of another, unidentified gene cannot be discounted.

All of the designed oligos except *MA*19 were targetted to the coding mRNA strand of the MRP1 gene, while *MA*19 was targetted to the gene itself. *MA*19 would be classified as an "antigene", or antisense targetted to the MRP1 DNA sequence. Such antigenes bind to the DNA, forming stable triple-stranded structures and are sometimes effective in downregulating expression of the targetted gene. *MA*19 was targetted to the transcriptional start site of the MRP1 gene, in which there are three Sp1 transcription factor recognition sites clustered together. This region of the MRP1 promoter sequence has previously been demonstrated to be important in regulation of the gene (Zhu and Center 1994 and 1996; Wang and Beck, 1998), while the importance of the Sp1 promoter in regulating MRP1 gene expression has also previously been determined (Zhu and Center, 1994). The importance of this area of the MRP1 promoter has also been demonstrated in the DLKP cell line in this study. It is therefore possible that *MA*19 may be exerting an inhibitory effect on MRP1 in DLKP-SQ, but that the targetting of the Sp1 promoter may be exerting additional inhibitory effects on Sp1-controlled essential factors.

4.6.2.5 Designed MRP1 antisense do not downregulate target sequences *in vitro*

The optimisation of an RNaseH cleavage assay for the antisense oligonucleotides established a reliable method of identifying the mRNA inhibitory properties of phosphorothioate oligos *in vitro*. However, when the designed Second Generation™ oligos were included for analysis in this assay, an incomprehensible banding pattern was observed. Despite the fact that this design of oligo has been demonstrated to recruit RNaseH to mediate cleavage, this has not been demonstrated here. It thus remains to be demonstrated whether or not these antisense oligos are capable of mediating gene inhibition in the manner described.

4.6.2.6 In conclusion: The results of gene therapy targetted against MRP1

The results from the various authors cited above demonstrate clearly the potential usefulness of using antisense oligonucleotides, whether modified or chimeric, to effectively down-regulate the expression of MDR-related genes.

However, it is also apparent that a large amount of work remains to be carried out in this area. In almost all of the studies carried out above, as well as the work carried out in this thesis, full reversion of multiple drug resistance was not achieved. In many cases, this is due to the MDR phenomenon being multifactorial, with a combination of proteins causing increased cytotoxic drug resistance, so that reducing the expression of any one individual gene will not eliminate the MDR phenotype.

Section 5.0

Conclusions and Future Work

5.1 Conclusions

A major objective of the work undertaken for this thesis was to contribute to the understanding of the molecular mechanisms by which BrdU alters the differentiation status of the epithelial lung cell lines DLKP (derived from a poorly differentiated carcinoma of the lung), and A549 (derived from an adenocarcinoma of the lung). The following points summarise the main findings of the study.

RT-PCR analysis carried out on the poorly differentiated cell line DLKP demonstrated dramatic effects on the gene expression profile at the mRNA level of the cell line following exposure to the differentiating agent, BrdU. Significant increases in expression of the multidrug resistance-associated gene (MRP1) and its MRP3 homologue, the breast cancer resistance protein (BCRP) gene, the pro-apoptotic genes BAXα and MRIT, an isoform of the cyclooxygenase gene (COX-2) and the eukaryotic initiation factor 2α (eIF-2α) genes were observed. These large increases were accompanied by less significant increases in mRNA expression for another MRP1 homologue, MRP2, and for α-catenin and E-cadherin. Expression of the anti-apoptotic Survivin gene was observed decreased in DLKP following BrdU-treatment, while preliminary Western blotting analysis indicated that expression of the Survivin protein may be decreased also.

DNA microarray analysis of BrdU-treated DLKP also detected increased gene expression of the transcription factors ETR103 and NSEP, the cell cycle protein p55CDC, the Ini1 tumour suppressor gene, the cell adhesion protein EB1, the RPS19 ribosomal protein and the activation antigen, FNRB. Decreased gene expression of transcription factors CREB2, c-*myc*, TBP, CNBP, HIP116 and GABP-β2 and of the ribosomal protein, RPL6, were also detected in BrdU-treated DLKP cells using DNA microarray analysis.

DLKP may resemble a stem cell line of the lung. The exposure of DLKP to the differentiating agent BrdU induces that cell line to differentiate. As such, the gene and protein expression changes observed here in response to that agent may represent expression changes accompanying differentiation in lung cells *in vivo*.
By contrast, exposure of the adenocarcinoma cell line, A549, to BrdU induced far fewer changes in gene expression when compared with DLKP. RT-PCR analysis detected increased gene expression of MRP1 and BCRP, as well as decreased MRP2, MRP4 and mdr-1 gene expression in this cell line following induced differentiation by BrdU. As the number of affected genes in BrdU-treated A549 was observed to be much lower, DNA microarray analysis was not carried out on BrdU-treated A549 cells.

Exposure of DLKP cells to the chemotherapeutic drugs cisplatin, taxol and VP16 also resulted in induction of gene expression. RT-PCR analysis of the DLKP cells identified significantly increased expression of the MRP1, MRP2 and BCRP genes following exposure to cisplatin, of MRP2, α-catenin and E-cadherin following exposure to taxol and of MRP1, MRP2 and α-catenin following exposure to VP16. These results suggested that the gene induction pattern obtained following exposure to an inducing agent is specific both for that type of agent and the length of exposure. Also, the morphological response of the cells to the different drugs was similar, and bore many of the same characteristics of differentiated cells. It is therefore possible that the process of differentiation in cells resembles in part the response of those cells to certain types of chemotherapy.

Selection of DLKP cell lines in a number of chemotherapeutic drugs also elicited a diverse profile of gene expression. It was observed that the gene expression profile for each drug differed significantly. Among the drugs used for selection were cisplatin and VP16; the selection process was observed to induce a different expression profile from that induced by the transient exposures detailed earlier. It is apparent from these results that gene induction is dependent not only on the type of inducing agent used, but also the length of time and schedule used for drug exposure.

It was considered likely that BrdU (in view of its structure and known metabolism), and possibly some of the chemotherapeutic drugs tested, may transcriptionally upregulate

gene and protein expression in the DLKP cell line. If this were the case, it is possible that these agents may mediate transcriptional upregulation of these genes using a common transcription factor, or set of factors.

Transfac™ transcription factor analysis of the 5' promoter regions of MRPs 1-3, BCRP, MRIT, COX-2, eIF-2α, α-catenin and E-cadherin identified seven transcription factors which may be shared by all nine genes. As all nine genes were upregulated in DLKP following induced differentiation by BrdU, it is possible that one, or more of these factors may have been utilised by BrdU in upregulating transcription of the genes. The seven factors identified in common were GATA-1, GATA-2, GATA-3, MZF1, Ik-2, CdxA and AML-1a. RT-PCR analysis using primers for each of these factors revealed increased GATA-2 and GATA-3 expression in BrdU-treated DLKP. The expression of the other factors was not detected by RT-PCR. No significant change was observed in BrdU-treated A549 cells. It is possible therefore, that BrdU upregulates the expression of some or all of the genes observed affected in DLKP at least in part, by inducing expression of one or both GATA-2 and GATA-3 transcription factors.

RT-PCR analysis on the drug-exposed DLKP cells revealed increased GATA-2 expression in VP16-treated DLKP. No change was observed in expression of GATA-2 in response to cisplatin or taxol. Expression of GATA-3 in DLKP cells was not significantly affected by exposure to any of the three chemotherapeutic drugs. This result indicates that VP16 may mediate some or all of its gene inducing effects in DLKP by inducing increased GATA-2 expression. However, VP16 does not appear to induce expression of the GATA-3 transcription factor. This result is connected with the difference in gene expression profiles induced in the DLKP cell line by the different agents. It is also apparent that in this cell system, the chemotherapeutic drugs cisplatin and taxol may mediate gene induction via alternate transcription pathways than those examined here.

Luciferase reporter plasmids attached to truncated regions of the MRP1 5' promoter region identified a fragment of the promoter as the major positive regulatory region in normal DLKP cells. This region comprised the positions –91 to +103 bases relative to the transcriptional start. This region has also previously been determined as the major

regulatory region of MRP1 in other cell systems (Zhu and Center, 1994). When the transfected DLKP cells were treated with BrdU, luciferase expression is highest further upstream of this region, between -91 and –411 bases relative to the transcriptional start. It is apparent from this result that exposure of DLKP cells to BrdU shifts the bulk of transcriptional activity to another area of the promoter sequence. This region of the MRP1 promoter contains a single GATA-2 transcription factor recognition sequence. However, luciferase expression levels over the whole 2kb fragment were not altered significantly between treated and untreated cells, indicating that the major regulatory target for BrdU lies outside this area.

Exposure to VP16 was observed to elicit a similar result in the luciferase promoter-transfected cells. Exposure of the transfected cells to the agent induced most luciferase production in the -91 and –411 bases fragment relative to the transcriptional start. It is important to note here that only VP16 increased GATA-2 expression in the drug-treated DLKP, and that this fragment contains a potential GATA-2 recognition site. Also, the expression levels for the plasmid containing the whole gene did not vary significantly if VP16 was added, indicating that, like BrdU, its major regulatory target also lies outside this area. Exposure of the transfected cells to cisplatin and taxol did not elicit any significant effect. This result was surprising, as cisplatin has been shown to induce MRP1 expression. It is apparent from this result that the major target for cisplatin-mediated MRP1 upregulation also lies outside 2kb from the transcriptional start site of the gene.

RT-PCR analysis on a number of human lung, breast and oesophageal tumour samples was carried out to examine the expression of MRPs 1-6, BCRP, mdr-1, mdr-3, COX-1, COX-2, MRIT, BAXα, Bcl-x_L, Bcl-x_S, Bcl-2α, Survivin, BAG, BAP, eIF-4E, eIF-2α and c-*myc*. This clinical tumour sample study demonstrated the ability to extract high quality, reproducible expression results from human tissue, using a basic sample collection procedure. Only the results from the primary lung tissue sample group were felt to be of significance due to the small sample sizes for the other two groups. Gene expression results obtained from the clinical study demonstrated the high level of expression of the MRP1 and MRP2 genes in most of the tissues analysed. The potential clinical relevance of the following genes; MRP4, MRP5, COX-2, MRIT, Bcl-x_L, Bcl-2α

and eIF-2α in primary lung tumour samples was also demonstrated. However, additional tissue samples are required to confirm these preliminary results. MRP1 gene expression was observed to be generally higher in tumour samples from male patients.

Expression of the MRP1 gene has been demonstrated to be of importance in the BrdU-mediated differentiation of both DLKP and A549 cells, in the drug-treated and drug-selected cell lines and in clinical tissue. A novel ribozyme to MRP1 was designed and was observed to cleave its intended target in an *in vitro* cleavage assay. The ribozyme was successfully transfected into MRP1-expressing DLKP-SQ cells. However, the transfections did not result in significant downregulation of MRP1 RNA or protein or significant changes in the drug resistance profiles of the cells.

RNaseH activity assays for antisense oligonucleotides analyses were also successfully set up using an mdr-1 antisense. However, the identification of effective MRP1 antisense oligonucleotides was not made by either *in vitro* or *in vivo* studies.

5.2 Future Work

The work described in this thesis has identified a number of key genes and proteins which may constitute novel differentiation-specific markers in lung epithelial cell lines. Comparisons with gene induction profiles from chemotherapeutic agents have identified that different agents may induce different responses in the same cell system. Transcription factor analysis of induced genes has indicated that these genes may be transcriptionally upregulated via a number of key transcription factors.

Further work on this area would include the following:

1. Examination by Nuclear run-on analysis of the stability of mRNA levels in cells induced to differentiate by BrdU. This would indicate if the change in mRNA levels observed by RT-PCR is due to increased gene expression, rather than mRNA instability.
2. Additional MRP1 promoter pGL2 Luciferase expression plasmids could be manufactured which concentrate more heavily on the promoter regions of interest determined by this study. These would include the regions between –91 and –660 bases from the transcriptional start and also further upstream than the 2kb sequence examined here. For instance, a number of extra plasmids could be generated with various lengths of the promoter sequence attached to the *luc* gene. DLKP cells transfected with these extra plasmids would then be exposed to BrdU and the chemotherapeutic agents already outlined to pinpoint the target promoter region.
3. Point mutation analysis of the postulated transcription factor binding sites already identified to determine more specifically if these sites are responsible for binding and hence, gene upregulation. Mutational analysis could be carried out on the additional PGL2 plasmids already mentioned, followed by transfection of these mutated plasmids into DLKP and subsequent exposure to BrdU and chemotherapeutic agents.

4. Transfection of expression plasmids coding for GATA-2 and GATA-3 cDNAs could also be carried out in DLKP cells, and their subsequent effect on gene expression monitored.
5. The targetting of additional ribozyme and antisense sequences directed against the mRNAs of the identified transcription factors GATA-2 and GATA-3 could also be attempted. The resultant effect on gene induction in response to BrdU could then be monitored.
6. The ability of other differentiating agents to induce differentiation and the consequent gene/protein upregulation could also be examined. The effect on the drug resistance profile of the differentiated cells could also be examined. Suggested differentiating agents would include Retinoic acid (RA), CdU, 5,5'-FdU and 5-Bur.
7. The ability of other chemotherapeutic drugs to induce gene/protein expression could also be examined. It would also be relevant to examine the effect on the drug resistance profile of the treated cells. Suggested drugs would include Taxotere, Carboplatin, Vinblastine, etc.
8. Expression of the protein product of each gene observed to be upregulated could also be examined.

Section 6.0

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Section 7.0

Appendices

7.1 Appendix A: Antisense/Primer sequences & conditions used in study

7.1.1 Primers used for RT-PCR gene expression analysis

Table 7.1A List of gene expression RT-PCR primers and conditions

Gene	Length (bases)	T_m (°C)	Size (bp)	Sequence
β-Actin (large)	29	55	383	GAA ATC GTG CGT GAC ATT AAG GAG AAG CT
	22			TCA GGA GGA GCA ATG ATC TTG A
β-Actin (small)	23	55	142	TGG ACA TCC GCA AAG ACC TGT AC
	22			TCA GGA GGA GCA ATG ATC TTG A
mdr-1	20	51	157	GTT CAA ACT TCT GCT CCT GA
	20			CCC ATC ATT GCA ATA GCA GG
mdr-3	24	55	321	ATT AGC AGT TGT TCC AAT TAT TGC
	24			TGT CCA TTC AGA ATG AGA TAT GCA
MRP1	21	55	202	GTA CAT TAA CAT GAT CTG GTC
	21			CGT TCA TCA GCT TGA TCC GAT
MRP2	20	53	241	CTG CCT CTT CAG AAT CTT AG
	19			CCC AAG TTG CAG GCT GGC C
MRP3	19	63	262	GAT ACG CTC GCC ACA GTC C
	21			CAG TTG GCC GTG ATG TGG CTG
MRP4	19	42	239	CCA TTG AAG ATC TTC CTG G
	18			GGT GTT CAA TCT GTG TGC
MRP5	18	49	381	GGA TAA CTT CTC AGT GGG
	20			GGA ATG GCA ATG CTC TAA AG
MRP6	21	68	324	ACA CCC ATT GGT CAC CTG CTA
	24			GGT CAC CTG GAG GGC AGC AGA GAC
BCRP	20	63	1113	AGA CTT ATG TTC CAC GGG CC
	20			CAA GGC CAC GTG ATT CTT CC
COX-1	24	57	952	TGC CCA GCT CCT GGC CCG CCG CTT
	24			GTG CAT CAA CAC AGG CGC CTC TTC
COX-2	24	61	569	GGT CTG GTG CCT GGT CTG ATG ATG
	22			GTC CTT TCA AGG AGA ATG GTG C

Table 7.1A Cont'd.

Gene	Length (bases)	T_m (°C)	Size (bp)	Sequence
BAG	20	52	251	TCC AGC TGG TTA GCT ATC TT
	20			AGC AGT GAA CCA GTT GTC CA
BAXα	21	50	230	GAC GAA CTG GAC AGT AAC ATG
	20			AGG AAG TCC AAT GTC CAG CC
BAP	20	50	338	ACC AAG GAG TAC GAC CGC TT
	20			CCG GAC TAC AAC TAA CTC GT
MRIT	20	57	314	GAG TGC TGA TGG CAG AGA TT
	20			TTG AAG GAT CCT TGA GAC TC
Bcl-X_L	20	50	396	CAC AGC AGC AGT TTG GAT GC
	19			CTC GGC TGC TGC ATT GTT C
Bcl-X_S	20	50	207	CAC AGC AGC AGT TTG GAT GC
	19			CTC GGC TGC TGC ATT GTT C
Bcl-2_α	22	50	306	TCA TGT GTG TGG AGA GCG TCA A
	24			CTA CTG CTT TAG TGA ACC TTT TGC
Survivin	19	54	647	AGA ACA AAA TTG CAA AGG A
	21			ACA CAC AAG TCA TGC ATC TAC
eIF-2α	24	67	282	AGA AGG CGT ATC CGT TCT ATC AAC
	24			TCC AGG TCT CTT GTA CTT GTC ATC
eIF-4E	21	55	323	GAT CAG ATC GAT CTA AGA TGG
	21			CCA CAT AGG CTC AAT ACC ATC
c-*myc*	20	54	336	GAC GGC TTC GAG GAT CAG AT
	21			ACC ACA TGG TGA CCG AGA ACT
α-catenin	21	57	300	GTC ATT CAC GTA GTC ACC TCA
	23			TTC TGA CAT CAA AAT CCT CTG TC
β-catenin	20	55	668	AAG GTC TGA GGA GCA GCT TC
	20			TGG ACC ATA ACT GCA GCC TT
E-cadherin	18	55	653	AGC CAT GGG CCC TTG GAG
	20			CCA GAG GCT CTG TCA CCT TC
pHβ Rz exp.	19	50	118	AGC ACA GAG CCT CGC CTT T
	17			TCT GGA TCC CTC GAA GC

7.1.2 Primers used for transcription factor RT-PCR

Table 7.2A List of transcription factor RT-PCR primers and conditions

Gene	Length (bases)	T_m (°C)	Size	Sequence
GATA-2	21	63	395	CCC TAA GCA GCG CAG CAA GAC
	21			GAT GAG TGG TCG GTG CTG GCC
GATA-3	21	63	260	GTA CAG CTC CGG ACT CTT CCC
	21			CTG CTC TCC TGG CTG CAG ACA
GATA-1	18	-	-	ACA TCG GTC TTA AGA CCT
	18			TTA GCC ACC TCA TGC CTT
MZF1	20	-	-	TTG CGC ATG CGC TTC TGC TC
	21			GCC ACA TAC ATC GCA ACG GCC
Ik-2	24	-	-	TAT GGA TGC TGA CGA GGG TCA AGA
	24			ACA GGC ACG CCC ATT CTC TTC ATC
CdxA	20	-	-	ACA GCC GTT ACA TCA CAA TC
	20			GCT ATG GCA GAA ACT CCT CT
AML-1a	20	-	-	GTT GAG AGT CGA CTG GAA AG
	20			GAG GGA AAA GCT TCA CTC GA

7.1.3 Primers used for MRP1 Ribozyme *in vitro* cleavage (IVC) assay

The novel MRP1 primer sequences used to amplify that segment of the MRP1 gene (MRP1 RT-PCR) containing the ribozyme cleavage site are listed in Table 7.2A. The primer sequences used to amplify the MRP1 ribozyme DNA (Ribozyme 1 & 2) are also included in Table 7.2A.

Table 7.3A Primer sequences used for *in vitro* cleavage (IVC) assay

Primer	Length (bases)	T_m (°C)	Size (bp)	Sequence
MRP1	21	55	477	TGC TCA TCA AGT TCG TGA ATG
RT-PCR	21			CGT TCA TCA GCT TGA TCC GAT
Ribozyme 1	40	50	-	TAA TAC GAC TCA CTA TAG GGA GAG CCA CTC TGA TGA GTC C
Ribozyme 2	32	50	-	TGC TTC GTT TCG TCC TCA CGG ACT CAT CAG AG

7.1.4 Primers used to optimise mdr-1 RNase H assay

Table 7.4A Primer pair used in amplification of mdr-1 target DNA

Gene	Sequence	No. of bases
RNaseH1	TAA TAC GAC TCA CTA TAG GGC GAA GGA GCG CGA GGT CGG G	40
RNaseH2	TCC CCT TCA AGA TCC ATC CCG ACC TCG CGC	30

7.1.5 Phosphorothioate Antisense/Sense oligonucleotide sequences

This table shows the oligonucleotide sequences selected from the literature. They include the mdr-1 antisense used to optimise the RNaseH assay (Alahari *et al.*, 1996), as well as the MRP1 antisense and sense oligonucleotides (Stewart *et al.*, 1996; Canitrot *et al.*, 1996).

Table 7.5A Selected Antisense/Sense oligo sequences

Gene	Length (bases)	Sequence
Mdr-1 Antisense	20	CCA TCC CGA CCT CGC GCT CC
MRP1 Antisense	20	TGC TGT TCG TGC CCC CGC CG
MRP1 Sense	20	CGG CGG GGG CAC GAA CAG CA

7.1.6 Primers used in Second Generation chimera™ RNaseH assay

These primers were used to amplify individual DNA fragments which were then used as templates for production of the RNA target for the designed MRP1 antisense oligonucleotides. T_ms in all cases were 50°C and the sequences are listed in Table 7.6A.

Table 7.6A Primers used in RNaseH assay for MRP1 antisense oligos

MRP1 Antisense	Length (bases)	Sequence
*MA*1	35	TAA TAC GAC TCA CTA TAG GGC GAT GGG TGC CTT GT
	30	CGG AAC AAA AAT GGA GAC CCG GAC AAA GGG
*MA*3	35	TAA TAC GAC TCA CTA TAG GGC GAC AAC TTC TGG TG
	30	AGA CCA CCT AGT GTC CCA ACT AAC AGG CCC
*MA*5	35	TAA TAC GAC TCA CTA TAG GGC GAA GAA AAC ATC CT
	30	TGT AGG AAA AAC CTA CAG TCG ACC TCC TTG
*MA*8	35	TAA TAC GAC TCA CTA TAG GGC GAA TTC GAG CCT TC
	30	TCG GAA GCT CCT CGT CCT CCG GAA GTA GGT
*MA*9	35	TAA TAC GAC TCA CTA TAG GGC GAT CAG CTG GAG AG
	30	ACC TCT CTC CTC TCC CTC AAG TCA GAA GTC
*MA*10	35	TAA TAC GAC TCA CTA TAG GGC GAA GCC AGA AAA TC
	30	CTT TTA GGA GGT GCC AGC CCC TCT AAC AGT
*MA*12	35	TAA TAC GAC TCA CTA TAG GGC GAC AAG GTG CTG GC
	30	ACG ACC GGT AGT CCG TCC TCC TCG ACT TCC
*MA*13	35	TAA TAC GAC TCA CTA TAG GGC GAA GGA AGA CGA AG
	30	CGT CTT CTA GGA ACA CAA CCT ACT CCG GTG
*MA*14	35	TAA TAC GAC TCA CTA TAG GGC GAA AAG TTG TCC CT
	30	ACA GGG ACG AGA GTC GGG AGA ACC GAC TCT

7.2 Appendix B: Clinical study gene expression levels and clinical data

This appendix contains all the gene expression data for the clinical study group, as outlined in Section 3.6.1. It also contains the Clinical Data Questionnaire.

Table 7.1B: RT-PCR gene expression results for Primary Lung tumour tissue

Sample no.	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MDR1	BCRP	MDR3	COX-1	COX-2
1 T	LOW	LOW	LOW	LOW	0	HIGH	LOW	MED	0	MED	0
2 T	MED	0	LOW	MED	HIGH	LOW	HIGH	MED	0	0	MED
3 T	0	LOW	LOW	LOW	HIGH	LOW	LOW	HIGH	0	LOW	0
12 T	LOW	LOW	LOW	MED	LOW	HIGH	LOW	HIGH	0	0	0
16 T	MED+	0	LOW	LOW+	LOW	LOW	LOW	HIGH	0	HIGH	MED
16 N	**MED**	**LOW**	**MED**	**LOW**	**MED**	**LOW**	**MED**	LOW	**0**	**MED**	**MED+**
17 T	MED+	LOW	LOW	LOW	HIGH	LOW	LOW	0	0	0	LOW
17 N	**MED**	**LOW**	**0**	**LOW+**	**MED**	**LOW+**	**LOW**	LOW	**0**	**LOW**	**0**
18 T	MED	LOW+	MED	LOW	HIGH	LOW	0	MED	0	MED	MED
18 N	**LOW**	**LOW**	**LOW**	**0**	**MED**	**LOW+**	**MED**	MED	**0**	**LOW**	**MED+**
19 T	MED	LOW+	MED	LOW+	MED	LOW	LOW	HIGH	0	0	MED
19 N	**LOW**	**LOW**	**LOW**	**LOW**	**LOW**	**MED**	**0**	MED	**0**	**MED**	**LOW**
21 T	MED	LOW	LOW	0	LOW	MED	0	MED	0	0	HIGH+
21 N	**MED+**	**LOW**	**LOW+**	**HIGH**	**LOW**	**LOW**	**HIGH**	HIGH	**0**	**0**	**HIGH**
22 T	MED	LOW+	MED	MED	MED	LOW	MED	HIGH	0	0	LOW
22 N	**MED+**	**LOW**	**LOW**	**0**	**LOW**	**0**	**HIGH**	HIGH	**0**	**0**	**0**
23 T	MED	MED	LOW	HIGH	0	0	HIGH	0	0	0	MED
23 N	**MED**	**LOW**	**LOW**	**LOW**	**LOW**	**HIGH**	**LOW**	0	**0**	**0**	**LOW**
25 T	HIGH	LOW	LOW	0	HIGH	0	LOW	LOW	0	HIGH	HIGH
25 N	**MED**	**MED**	**HIGH**	**0**	**LOW**	**LOW**	**MED**	LOW+	**0**	**0**	**0**
B5 T	LOW	0	MED	0	LOW	LOW	LOW	0	0	0	0
B5 N	**MED**	**LOW**	**MED**	**0**	**LOW+**	**MED**	**LOW**	LOW	**0**	**0**	**0**
B6 T	0	0	LOW	0	MED	MED	MED	MED	0	0	0
B6 N	**MED**	**0**	**LOW**	**0**	**LOW**	**HIGH**	**LOW**	LOW	**0**	**0**	**0**

Table 7.1B: Cont'd.

Sample no.	BAP	BAX	MRIT	Bcl-xs	Bcl-xl
1 T	MED	MED	LOW	MED	HIGH
2 T	HIGH	HIGH	LOW	MED	MED
3 T	MED	HIGH	HIGH	HIGH	HIGH
12 T	HIGH	MED	MED	MED	HIGH
16 T	HIGH+	HIGH+	HIGH	MED	MED
16 N	**HIGH**	**HIGH**	**LOW**	**LOW**	**LOW**
17 T	HIGH+	MED	LOW	LOW	HIGH
17 N	**HIGH**	**HIGH**	**LOW+**	**MED**	**LOW**
18 T	MED	HIGH+	LOW	LOW	MED
18 N	**HIGH**	**HIGH**	**MED**	**HIGH**	**MED**
19 T	HIGH	HIGH+	LOW	HIGH	HIGH+
19 N	**MED**	**HIGH**	**MED**	**LOW**	**HIGH**
21 T	MED	HIGH	0	HIGH	HIGH
21 N	**MED+**	**HIGH+**	**LOW**	**LOW**	**MED**
22 T	MED	HIGH+	LOW	MED	MED
22 N	**MED+**	**MED**	**MED**	**0**	**LOW**
23 T	MED+	HIGH	LOW	MED	MED
23 N	**MED**	**MED**	**MED**	**MED**	**HIGH**
25 T	MED	HIGH	LOW+	HIGH	LOW
25 N	**LOW**	**LOW**	**LOW**	**MED**	**LOW+**
B5 T	MED	LOW	LOW	0	0
B5 N	**HIGH**	**MED**	**MED**	**0**	**0**
B6 T	MED	HIGH	LOW	0	0
B6 N	**MED**	**HIGH+**	**MED**	**0**	**0**

Bcl-2	BAG	Survivin	eIF 4E	eIF 2	c-myc
LOW	MED	LOW	MED	HIGH	0
LOW	HIGH	HIGH	HIGH	MED	LOW
HIGH	HIGH	HIGH	HIGH	MED	0
HIGH	MED	MED	HIGH	MED	LOW
MED+	HIGH	LOW	HIGH	LOW+	0
MED	**MED**	**LOW+**	**0**	**LOW**	**0**
LOW	MED	LOW+	0	LOW	0
LOW	**HIGH**	**LOW**	**0**	**MED**	**0**
LOW+	LOW	LOW	MED	MED+	0
LOW	**MED**	**0**	**MED+**	**MED**	**0**
MED	HIGH	0	LOW	HIGH+	0
LOW	**MED**	**LOW**	**HIGH**	**HIGH**	**0**
LOW	LOW	LOW+	LOW	MED	0
LOW	**LOW+**	**LOW**	**LOW+**	**LOW**	**LOW**
MED	MED	LOW	LOW	MED	LOW
LOW	**MED+**	**LOW**	**LOW**	**LOW**	**0**
LOW	MED	LOW	LOW	HIGH	LOW
LOW	**MED**	**LOW+**	**MED**	**HIGH+**	**LOW**
HIGH	LOW	LOW	MED	MED	LOW
MED	**0**	**0**	**HIGH**	**LOW**	**0**
LOW	LOW	LOW+	HIGH	LOW	LOW
MED	**HIGH**	**LOW**	**MED**	**MED**	**MED**
HIGH	LOW	LOW	LOW	MED+	MED
LOW	**MED**	**LOW+**	**MED**	**MED**	**MED+**

Table 7.2B: Gene expression results for Primary Breast tissue

Sample no.	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6
B1 T	LOW	LOW	MED	0	LOW	LOW
B2 T	LOW	LOW	MED	0	LOW	LOW

Table 7.2B: Cont'd.

Sample no.	BAP	BAX	MRIT	Bcl-xs	Bcl-xl	Bcl-2
B1 T	HIGH	MED	HIGH	0	0	MED
B2 T	HIGH	HIGH	MED	0	0	HIGH

MDR1	BCRP	MDR3	COX-1	COX-2
MED	MED	0	0	0
LOW	MED	0	0	0

BAG	Survivin	eIF 4E	eIF 2	c-myc
HIGH	HIGH	HIGH	HIGH	MED
HIGH	MED	HIGH	HIGH	HIGH

Table 7.3B: RT-PCR gene expression results for Oesophageal Primary tissue

Sample no.	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MDR1	BCRP	MDR3	COX-1	COX-2
9 O	LOW	HIGH	MED	LOW	LOW	LOW	HIGH	MED	0	LOW	0
13 O	LOW	LOW	LOW	LOW	LOW	LOW	MED	HIGH	0	0	0

Table 7.3B: Cont'd.

Sample no.	BAP	BAX	MRIT	Bcl-xs	Bcl-xl	Bcl-2	BAG	Survivin	eIF 4E	eiF 2	c-myc
9 O	HIGH	MED	LOW	HIGH	MED	LOW	MED	MED	MED	LOW	LOW
13 O	MED	MED	HIGH	HIGH	MED	MED	MED	LOW	MED	MED	LOW

Table 7.4B: RT-PCR gene expression results for Metastatic tissue

Sample no.	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MDR1
4 T	MED	LOW	LOW	MED	LOW	HIGH	HIGH
5 T	MED	LOW	LOW	LOW	MED	HIGH	LOW
6 T	LOW	LOW	LOW	LOW	MED	HIGH	LOW
7 T	LOW	LOW	LOW	LOW	LOW	MED	HIGH
8 T	MED	HIGH	HIGH	MED	LOW	LOW	MED
10 T	MED	LOW	MED	HIGH	HIGH	LOW	HIGH
B4 T	HIGH	0	MED	0	MED	LOW	LOW
14 LN	MED	LOW	LOW	HIGH	HIGH	0	HIGH
15 LN	LOW	LOW	LOW	LOW	LOW	0	MED
15 T	MED	LOW+	LOW	MED	MED	LOW	LOW
15 N	**LOW**	**LOW**	**MED**	**LOW**	**0**	**MED**	**0**
24 T	HIGH	MED	0	LOW	0	LOW	LOW
24 N	**0**	**MED**	**0**	**HIGH**	**0**	**MED**	**0**
27 T	MED	LOW	LOW	0	LOW	0	0
27 N	**0**	**LOW**	**MED**	**0**	**LOW**	**LOW**	**LOW**
B7 T	MED	LOW	LOW	0	LOW	LOW+	LOW
B7 N	**MED**	**0**	**HIGH**	**0**	**LOW**	**LOW**	**0**

BCRP	MDR3	COX-1	COX-2
MED	0	LOW	LOW
LOW	0	MED	MED
MED	0	LOW	LOW
0	0	MED	0
LOW	0	0	LOW
HIGH	0	HIGH	0
LOW	0	0	0
HIGH	0	LOW	0
0	0	LOW	LOW
0	0	MED	MED
LOW	**0**	**MED+**	**MED+**
0	0	MED	LOW
LOW	**0**	**0**	**MED**
0	0	LOW	0
0	**0**	**0**	**0**
LOW	0	0	0
MED	**0**	**0**	**0**

Table 7.4B: Cont'd.

Sample no.	BAP	BAX	MRIT	Bcl-xs	Bcl-xl
4 T	MED	HIGH	LOW	MED	MED
5 T	MED	HIGH	MED	MED	LOW
6 T	HIGH	MED	HIGH	LOW	HIGH
7 T	MED	LOW	LOW	MED	LOW
8 T	HIGH	MED	HIGH	LOW	MED
10 T	0	HIGH	MED	HIGH	HIGH
B4 T	HIGH	HIGH	LOW	0	0
14 LN	MED	HIGH	HIGH	HIGH	MED
15 LN	HIGH	LOW	LOW	MED	HIGH
15 T	MED	HIGH	LOW	HIGH	MED
15 N	**LOW**	**0**	**0**	**HIGH+**	**HIGH**
24 T	LOW	LOW	0	HIGH	0
24 N	**LOW**	**MED**	**LOW**	**0**	**LOW**
27 T	MED	HIGH	LOW+	0	LOW+
27 N	**LOW**	**0**	**LOW**	**0**	**LOW**
B7 T	HIGH	MED	MED	0	0
B7 N	**MED**	**MED**	**MED**	**0**	**0**

Bcl-2	BAG	Survivin	eIF 4E	eIF 2	c-myc
LOW	HIGH	LOW	LOW	MED	LOW
MED	MED	LOW	MED	HIGH	LOW
MED	LOW	MED	HIGH	MED	0
HIGH	LOW	LOW	HIGH	LOW	0
LOW	HIGH	LOW	HIGH	LOW	LOW
HIGH	MED	HIGH	HIGH	MED	HIGH
MED	MED	LOW	LOW	MED	MED
HIGH	MED	HIGH	MED	LOW	0
MED	LOW	LOW	HIGH	LOW	0
HIGH	MED	HIGH	LOW	LOW+	0
0	**LOW**	**LOW**	**LOW**	**LOW**	**0**
0	MED	0	0	0	0
MED	**LOW**	**0**	**0**	**LOW**	**0**
MED	LOW	LOW	0	LOW	LOW
0	**0**	**LOW+**	**MED**	**0**	**0**
LOW	MED	LOW	0	MED	MED
LOW+	**MED+**	**0**	**LOW**	**MED**	**LOW**

Table 7.5B: RT-PCR gene expression results for non-carcinoma tissue

Sample no.	MRP1	MRP2	MRP3	MRP4	MRP5	MRP6	MDR1	BCRP	MDR3	COX-1	COX-2
11 E	LOW	LOW	0	0	LOW	MED	LOW	0	0	0	LOW
20 T	0	LOW	LOW	0	0	LOW	0	LOW	0	HIGH	LOW
B3 D	MED	LOW	MED	0	LOW	LOW	MED	MED	0	0	0
26 T	LOW	LOW	MED	0	0	LOW	0	0	0	0	0
26 N	**MED**	**LOW**	**LOW**	**0**	**LOW**	**0**	**LOW**	**0**	**0**	**0**	**LOW**

Table 7.5B: Cont'd.

Sample no.	BAP	BAX	MRIT	Bcl-xs	Bcl-xl	Bcl-2	BAG	Survivin	eIF 4E	eIF 2	c-myc
11 E	MED	LOW	LOW	LOW	MED	MED	LOW	0	HIGH	LOW	0
20 T	HIGH	HIGH	LOW	HIGH	HIGH	LOW	MED	HIGH	LOW	MED	0
B3 D	HIGH	HIGH	HIGH	0	0	MED	HIGH	MED	HIGH	HIGH	HIGH
26 T	LOW	LOW	0	HIGH	LOW	0	0	0	LOW	LOW	0
26 N	**MED**	**HIGH**	**HIGH**	**LOW**	**HIGH**	**LOW**	**MED**	**LOW**	**MED**	**LOW+**	**LOW**

Table 7.6B: Clinical data for Primary Lung tumour tissue

Sample nc	C. Type	Radiation	LN Status	Different	Recurrenc	Add. Info
1 T						
2 T						
3 T						SCLC
12 T	Carb, vp16					SCLC
16 T/N			Neg.	Moderate		Colonic
17 T/N		No	N0	Poor	Nil	
18 T/N				Moderate		
19 T/N		No	N1	Well	Nil	
21 T/N			0/4	Moderate		Bladder
22 T/N		No	N0		Nil	Prostrate
23 T/N		No	12-Feb		Nil	
25 T/N		No	Three/12	Moderate		
B5 T/N			N2	Moderate		M.SCLC
B6 T/N				Moderate		NSCLC

Description
Left upper lobe mass. Bloodstained smears present
Poorly differentiated squamous bronchogenic carcinoma
Left lung, upper lobectomy
Small-cell undifferentiated carcinoma; extensive necrosis
Moderately differentiated adenocarcinoma

Right upper lobectomy, difficult dissection

Moderately differentiated adenocarcinoma with necrosis

Left lower lobectomy, benign carcinoid

Moderately differentiated adenocarcinoma

Papillary adenoma of type II pneumocytes

Carcinoid tumour, multiple adhesions

Non-small cell tumour, right pneumonectomy

Invasive moderately differentiated suamous cell carcinoma

High-grade malignant neoplasm

Table 7.6B: Cont'd.

Sample no.	Spec. Type	Gender	Age	Size (cm)	Metastasis	Primary
1 T	Lung	M	70	2.2		P
2 T	Lung	F	62	7.5	M0	P
3 T	Lung	F	47	2.5		P
12 T	Lung	M	48	0.3		P
16 T/N	Lung	M	56	2.5	M0	P
17 T/N	Lung	M	61	3	M0	P
18 T/N	Lung	M	67	1.2	M0	P
19 T/N	Lung	F	41	3.5	M0	P
21 T/N	Lung	F	54	2.5		P
22 T/N	Lung	M	71	3.5	M0	P
23 T/N	Lung	F	54	5		P
25 T/N	Lung	M	65	1.8	M1	P
B5 T/N	Lung	F	53	4		P
B6 T/N	Lung	F	71	13		P

Smoker	Menopaus	Last seen	Stage	Chemo
Yes				
Yes		RIP		Yes
Yes				
		RIP 28/4/9	T3	No
	Pre	12/12/00		No
Yes			T2	
		22/12/00		No
		28/11/00		No
		RIP 7/4/00	T4	No
		08/05/99	T2	
		07/04/99		

Table 7.7B: Clinical data for Primary Breast tissue

Sample no.	Age (yrs)	Size (cm)	Metastasis	Menop	Last seen	Stage	Endocrine	LN Status	Different	Recurrence	Oestrogen	Progest.
B1 T	52	0.5	M0		03/11/99			Pos	Poor	Nil	Pos	
B2 T	76	2.2	M0	Post	May-99	T2	Yes-Tamox	N0		Nil	Pos	Pos

Table 7.7B: Cont'd.

Sample no.	Description
B1 T	Left masectomy for breast, bronchial tumour visible, nodes positive, 90% staining show strong positive for oestrogen
B2 T	Modified radical masectomy left breast

Table 7.8B: Clinical data for Oesophageal Primary tissue

Sample nc	Spec. Typ	Gender	Age	Size (cm)	Different	Description
9 O	Oesoph	M	79	3	Poor	Multiple fragments of oesophageal adenocarcinoma
13 O	Oesoph	M	74	0.8	Poor	Poorly differentiated adenocarcinoma

Table 7.9B: Clinical data for Metastatic tissue

Sample no.	Spec. Type	Gender	Age	Size (cm)	Metastasis	Primary
4 T	Lung	M	54	1.8	Multiple	Unknown
5 T	Lung	F	79			
6 T	Lung	F	69		M0	
7 T	Lung	M	71			
8 T	Lung	M	82			
10 T	Breast	F	44	1.8	soft tissue sarcoma	
B4 T	Breast	F	64	5.5	M0	
14 LN						
15 LN/T/N	LN	M	80	3.5	M0	
24 T/N						
27 T/N	Lung	M	59	5		
B7 T/N	Breast	F	45			

Smoker	Menopausal	Last seen	Stage	Chemo
	Post	18/2/01	T3/T4	Yes
				Yes
		06/06/00	T1	Yes
	Post	28/02/00	T3	No
		RIP 26/2/0	T2	No
		06/04/99		

Table 7.9B: Cont'd.

Sample no.	C. Type	Radiation	LN Status	Different	Oestrogen	Recurrence	Add. Info	Description
4 T								Multiple metastatic lung disease, unknown primary
5 T								
6 T	Carb, VP16	Yes	N1	Undiff.		Nil		Small cell carcinoma with neuroendocrine features
7 T								Unknown tissue, previous reactive anthracotic LNs
8 T	Dexam						History	Secondry lung carcinoma
10 T	CMF, Tamox	Yes	0/29		Pos.		No history	Nodal deposit of ductal carcinoma
B4 T	tamoxifen	Yes	N1		Pos.+Prog.	Nil		Modified radical masectomy left breast
14 LN								Mediastenial lymph node
15 LN/T/N		No		Moderate		13/2/01		Mediastinoscopy of squamous cell carcinoma
24 T/N								
27 T/N								Left lower lobe tumour, left pneumonectomy
B7 T/N								

Table 7.10B: Clinical data results for non-carcinoma tissue

Sample nc	Spec. Typ	Gender	Age (yrs)	Size (cm)	Smoker	Last seen	Description
11 E	ESD	M	47	1.5	Yes		Empyema strip decortication, no evidence of neoplasm
20 T	Lung?	F	53	1.5	Yes	21/12/00	Bronchial resection margin
B3 D	Duodenum	F	47	0.4		15/04/99	Duodenal biopsy
26 T/N	Lung?	M	77	5			Lobe of lung, no evidence of malignancy

CLINICAL SURVEY SAMPLE SHEET DATE: _____

Hospital Patient No.: _____ Male ☐ Female ☐

1. DOB of Patient: _____
2. Date of diagnosis: _____
3. Date of operation: _____
4. Menopausal status of patient: _____
5. Last seen alive: _____
6. Tumour size: _____
7. Operation details:
8. Diagnosis:
9. Histological grade of tumour: _____
10. Metastatic grade of tumour: _____
11. Tumour stage: _____
12. Pre-operation Chemotherapy:
13. Post-operation Chemotherapy:
14. Other therapy (e.g. Radiation)
15. Oestrogen Receptor: POS. ☐ NEG. ☐
16. Progesterone Receptor: POS. ☐ NEG. ☐
17. Lymph Node status: _____
18. Endocrine therapy:
19. Differentiation grade of tumour: _____
20. Adjuvant treatment:
21. Recurrence:
22. Additional information: