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MULTIFACTORIAL RESISTANCE TO DOXORUBICIN IN HUMAN MULTIPLE MYELOMA RPMI 8226

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree of Philosophy in the Graduate
School of The Ohio State University

By
Tasneem Z. Vasowalla, M.S.

The Ohio State University
2001

Dissertation Committee: Approved by
Dr. Robert M. Snapka, Adviser
Dr. Michael C. Ostrowski, Co-Adviser
Dr. Gary D. Stoner
Dr. Altaf A. Wani

Robert M. Snapka
Adviser

Michael C. Ostrowski
Co-adviser

Molecular, Cellular, and Developmental Biology Graduate Program

Molecular, Cellular, and Developmental Biology Graduate Program
ABSTRACT

Multiple Myeloma (MM) is an incurable B-cell neoplasm characterized by bone marrow infiltration with malignant plasma cells, which synthesize and secrete monoclonal immunoglobulin (Ig) fragments. The cell line used in this study (Dox IV) is a doxorubicin resistant variant of human multiple myeloma RPMI 8226. Multi-drug resistance (MDR) is the most common and well studied cause of resistance to antitumor agents. It results from overexpression of the mdrl gene which codes for a transmembrane protein called P-glycoprotein (P-gp). In addition to P-gp, another protein called multi-drug resistance protein (MRP) has been identified to be responsible for MDR. However, the use of verapamil, a competitive inhibitor of P-gp and MRP, in the selection of these drug resistant cells results in a low level of doxorubicin resistance in the absence of the classical-MDR phenotype.

In vitro selection of drug-resistant cells usually results in a multimodal drug resistant phenotype. Historically, drug resistant cell lines are characterized after prolonged exposure to increasing concentrations of drug. Because of the narrow range of efficacy for cytotoxic drugs, a 2-fold increase in drug resistance could theoretically be sufficient
to develop clinical drug resistance. Thus, it is critical to identify mechanisms that confer low levels of drug resistance.

Doxorubicin belongs to the anthracycline class of anti-tumor agents which are DNA topoisomerase II inhibitors. Topoisomerase II is an essential nuclear enzyme which changes the topology of DNA by passing an intact helix through a transient double-stranded break made in a second helix followed by religation of the DNA break. Since they are usually expressed at higher levels in tumor cells than in normal cells they make attractive targets for anti-tumor agents. Topoisomerase II inhibitors can stabilize the so-called cleavable complex i.e. the transient intermediate of double-strand nicked DNA, covalently bound to the enzyme. Stabilization of this complex is either sufficient to inhibit cell proliferation or is perceived as a lethal signal for cells which enter apoptosis as a response to this aggression.

Earlier characterization of Dox IV cells for their cause of drug resistance had identified a reduction in topoisomerase II activity and expression in nuclear extracts of these cells compared to the parent 8226 cells. This reduced expression was found in the absence of mutations in the topoisomerase II gene and promoter. Further, it was found that NF-Y, a ubiquitous transcription factor which binds to CCAAT boxes present in the topoisomerase IIα promoter was reduced in nuclear extracts of the drug resistant Dox IV cells. Thus, it was suggested that NF-Y could be responsible for regulating topoisomerase IIα expression in the context of drug resistance.
In Chapter 2, I cloned the three NF-Y subunits in order to transfet them in the Dox IV cells to check whether ectopic expression of NF-Y increased sensitivity of Dox IV cells to doxorubicin. In the process of cloning, I identified two novel isoforms of NF-YA in addition to two which were already defined. Since there are multiple isoforms of NF-YA, I studied the relative distribution of these isoforms in the Dox IV and RPMI 8226 cells to check if changes in abundance of isoforms could be responsible for reduced NF-Y activity in Dox IV cells. The results obtained do not indicate any difference in abundance of the different isoforms. In addition, I maintained a Dox IV cell line in the absence of doxorubicin and verapamil (Dox IV-). Cytotoxicity assay showed that resistance was stable in the Dox IV cells, even in the absence of doxorubicin and verapamil.

In Chapter 3, I initially determined whether topoisomerase activity was reduced in nuclear extracts of Dox IV- cells as in the Dox IV+ cells by the decatenation assay. Since there was a decrease in topoisomerase II activity I went on to determine which of the three NF-Y subunits was reduced to result in reduced NF-Y activity. Since there was not much difference in protein levels of the three subunits, I studied the NF-Y binding activity in nuclear extracts of the parent and drug resistant cell lines. The activity of NF-Y in the nuclear extracts of Dox IV- cells was comparable to that in the parent RPMI 8226. The presence of doxorubicin could therefore be responsible for the reduced NF-Y activity rather than NF-Y being the cause of drug resistance. Thus, there may be other
transcription factors responsible for regulation of topoisomerase IIα transcription in Dox IV.

In Chapter 4, I considered other transcription factors that have binding sites in the topoisomerase IIα promoter. Of these myb and Spl were studied by electrophoretic mobility shift assays. A reduction in binding activity at the myb site was observed in nuclear extracts of both Dox IV+ and Dox IV-. However, due to the extreme instability of the binding factor it was difficult to perform supershift assays to confirm the identity of the factor and also to study the protein levels by Western blot. Binding at the Spl site was also reduced equally in both Dox IV+ and Dox IV- cells compared to the parent RPMI 8226. This reduction in binding activity corresponded to a similar reduction in Spl protein as studied by Western blotting. Results suggest that Spl and myb may be reduced in Dox IV cells and thus may be involved in lower levels of topoisomerase IIα. I also studied the effects of methylation and acetylation on transcription in the three cell lines.

A topoisomerase II poisoning assay was performed (Chapter 5) to determine whether the reduced topoisomerase activity observed in the nuclear extracts was the actual reduction in active topoisomerase II in the cells or a difference in extractability. This assay measures the level of active topoisomerase II and results indicated that there was only about 5-9% reduction in active topoisomerase II in the Dox IV cells compared to the parental 8226 cells. This decrease can account for the low level of resistance in these cells.
Since drug-resistance could be multifactorial, in Chapter 6 I studied global changes in proteins between the drug resistant and parent cells by 2-D electrophoresis. Eight protein spots were identified that were significantly altered. Four of these were increased or expressed while the other four were decreased in Dox 1V cells. MALDI-MS analysis of these spots identified nine proteins. Of these, aldose reductase, protein disulfide isomerase and Rho-GDI have already been implicated in drug resistance. Others, hnRNP A2/B1, UDP-N-acetylhexosamine pyrophosphorylase and human homolog of RAD23 (hHR23) can be implicated in drug resistance. hnRNP H is a pre-mRNA processing factor and does not seem to have any identifiable role in drug resistance. Two remaining proteins have no known functions and therefore cannot be analyzed for their contribution to drug resistance.
Dedicated to my mother

who lost her fight against cancer
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VITA

December 29, 1972..............Born – Bombay, India

1994......................................B.S. Microbiology/Biochemistry, University of Bombay

1996......................................M.S. Biochemistry, University of Bombay

1996 – present......................Graduate Teaching and Research Associate,
                                 The Ohio State University

FIELDS OF STUDY

Major Field: Molecular, Cellular, and Developmental Biology
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Multiple Myeloma

Multiple myeloma (MM) is a tumor of immature, isotype switched, immunoglobulin secreting plasma cells derived from B-lineage clonogenic cells. (Kastrinakis, et al., 2000). MM presently accounts for almost 10% of all hematologic malignancies and approximately 1% of cancer-related deaths in Western countries (Carli, et al., 1998; Kastrinakis, et al., 2000). The frequency and extent of karyotypic abnormalities correlates with the stage, prognosis, and response to therapy (Hallek, et al., 1998). The characteristic numerical abnormalities are monosomies of chromosomes 1 and 13, trisomies of chromosomes 3, 5, 7, 9, 11, 15, and 19, lp deletions, 1q duplications, rearrangements in chromosomes 2, 6, 8 and 11 (Ferti, et al., 1984; Hallek, et al., 1998; Kastrinakis, et al., 2000; Lai, et al., 1995; Mitelman, 1994).

The major pathophysiological consequences are diffuse osteoporosis and numerous lytic bone lesions that result in loss of height, bone pain and pathologic fractures. On examination of the bone marrow patients have >30% plasma cells and they usually have
an M-protein in the serum or urine. Frequently they will have anemia, hypercalcemia, recurrent bacterial infections, and renal insufficiency (Kastrinakis, et al., 2000). Asymptomatic patients presenting with a small monoclonal M-spike without bone lesions and with less than 10% plasma cells in the bone marrow have a very good prognosis. By definition they have Monoclonal Gammopathy of Undetermined Significance (MGUS). Most such patients never have any symptoms related to their M-protein; approximately, 1% per year progress to develop myeloma.

The most characteristic feature of MM is the presence on protein electrophoresis of an M-protein, also called M-spike (the ‘M’ stands for monoclonal, not for IgM) that is the immunoglobulin secreted by the MM cells. In 57% of patients it is IgG, in 21% an IgA, in 2% an IgD, only extremely rarely (>1%) IgM or IgE, in 18% only a light chain is secreted (called a Bence-Jones protein, which because of its low molecular weight is excreted in the urine), and in 2% no immunoglobulin is secreted.

While none of the treatment protocols currently available against MM can be characterized as curative, disease prognosis has clearly improved over the last decade. High-dose chemotherapy followed by autologous hemopoietic stem-cell transplantation has proven significantly more effective than conventional chemotherapy, particularly in younger patients (Attal, et al., 1996; Kastrinakis, et al., 2000).
Topoisomerase targeting drugs and Doxorubicin

DNA topoisomerases are nuclear enzymes which make transient DNA strand breaks allowing the cell to manipulate the topology of its DNA. DNA topoisomerase II is a ubiquitous enzyme that is essential for the survival of all eukaryotic organisms and plays critical roles in virtually every aspect of DNA metabolism: replication, transcription, chromosomal segregation and DNA recombination (Burden, et al., 1998; Froelich-Ammon, 1995; Hande, 1998; Liu, et al., 1994). The enzyme catalyzes interconversions between DNA topological isomers in an ATP-dependent fashion through transient cleavage, strand passing, and religation of double-stranded DNA, resulting in relaxation of supercoiled DNA, catenation, decatenation, knotting or unknotting (Fig 1.1) (Larsen, et al., 1996; Nitiss, et al., 1996).

In mammalian cells, there are two isozymes of topoisomerase II, designated α and β. Topoisomerase IIα and β share extensive amino acid sequence identity (~70%), but are encoded by separate genes and can be distinguished by their protomer molecular masses (~170 kDa and ~180 kDa respectively) (Austin, et al., 1993; Beck, et al., 1994; Burden, et al., 1998; Nitiss, et al., 1996). These isoforms are expressed differently through the cell cycle: topoisomerase IIα is preferentially expressed in proliferating cells during S phase, whereas topoisomerase IIβ appears to be expressed at all points in the cell cycle, with no appreciable differences between proliferating and non-proliferating cells (Hwang, et al., 1994; Nitiss, et al., 1996; Woessner, et al., 1991).
Cancer cells often have a higher level of topoisomerase II protein and topoisomerase II activity as compared to normal cells, during all stages of the cell cycle. (Larsen, et al., 1996; Larsen, et al., 1998; Nelson, et al., 1987). Thus, topoisomerase II is a good target for chemotherapeutic agents. In addition, the topoisomerase II content in cancer and transformed cells is less regulated by growth conditions which might explain why the sensitivity of different tumors to topoisomerase II inhibitors is usually correlated with topoisomerase II protein expression and activity, but not necessarily with the cell doubling time (Larsen, et al., 1996; Larsen, et al., 1998).

Topoisomerase II inhibitors do not kill cells by blocking topoisomerase catalytic function. Rather they poison these enzymes by increasing the steady-state concentration of their covalent DNA cleavage complexes. This action converts topoisomerases into physiological toxins that introduce high levels of transient protein-associated breaks in the genome of treated cells (Burden, et al., 1998; Froelich-Ammon, 1995; Hande, 1998; Liu, et al., 1992; Liu, et al., 1994).

The potential lethality of these drug-induced cleavage complexes rises dramatically when replication machinery or helicases attempt to traverse the covalently bound topoisomerase roadblock in the DNA. This disrupts the cleavage complex and converts transient single- or double-stranded breaks into permanent double-stranded fractures which are no longer held together by proteinaceous bridges. These breaks become targets for recombination, sister chromatid exchange, the generation of large insertions and
deletions and the production of chromosomal aberrations and translocation. When these permanent DNA breaks are present at sufficient concentration, they trigger a series of events that ultimately culminates in cell death by apoptosis (Burden, et al., 1998; D'Arpa, et al., 1990; Ferguson, et al., 1994; Hande, 1998; Holm, et al., 1989; Howard, et al., 1994).

Topoisomerase II has been recognized as the target of a variety of anticancer drugs belonging to different drug classes: anthracyclines (doxorubicin, daunorubicin and analogues), epipodophyllotoxins (etoposide, teniposide), amino-acridines (amsacrine), ellipticines (ellipticinium), anthracene-diones (mitoxantrone) and several others having not yet been introduced in clinical use (Corbett, et al., 1993a; Liu, 1989; Liu, et al., 1994; Robert, et al., 1998; Wang, 1994).

Anthracycline antibiotics, originally isolated from fermentation products of *Streptomyces peucetus*, were found to have antineoplastic activity over 4 decades ago, long before the identification of topoisomerase enzymes (Hande, 1998). In addition to topoisomerase inhibition, anthracyclines produce a wide range of biological reactions like intercalation into DNA with consequent inhibition of macromolecular biosynthesis (Fritzsche, et al., 1987; Gewirtz, 1999; Hande, 1998; Kım, et al., 1972; Zunino, et al., 1972), interference with DNA unwinding or DNA strand separation and helicase activity (Bachur, et al., 1992; Fornari, et al., 1994; Gewirtz, 1999; Hande, 1998; Tuteja, et al., 1997), damaging macromolecules and lipid membranes via formation of free radicals (Gewirtz, 1999;...
Doxorubicin (Adriamycin®) is the most commonly used anthracycline. It has antineoplastic activity when used as therapy for a wide variety of cancers like lymphomas, myelomas, breast cancer, sarcomas, leukemias, etc. (Hande, 1998; Gewirtz, 1999).

**Drug Resistance**

Resistance to cytotoxic chemotherapy is a major problem in the treatment of patients with multiple myeloma. The patients often respond to cytotoxic therapy initially, but later relapse in spite of treatment (Wyler, et al., 1997).

Natural and acquired resistance to antitumor agents may be based on the same molecular mechanisms and are usually multifactorial (Larsen, et al., 1998; Prost, 1995). The different mechanisms may be divided into three major categories: (1) pre-target events, including drug uptake, metabolism and intracellular distribution; (2) drug-target interactions; and (3) post-target events, which include macromolecular synthesis, DNA repair, cell cycle progression and mechanisms involved in the regulation of cell death (Larsen, et al., 1998).
Pre-target events

The best described mechanism of resistance to anticancer drugs is reduced drug accumulation and/or an altered intracellular drug distribution (Larsen, et al., 1998; Prost, 1995). Drug transport is mediated by a transmembrane protein, Pgp 170, encoded by the MDR1 gene, which shows increased expression/activity in resistant cell lines and primary tumors. This protein acts as an energy-dependent pump mediating drug efflux. Resistance involving the expression of this 170-kD membrane protein is called the “typical multidrug resistance” (MDR) phenotype. Resistance to topoisomerase inhibitors does not always involve this transporter. Recently, other membrane proteins such as the multidrug resistance-associated protein (MRP) have been shown to be able to promote an active efflux of compounds and can also confer resistance to doxorubicin or etoposide (Prost, 1995).

An altered intracellular drug distribution with drug sequestration in cytoplasmic organelles has been described for several resistant cell lines in vitro as well as in patients (Larsen, et al., 1998).

Another drug-resistance mechanism is through the cellular detoxification system associated with the overexpression of proteins such as glutathione conjugating enzymes. These enzymes catalyze the conjugation of cytotoxic compounds with reduced glutathione followed by drug efflux mediated by the putative ATP-dependent glutathione-S-conjugate export pump. Quantitative and qualitative changes in the pattern
of glutathione-dependent enzymes may thus be important in the resistance phenotype (Larsen, et al., 1998; Prost, 1995).

**Drug-target interactions**

Though cleavable complexes are only potentially lethal lesions and seem not to be sufficient for cell killing, the major decisive determinants of cell sensitivity to topoisomerase II-interfering drugs is the number of cleavable complexes that are formed and stabilized in the presence of the drug. Therefore, a high expression of topoisomerase II will correspond to a high sensitivity of the cells to the drug. The level of DNA damage exerted by topoisomerase II inhibitors depends both on the amount of drug available and on the amount of enzyme present. As a consequence, decrease in topoisomerase II expression or capacity to interact with anticancer drugs can be responsible for the resistance to these drugs (Prost, 1995; Robert, et al., 1998).

Previous studies with adriamycin-resistant cells and with cell lines resistant to topoisomerase II inhibitors in general have suggested that cellular resistance is correlated with qualitative and quantitative changes in topoisomerase II enzyme activity (Prost, 1995; Son, et al., 1998). These changes will be discussed later (see below).

**Post-target interactions**

It is proposed that the collision between the replication or the transcription machinery is necessary to convert the stabilized double-stranded breaks into permanent breaks for cell
death. Thus, slow growing cell populations with a low S-phase fraction or prolonged G1 phase tend to be resistant to topoisomerase II poisons (Larsen, et al., 1998).

Expression of certain oncogenes commonly associated with human tumors can modulate cell cycle check points. Cells with strong cell cycle check points have the opportunity to repair the damage caused upon exposure to genotoxic agents and hence become resistant to the drugs (Larsen, et al., 1998).

Since topoisomerase poisons have been shown to induce apoptotic cell death, the ability of tumor cells to undergo apoptosis following treatment with anticancer drugs would be a major factor in the ultimate success of the treatment (Prost, 1995). The mode of cell death is probably an important determinant of the final outcome. It appears that the different cell death processes, apoptosis, mitotic cell death and necrosis, are not equally efficient in cell killing and therefore associated with different risk for re-growth of surviving cells (Larsen, et al., 1998).

Though the basis for drug resistance in many cases is multifactorial, there is a significant role for altered topoisomerase IIα gene activity and expression which is shown to occur in the absence of other alterations in at least some resistant isolates (Isaacs, et al., 1998). Thus, these alterations will be discussed in greater detail.
Regulation of topoisomerase IIα gene expression & activity in drug resistance

In tumor cells selected for resistance to topoisomerase II-targeted drugs, the most common mechanism of drug resistance involves reduced formation of cleavable complexes due to the decreased expression or activities of topoisomerase IIα or topoisomerase IIβ (Evans, et al., 1994; Mirski, et al., 1993; Son, et al., 1998). While abundant evidence is available to link the topoisomerase IIα isoform to sensitivity of topoisomerase II drugs, only a few studies showed reduced expression of topoisomerase IIβ in cell lines selected for resistance to topoisomerase II inhibitors (Dingemans, et al., 1998; Markovits, et al., 1995). Thus, the exact role of the two topoisomerase II isoforms in drug sensitivity to topoisomerase II inhibitors is still not settled.

Previous studies with adriamycin-resistant cells have suggested that cellular resistance to adriamycin is correlated with quantitative and qualitative changes in topoisomerase II enzyme activity (De Jong, et al., 1990; McPherson, et al., 1993; Son, et al., 1998). Resistance to adriamycin in MKN/ADR cells was found to be associated with altered expression of the topoisomerase IIα isozyme form (Son, et al., 1998). However, resistance to adriamycin might well be due to topoisomerase IIβ.

Activity/qualitative changes

Qualitative changes in topoisomerase II responsible for resistance could result from mutations or post-translational modifications. These alterations lead to production of
qualitatively altered protein with modified activity or ability to react with the inhibitors (Prost, 1995). Several mutations have been reported in resistant cell lines in which the amount of topoisomerase II protein was unchanged but the biochemical properties of the enzymes were altered. These mutations appear to cluster in two “hotspot” regions of the enzyme: 1) the nucleotide-binding domain (aa 420-495); and 2) the domain including the tyrosine (804) involved in the covalent binding of topoisomerase II to the nicked DNA (aa 740-810). One characteristic feature of several topoisomerase II alterations leading to drug resistance is the increase of ATP requirement of the resistant cells (Robert, et al., 1998; Prost, 1995). One of the various mutations identified is a single mutation in the codon 493 (nucleotide binding domain) in the topoisomerase IIα gene of VpmR-5 cell line (established from a CHO cell line by culturing in the presence of teniposide) in which resistance was due solely to a qualitative alteration of the enzyme (Chan, et al., 1993). Other changes have been reviewed by Prost (1995) (Fig., 1.2).

Post-translational modifications of the protein can also permit modification of topoisomerase II activity and of the reaction with the poisons (Prost, 1995). Topoisomerase II can be modified by poly-ADP ribosylation and phosphorylation. Poly-ADP ribosylation has been shown to inhibit the catalytic activity of topoisomerase II in vitro and in vivo (Darby, et al., 1985; Larsen, et al., 1996; Scavassi, et al., 1993). Phosphorylation of purified topoisomerase II stimulates its ATPase activity, and thereby its catalytic functions (Corbett, et al., 1992; Corbett, et al., 1993; Larsen, et al., 1996). Topoisomerase II enzyme has been shown to be phosphorylated on multiple serine and
threonine residues. This post-translational modification is mediated by at least three different protein kinases: casein kinase II, protein kinase C and a proline-directed kinase p34^cd^2 (Isaacs, et al., 1998; Larsen, et al., 1996). A decrease in the phosphorylation of topoisomerase IIα has been associated with drug resistance in a number of mammalian cell lines, including HL60 leukemic cell lines and K562 cells (Ganapathi, et al., 1996; Isaacs, et al., 1998; Ritke, et al., 1995). On the contrary increased phosphorylation of topoisomerase II has been associated with drug resistance in a human KB cell line (Isaacs, et al., 1998; Takano, et al., 1991). Thus, it is not yet clear whether phosphorylation contributes directly to drug resistance in vivo. It is possible that hyper- and hypo-phosphorylation are both associated with resistance to topoisomerase II poisons depending on the specific sites that are phosphorylated. Alternatively, alterations in phosphorylation seen in drug resistant cell lines could reflect either indirect effects, such as through altered cell cycle parameters, or the necessity of maintaining the enzymatic activity of this essential enzyme in spite of mutations giving drug resistance (Isaacs, et al., 1998).

A decrease of topoisomerase protein may also explain the occurrence of low level resistance towards topoisomerase inhibitors. However, higher levels of resistance are usually associated with several additional modifications of the target enzyme. Regulation of topoisomerase II gene expression is complex, with positive and negative controls in evidence at the level of both promoter activity and mRNA stability (Isaacs, et al., 1998).
Expression/quantitative changes

Two basic factors play key roles in regulating gene expression: (a) changes in cis elements of the promoter region, such as mutation, altered methylation or histone acetylation (Mo, et al., 1997). (b) changes in levels of trans-acting factors (either transactivators or repressors) (Mo, et al., 1997; Nitiss, et al., 1996). Another means of regulating the level of a transcript is to modulate the stability of the mRNA in the cytoplasm. Changes in the stability of particular mRNAs are often accompanied by a parallel alteration in the transcription rate of the corresponding gene (Isaacs, et al., 1998).

The topoisomerase II gene could also be down-regulated by either altered binding or by altered regulation of trans-acting factors such as NF-Y, Sp1 and myb (Takano, et al., 1999). Decreased topoisomerase II expression has been shown in the doxorubicin resistant derivatives of human multiple myeloma RPMI 8226 cells (Dox IV) (Wang, et al., 1997). Since a correlation with drug resistance and down-regulation of topoisomerase IIα was observed, topoisomerase IIβ levels were not determined in the study by Wang, et al. (1997). It is thus important to identify the mechanism of topoisomerase IIα down-regulation in the Dox IV cells in order to understand the basis of resistance. Once the mechanism of topoisomerase II down-regulation responsible for drug resistant phenotype of Dox IV cells is established, a possible method of modulating it can be developed to sensitize the cells to doxorubicin treatment. Studying the mechanisms of doxorubicin resistance in the Dox IV cell line is the subject of this thesis.
Regulation of transcription

a. Cis-regulatory elements

Modifications of both DNA and chromatin can affect gene expression and lead to gene silencing. Large-scale silencing of genes is mediated by the packing of DNA in highly condensed heterochromatin structures and DNA methylation at cytosines in defined guanine-cytosine (CG)-sequences (Beyersmann, 2000).

Dynamic changes in chromatin structure are directly influenced by post-translational modifications of the amino-terminal tails of the histones (Luger, et al., 1998; Rice, et al., 2001; Wolffe, et al., 1999). Specific amino acids within these histone tails are targets for a number of post-translational modifications, including acetylation, phosphorylation, poly(ADP-ribosylation), ubiquitination and methylation (Rice, et al., 2001; Spencer, et al., 1999; Wu, et al., 2000). These covalent modifications may alter the interaction of the histone tail with DNA and thus regulate gene transcription.

Alterations in core histone acetylation status has a causal role in determining transcriptional activity in chromatin. Each core histone has two domains: a histone fold domain, which is involved in histone-histone interactions and in wrapping DNA in nucleosomes, and an amino-terminal tail domain that lies on the outside of the nucleosome, where it can interact with other regulatory proteins and with DNA (Jones, et al., 1999; Pruss, et al., 1995; Wolffe, 1998). The amino-terminal tail domains are lysine rich and are targets for acetylation. Acetylation of histones decreases their overall
positive charge and greatly reduces their affinity for the negatively charged DNA. This facilitates the binding of transcription regulatory proteins to the chromatin templates (Lee, et al., 1993; Rice, et al., 2001; Vettese-Dadey, et al., 1996; Wolffe, 1998).

Recent findings suggest that lysine and arginine-specific methylation of histones may cooperate with other types of post-translational histone modification to regulate chromatin structure and gene transcription (Stallcup, 2001). In contrast to acetylation, methylation of histone does not alter its overall charge. However, increasing methyl addition (mono, di or tri) does increase its basicity and hydrophobicity. Furthermore, increased methyl addition on histone tails increases their affinity for anionic molecules i.e. DNA (Rice, et al., 2001), thus hindering the binding of transcription factors to gene regulatory elements.

DNA methylation, or covalent addition of a methyl group to cytosine within the context of the CpG dinucleotide, is a major determinant in the epigenetic silencing of genes. Detailed studies of the effect of DNA methylation on promoter activity have revealed that DNA methylation is a potent suppressor of gene activity (Jones, et al., 1999; Robertson, et al., 2000). Two mechanisms have been proposed for this repression. (i) direct inhibition of binding of sequence-specific transcription factors whose binding sites contain CpG sites and (ii) indirect via methyl-CpG-binding proteins which may compete with transcription factors for their binding sites in methylated DNA or reorganize DNA into tightly packed chromatin structures incompatible with transcription (Boyes, et al.,
The exact mode of transcriptional repression in vivo most likely results from a combination of these two mechanisms and is also dependent on the CpG density and regulatory element composition of the specific promoter (Robertson, et al., 2000).

Evidence of links between DNA methylation and histone hypoacetylation is accumulating. Inactive regions of DNA which were demonstrated to be heavily methylated were also found to be enriched in hypoacetylated histones. Studies have found that several proteins that specifically bind to methylated DNA are associated with complexes that include histone deacetylases (HDACs) (Dobosy, et al., 2001; Jones, et al., 1998; Nan, et al., 1998; Robertson, et al., 2000). Thus, HDACs are responsible for part of the repressive effect of DNA methylation (Dobosy, et al., 2001).

**b. Trans-acting factors**

The human topoisomerase IIα gene promoter is GC-rich and lacks a TATA box (Fig 1.3). The most proximal binding motif relative to the transcription initiation site is a Myb binding site at positions -12 to -17. Myb proteins can bind a variety of sequences but the consensus that has emerged is TAAC(G/T)G or TAACNG (Biedenkapp, et al., 1988; Brandt, et al., 1997; Deng, et al., 1996; Howe, et al., 1991). In human cell lines, c-Myb-regulated expression of topoisomerase IIα has been shown in haematopoietic cell lines (Fraser, et al., 1995; Isaacs, et al., 1998). Expression of c-Myb causes trans-activation of the topoisomerase IIα promoter in lymphoid- and myeloid-cell lines (Brandt, et al., 1997;
Isaacs, et al., 1998) whereas B-Myb causes promoter upregulation in HeLa cells as well as in haematopoietic cells (Isaacs, et al., 1998).

Just upstream from the Myb site is a partial consensus Myc/Max site to which the proto-oncogene product c-Myc binds in heterodimeric association with a second transcription factor Max. Whether this motif is functional in the case of the topoisomerase IIα gene promoter remains to be confirmed (Isaacs, et al., 1998).

G-rich elements called the GC-boxes (GGGGCGGGG) were found at positions -46 to -52 and -551 to -559. With its proximity to the transcription start site, it seems likely that the GC-1 motif is involved in the regulation of topoisomerase IIα promoter. However, there is no direct evidence to support this contention (Isaacs, et al., 1998). For some time it was thought that the general transcription factor Sp1 (Specificity protein 1) was the only factor able to bind GC boxes and subsequently either activate or repress gene transcription (Isaacs, et al., 1998; Suske, 1999). However, it is now known that Sp1 simply represents the first identified and cloned protein of a small protein family consisting of Sp1, Sp2, Sp3 and Sp4 (Suske, 1999). Mo, et al. (1997) have shown that reduced expression of Sp3 is associated with decreased topoisomerase IIα expression and therefore drug resistance in merbarone (a catalytic inhibitor of topoisomerase II) resistant human leukemic CEM cells (CEM/B12) suggesting that Sp3 acts as an activator for topoisomerase IIα. However, the etoposide-resistant KB cells express higher levels of Sp3 than the drug-sensitive parental cell line, implying that Sp3 was a transcriptional
repressor (Kubo, et al., 1995; Mo, et al., 1997). One explanation could be that the Sp3 in CEM cells has mutation in the inhibitory domain. It has been found that a conserved glutamic acid-rich sequence in the inhibitory domain at the COOH terminus of Sp3 is essential for its inhibitory function. If this region is deleted, or some of the conserved amino acids are replaced by others, this inhibitory function is abolished, thus converting Sp3 into a transcriptional activator (Dennig, et al., 1996; Mo, et al., 1997). Thus, Sp3 can function as either a repressor or activator of transcription.

Many eukaryotic promoters include a transcription regulatory element that contains the penta-nucleotide sequence CCAAT in a sense or inverted orientation approx. 50-225 bp upstream from the transcription initiation site. In the human topoisomerase IIα promoter there are five inverted orientation CCAAT boxes (ICBs) at positions -65 to -69, -105 to -109, -172 to -176, -254 to -258 and -386 to -390 (Isaacs, et al., 1998). Even though the CCAAT sequence is conserved in many promoters, it is not a target for a specific ubiquitous factor. Rather, CCAAT sequences of different genes are likely to serve as binding sites for distinct factors which include CBP, CTF/NFI and CBF/NF-Y (Raymondjean, et al., 1988). Thus, a number of different proteins can specifically bind the CCAAT sequence, but their binding affinities are influenced by the nature of the adjacent flanking DNA sequence (Dorn, et al., 1987; Isaacs, et al., 1998, Raymondjean, et al., 1988). Isaacs, et al. (1996) have demonstrated that the ICBs in the topoisomerase IIα promoter is recognized by the ubiquitous transcription factor NF-Y (also called CBF, ACF and CP1). The binding of NF-Y to DNA is thought to induce
distortion of the double helix by 62-82° depending upon the position of the CCAAT motif in a particular promoter fragment. This may indicate that the role of NF-Y is to facilitate interactions between different transcription factors. Thus, the sequences flanking the CCAAT box may influence the degree of DNA distortion and the effectiveness of this role for NF-Y (Isaacs, et al., 1998).

In an attempt to identify elements involved in the transcriptional activation of topoisomerase IIα by trichistatin A (TSA), it was found that mutation of ICB resulted in a dramatic decrease in the TSA-induced activation (Adachi, et al., 2000). These results suggest that the TSA-induced activation also requires the ICB elements and NF-Y. Currie (1998) has reported that the NF-Y complex shows histone acetyl transferase (HAT) activity in vivo through physical association with the related HAT enzymes, GCN5 and PCAF. This suggests a model in which NF-Y recruits a HAT enzyme(s) to the topoisomerase IIα promoter, thereby stimulating histone acetylation and activating transcription (Adachi, et al., 2000).

Between the second and third ICBs lies an inverted cell cycle-dependent element (CDE/CHR). The functional significance of this element has yet to be analyzed (Isaacs, et al., 1998). Upstream from the CDE/CHR lies a site with 80% homology to the consensus for an activating transcription factor (ATF) binding site (Isaacs, et al., 1998).
The region of the promoter up to -617 bp, which includes all of the motifs described above, has been found to direct maximal expression of reporter genes in transient transfection studies (Hochhauser, et al., 1992; Isaacs, et al., 1998). However, the full length promoter (approx. 2 kb) shows significantly lower activity than this 'core promoter' fragment, suggesting the possibility that an upstream negative regulatory element controls topoisomerase IIα gene activity under certain conditions (Isaacs, et al., 1998). So far there has been no characterization of this upstream region to identify the negative regulatory elements.
Figure 1.1 Catalytic cycle of topoisomerase II (adopted from Osheroff, et al., 1994). (a) The enzyme binds to double-stranded DNA. (b) The enzyme cleaves DNA in the presence of Mg$^{2+}$ forming a covalent complex via a phosphotyrosine linkage. (c) Upon ATP binding the enzyme undergoes a conformational change allowing DNA strand passage to occur. (d) Following strand passage, the enzyme reverses the cleavage reaction, and utilizes the energy of the phosphotyrosine bonds to restore the phosphodiester linkages in both DNA strands. (e) After religation of the strand breaks, ATP hydrolysis occurs which is coupled with a conformational change restoring enzyme conformation for another reaction cycle.
Figure 1.2 Point mutations in the topoisomerase IIα gene described in cell lines resistant to topoisomerase II inhibitors (adopted from Prost, 1995)
Figure 1.3 Human topoisomerase IIα promoter (adopted from Isaacs, et al., 1998) (a) Sequence of the human topoisomerase IIα promoter. The sequence of the promoter is shown to 617 bp upstream from the major transcription start site. Bases are numbered (as indicated on the left) with respect to the major transcription start site, which is designated +1 and marked with an arrow. Putative cis-acting elements, underlined and annotated above, include two GC boxes (Spl sites), five ICB's, one ATF site, one Myc/Max site, and one Myb site. The ATG translation start codon is double underlined. (b) Schematic representation of putative transcription factor binding motifs in the topoisomerase IIα promoter. The major transcription start site is indicated by an arrow. The positions of the GC boxes (GC1 and GC2), inverted CCAAT boxes (ICB1-5), and ATF binding site (ATF) are indicated.
GC2

-617  AGCCACCGCACACAGCCTACTTTTATTTCTTTGAAAAATGAATTCGAGGGTAAAGGGG
-557  GGGTTGAGGCAGATGCCAGAATCTGTTCGCTTCAACCAAGCAGCCAGGCTGCCTGTCCAG
-497  AAAGCCGGCACTCAGTTTCCTCAGGAAAACGAAGCTAAGGCTCCCATTCCCCTCGCTAAC
-437  AACGTCAAGACAGAGACAGTTTTTAGATTTCAGGGATCTTAAATAGATTGGCAGTTCCT
-377  GGAGAATAAACACATCTTTTGCTTTTCTCAGCACACTTTTTCCTCAGGCCACCCCCCTTCCCG

ICB5

-317  CTTCCAAGCCCATCTCTTCAAGCTTTTCGAGAAGAAACAAATGAGGCCCTTCTTCTATT

ATF

-257  GGCCAGATTCCTGTAATCTCTCCTGCTATGACGCCGAGTGGTCCTTTTGAAGCTCTCTC

ICB3

-197  TAGTCCCCCCTCTCAACTGATTTCTATTTAAACAAACAACCCCCCGCAACTCAAGCGGT

ICB4

-137  CATAGGTGATATAAAAAGCCAAGCTACATTTTGCTTTCTCTGACGGAGACGGTGAGACGG

ICB1

-77   AGTCAGGGATTGGCTGGTCTGCTTCGGGCGGGCTAAGGAAGGTTCAAGTGGAGCTCTCC

MYC/MAX

-17   TAAACCCACCCGCTCTGGAAGACCCGCTTTGTCGGGTCGTCCGGGGGGCTGCCTGCCC

+44   TGTGCTGCTTTCCAGGTTCTTGAAGCCCCCTCCAGGCCGCCTCAGCATC

(a)

(b)
CHAPTER 2

CLONING OF THE THREE SUBUNITS OF NF-Y

INTRODUCTION

Drug resistance remains one of the major obstacles to curing cancer today. The multi-drug-resistant (MDR) phenotype, due to over-expression of the MDR1 gene, is a major form of drug resistance (Futscher, et al., 1996; Kaye, et al., 1985; Shustik, et al., 1995). The MDR1 gene encodes for an integral membrane protein, P-glycoprotein (P-gp), which acts by decreasing intracellular drug concentrations through an ATP-dependent, verapamil-sensitive efflux of unmodified drug from the cell (Endicott, et al., 1989; Futscher, et al., 1996). Over-expression of P-gp has been associated with clinical drug resistance in a variety of tumors, including multiple myeloma, acute myelocytic leukemia and malignant lymphoma (Futscher, et al., 1996; Marie, et al., 1991; Miller, et al., 1996).

The cell line used in this study, Dox IV was derived from human multiple myeloma 8226 by continuous exposure to $10^{-5}$ M verapamil and stepwise increases in doxorubicin from $10^{-9}$ to $4 \times 10^{-8}$ M (Futscher, et al., 1997; Wang, et al., 1997). Verapamil is a competitive
inhibitor of P-glycoprotein (P-gp) and MRP activity and mRNA expression (Ax, et al., 2000; Muller, et al., 1994; Tsuruo, et al., 1981). In the study by Futscher, et al. (1996), 8226/S cells selected with doxorubicin alone (8228/Dox4) became P-gp positive with no changes in the expression of MRP or topoisomerase II. However, when doxorubicin and verapamil were combined from the beginning of drug selection of 8226/S, a non-P-gp drug-resistant cell line emerged (8226/Dox4V). This cell line did not express increased levels of MRP but decreased topoisomerase II protein and activity were found by immunoblot analysis and decatenation assay, respectively. Thus, verapamil was added to prevent doxorubicin resistance due to MDR. MDR is a well-studied mechanism of resistance and we wanted only resistance due to changes in the drug target topoisomerase II.

The selected cell line has acquired a low (i.e. 2-fold) and probably clinically relevant level of resistance to doxorubicin in the absence of MDRI overexpression (Futscher, et al., 1996; Wang, et al., 1997). This cell line has been shown to express atypical MDR (at-MDR) phenotype; i.e., compared to their respective parent cell lines, they are resistant to the cytotoxicity by inhibitors of topoisomerase II (Beck, et al., 1991; Danks, et al., 1993). At-MDR is generally associated with alterations in levels and/or activity of topoisomerase II. No mutations have been found in the topoisomerase II gene of Dox IV cells (Danks, et al., 1993; Wang, et al., 1997) probably because of the absence of a mutagen in the selection pressure. Previous work in our laboratory has reported a 2- to 3-fold decrease in topoisomerase II activity in nuclear extracts to be the only alteration
consistent with its drug resistant phenotype suggesting that decreased topoisomerase II activity is the major factor attributable to the drug resistant phenotype of Dox IV cells (Wang, et al., 1997). This decreased topoisomerase II activity quantitatively correlated with a decrease in transcription of topoisomerase IIα. In addition, topoisomerase IIα transcriptional down-regulation in Dox IV correlated well with a decrease in the extractable activity of a ubiquitous transcription factor CP-1 (NF-Y) (Wang, et al., 1997). Transient gene expression assays of reporter gene fused to the RPMI 8226 topoisomerase IIα promoter demonstrated that parent cells transfected with the reporter plasmid consistently expressed 2.5-fold higher reporter activity than the Dox IV cells transfected with the same plasmid. Thus, transcriptional down-regulation of topoisomerase IIα in Dox IV cells was found to be independent of the endogenous promoter sequence and dependent on other cellular factors (Wang, et al., 1997).

Since reduced NF-Y activity was thought to be an indirect cause of the resistant phenotype in Dox IV cells the initial aim of this study was to check if ectopic expression of NF-Y would affect the chemosensitivity of Dox IV cells to topoisomerase II inhibitors. NF-Y is a ubiquitous heteromeric protein formed by three subunits, NF-YA, NF-YB and NF-YC, all of which are required for DNA binding (Bellorini, et al., 1997; Kim, et al., 1990; Sinha, et al., 1995). Thus, initially all the three subunits were cloned into pCEP4 for transfection into Dox IV cells. In the process of cloning the three subunits multiple isoforms of NF-YA were identified; while some of which showed
variations in the regions of amino acids 25-55 or 182-189 as previously reported (Li, et al., 1992a; Li, et al., 1992b), others represent novel isoforms of NF-YA.

In addition to the Dox IV cells (henceforth to be referred to as Dox IV+) which were maintained as required in the presence of doxorubicin and verapamil, a cell line was maintained in the absence of the drugs (henceforth to be referred to as Dox IV-) to check if selection pressure was required to maintain the drug resistant phenotype of Dox IV cells.

MATERIALS AND METHODS

Cell culture

The parent RPMI 8226 and the doxorubicin resistant variant Dox IV were maintained as described (Futscher, et al., 1996) in RPMI 1640 supplemented with 5% fetal bovine serum (Gibco BRL). The Dox IV cells were maintained in $10^{-8}$ M doxorubicin (Adria Labs) and 10 $\mu$g/ml verapamil (Sigma). These cells will now be called Dox IV+. A culture of Dox IV cells was also maintained in the absence of doxorubicin and verapamil for 8 months prior to use in any experiments. These cells will be referred to as Dox IV-. Cells were cultured at 37°C in humidified 5% CO$_2$-95% air atmosphere and passaged in 5- to 6-day intervals.
Determination of mid-log phase

The cells were seeded at $1 \times 10^5$ cells/ml and viable cell numbers were counted by trypan blue exclusion test at intervals of 24 hrs. A growth curve was charted and the mid-log phase was determined as the density at the mid-point of exponential growth of the cells.

Cytotoxicity test

The parent RPMI 8226 and its drug resistant variants Dox IV+ and Dox IV- cells were seeded in a 12 well plate at a density of $1.5 \times 10^5$ cells/ml. Cells were treated with increasing concentrations ($1 \times 10^{-8}$ M to $128 \times 10^{-8}$ M) of doxorubicin and incubated in a humidified CO₂ incubator for 2 hrs. The drug was washed off with 1X PBS and the cells allowed to recover in 1.5 ml fresh RPMI 1640 supplemented with 5% fetal bovine serum. After an incubation of 72 hrs at 37°C in the humidified CO₂ incubator, the viable cell numbers were counted by trypan blue exclusion test. The experiment was performed in triplicate. In each experiment, each individual concentration was performed in duplicate. IC₅₀ was defined as a 50% reduction in viable cells with respect to the control cells which were not treated with doxorubicin.

Extraction of RNA

RNA from HeLa cells and WI-38 cells was extracted using the TRIzol reagent (Gibco BRL). A cell pellet of $2 \times 10^6$ HeLa cells or $1 \times 10^7$ WI-38 cells was suspended in 1 ml TRIzol reagent and incubated at R.T. for 5 mins. The DNA and protein were separated from the aqueous phase by the addition of chloroform and centrifugation at 12,000xg for
15 mins at 4°C. The RNA from the aqueous phase was then precipitated with isopropanol, washed with 85% ethanol in DEPC water, vacuum dried and resuspended in DEPC water. The RNA concentration was determined by measuring the absorbance at 260nm.

**PCR amplification of NF-YA, NF-YB and NF-YC**

The cDNA for NF-YA and NF-YB were synthesized using specific primers (αhNF-YA: 5'-TCAGCTCGAGCACATGGCGTGGGGTTAGGACAC-3’ and αhNF-YB: 5’-TAGTCTCGAGTCATGAAAACTGAATTTGCTGGACACC-3’ respectively). The positions at which these and other primers referred to later anneal to the NF-YA, NF-YB and NF-YC gene is indicated on the respective sequences (Figs. 2.2, 2.3 and 2.4). Oligo(dT) was used for NF-YC cDNA synthesis. RNA (5 μg for HeLa or 10 μg for WI-38) was incubated with 0.2 μM primer at 70°C for 10 min. The mixture was then transferred to ice and 5X PCR buffer (50 mM Tris-HCl, pH 8.3, 250 mM KCl and 0.5% Triton X100), 0.5 mM dNTP and 10 mM DTT were added. After 5 min of incubation at 42°C, superscript RT II (Gibco BRL) was added and the reaction mix was incubated at 42°C for 90 min. The enzyme was then denatured at 70°C for 15 mins followed by incubation with RNase H at 37°C for 20 min. cDNA was heated to 99°C for 5 mins and placed on ice before adding 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 at 25°C, 1% Triton X-100), 2.5 mM MgCl₂, 0.2 mM dNTP, 0.5 μM each primer (see below) and 2.5 units Taq DNA polymerase (Promega).
The PCR was carried out in the Amplitron II Thermolyne cycler using the following cycle: denaturation at 94°C for 45 secs, annealing at 57°C for 45 secs and primer extension at 72°C for 2.5 mins (3 mins for NF-YC). After 10 cycles the annealing temperature was increased to 72°C for another 20 cycles. The PCR product was then purified and concentrated using microcon-30.

**Cloning of NF-Y subunits**

The forward primers hNF-YA (5'-CCTCACAAGCTTCTAGGATCTCCAGAGTGACGAG-3'), hNF-YB (5'-TATCAAGCTTATGACAATGGACGGCACGACAGCTCTAC-3') and hNF-YC (5'-ATCAAAGCTTATGTCCACAGAAGGAGGGTTTGAGG-3') were designed to carry a Hind III restriction site (underlined) while the reverse primers chNF-YA, chNF-YB (see above) and chNF-YC (5'-TATACTCGAGTCAGTCTCCAGTCACCTGAGGGGGCC-3') were designed to carry the Xho I restriction site (underlined). The PCR products of NF-YA, NF-YB, NF-YC and pBluescript ksII+ (Stratagene) were digested with cloning enzymes Hind III (New England Biolabs) and Xho I (New England Biolabs). The digested products were isolated and purified from low melting agarose (FMC). The gel segments containing the DNA fragments of interest were isolated and melted at 65 °C for 5–10 min. The volume was adjusted to 0.5 ml with TE and an equal volume of buffer saturated phenol was added. The mixture was mixed using a vortex mixer, then incubated on ice for 10-15 min. The upper aqueous phase was harvested after centrifugation. Phenol extraction was repeated 3 times followed by a chloroform
extraction. The DNA was then precipitated with 0.3 M NaOAc and isopropanol, washed with 70% ethanol, dried under vacuum and resuspended in TE.

The purified NF-YA, NF-YB and NF-YC fragments were ligated to pBluescript ksII+ with T4 DNA ligase (New England Biolabs, Inc) and transformed into BB4 competent cells using the BioRad Gene Pulser II. After the sequences of the NF-YA, NF-YB and NF-YC cloned into bluscript were analyzed, they were sub-cloned into pCEP4 (Invitrogen) for transfection.

Plasmid minipreps

Plasmid minipreps were prepared by the alkaline lysis method. Bacterial culture (1 ml) was centrifuged at 14,000 rpm for 1.5 min. Bacteria cell pellets were resuspended in 100 μl lysis buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0). Bacteria were lysed by the addition of 200 μl alkaline SDS (1% SDS in 0.2 N NaOH) with thorough mixing. Ice cold 3 M KOAc, pH 4.8 (200 μl) was then added followed by mixing and centrifugation. The plasmid was precipitated from the supernatant with isopropanol. The precipitated plasmid was washed with 70% EtOH, dried under vacuum and dissolved in TE.

Plasmid Analysis

The purified plasmids were analyzed by restriction digestion. Initially, they were digested with the cloning enzymes, Hind III and Xho I to select recombinants containing inserted
fragments of expected size. Selected recombinants were then digested with an enzyme which cuts once in both the vector and the plasmid to confirm that the insert was correct and in the correct orientation. To achieve this, BamHI (Promega), Pst I (Promega) and Eco RV (New England Biolabs, Inc) were used for NF-YA, NF-YB and NF-YC, respectively.

**Sequencing**

DNA sequencing was performed using the Thermo Sequenase kit from Amersham as per the manufacturer's protocol. The termination mix was first prepared by mixing 2 μl dGTP termination master mix (7.5 μM dATP, dCTP, dGTP, dTTP) with 0.5 μl of 32P labeled ddATP, ddCTP, ddGTP or ddTTP in separate tubes for each sequence. The reaction mixture was prepared by mixing 10X reaction buffer (260 mM Tris-HCl, pH 9.5, 65 mM MgCl2), plasmid DNA (0.2 μg/reaction), primer (approx 2 pmol/reaction) and 2 μl Thermo Sequenase DNA Polymerase in a total of 20 μl. 4.5 μl of this mixture was transferred to each of the 4 tubes of termination mix. This was then overlayed with 20 μl mineral oil and the tubes placed in the Amplitron II Thermolyne Cycler and cycled 35 times as follows: denaturation at 95 °C for 30 secs, annealing at 64 °C for 30 secs (54 °C for hNF-YB 194, 44 °C for hNF-YA 254 & hNF-YA 547) and primer extension at 72 °C for 1 min. The reaction was stopped by adding 4 μl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The samples were centrifuged briefly (~10 secs) and heated at 70 °C for 3 mins just before loading onto a 6% glycerol tolerant sequencing gel. The gel was run in 1X glycerol tolerant gel buffer
(Amersham) at 110 W and 50 °C. At some occasions, single lane sequencing was performed using only one of the ddNTP termination mixes for each sequencing reaction. After electrophoresis, the gel was dried and used to expose a Kodak X-OMAT film overnight.

RESULTS

Resistance of Dox IV is not dependent on selection pressure

The Dox IV+ cells have acquired a low level of resistance upon selection in the presence of doxorubicin and verapamil. In order to test whether the Dox IV- cells retained the low level of resistance in the absence of selection, a cytotoxicity test was performed.

A viable count of all three cell lines; i.e., Dox IV+, Dox IV- and 8226 was taken 72 hrs after a 2 hr treatment with increasing concentrations of doxorubicin (Fig. 2.1). The IC₅₀ value for 8226, Dox IV+ and Dox IV- was determined to be 1.1 X 10⁻⁷ M, 2.4 X 10⁻⁷ M and 2.0 X10⁻⁷ M, respectively. Thus, Dox IV+ is approx 2-fold resistant to doxorubicin compared to the parental 8226 cell line (P=0037). Also, there is no significant difference between the level of resistance of Dox IV- and Dox IV+ (P=0.138) showing that the resistance is stable and not dependent on the selection pressure.
NF-YA cloning and characterization

NF-YA was initially cloned from HeLa cells. Suitable clones selected after restriction digestion analysis (HeLa A5, HeLa A6) were sequenced in completion with hNF-YA, αhNF-YA, hNF-YA 254 (5'-CAGGCTGTCCCTGGTGG-3') and hNF-YA 547 (5'-GCACCATTCTCCAGCAAG-3') primers to confirm the sequence as wild type. When the sequence was compared with the published sequence for hNF-YA (Fig. 2.2) (Li, et al., 1992b) a codon encoding for a glutamine in a sequence of 5 glutamine repeats between amino acids 22 and 28 was missing. This sequence corresponded to the published sequence for mouse NF-YA (Li, et al., 1992b) which contains only 4 glutamine residues (Table 2.1). To further investigate the correct sequence of human NF-YA, it was cloned from WI-38 cells. Two clones (WI-38 A4 & WI-38 A21) were selected for sequencing with hNF-YA primer. WI-38 A4 showed a 4 glutamine repeat while WI-38 A21 showed a 5 glutamine repeat. Thus, WI-38 A21 was used for complete sequencing with the primers hNF-YA 254, hNF-YA 547 and αhNF-YA. This clone had a 6 amino acid deletion between amino acids 182 and 189 (Table 2.1). This alternative sequence has been previously shown to result from alternative splice acceptor sites at position 183 or 189 (Li, et al., 1992a; Li, et al., 1992b). Since a clone of NF-YA which completely matches the published sequence had not been obtained, more clones from WI-38 were picked and single lane sequencing with ddGTP was performed using the primer hNF-YA to eliminate clones with only 4 glutamine repeats. WI-38 A2, WI-38 A9, WI-38 A10, WI-38 A11, WI-38 A15, WI-38 A16, WI-38 A18, WI-38 A20 and WI-38 A40 which had the 5 glutamine repeats were then subjected to another single lane sequencing with
ddTTP and primer hNF-YA 254 (long run) to eliminate clones with the 6 amino acid deletion. After the second round of elimination, WI-38 A9, WI-38 A15 and WI-38 A18 were selected for complete sequencing. However, all of them had some random changes. Thus, the ~400bp Hind III/Sfi I fragment from WI-38 A21 carrying the 5 glutamine repeat was used to replace the Hind III/Sfi I fragment carrying the 4 glutamine repeat in HeLa A6. The resulting plasmid was transformed into BB4 competent cells. The selected clone was then analyzed by restriction digestion and sequenced with hNF-YA and hNF-YA 254 to confirm the presence of 5 glutamine repeats and an intact Sfi I site. This completely intact NF-YA sequence was subcloned from pBluscript ks II+ into pCEP4 (Invitrogen) for transfection.

NF-YB and NF-YC

NF-YB was also cloned from HeLa cells into pBluescript ks II+ and transformed into BB4 competent cells. The selected clones were analyzed by restriction digestion and a few positive clones were sequenced in completion using the primers hNF-YB and hNF-YB 198 (5'-TCCAATAGCAAACGTGGCTAGG-3') to confirm the sequence as wild type. Since the obtained sequence was in accordance with the published sequence for hNF-YB (Fig 2.3) (Li, et al., 1992a; Li, et al., 1992b) it was subcloned into pCEP4 for transfection.

NF-YC was similarly cloned from HeLa cells into pBluescript ks II+ using the enzymes Hind III and Xho I. The suitable clones selected after restriction digestion analysis were
NF-YA from Dox IV and 8226

Since several forms of NF-YA were identified in human cells, the possibility of decreased NF-Y activity in the doxorubicin resistant Dox IV due to a change in abundance between different NF-YA isoforms was investigated. The PCR amplified NF-YA fragment from Dox IV+, Dox IV- and 8226 were cloned into pBluscript KSII+, transformed into BB4 competent cells and the clones analyzed by restriction digestion. Most clones analyzed gave the required insert but the insert differed slightly in size. Thus, an internal cutter, Bgl I, which has 3 restriction sites in NF-YA (at nucleotides 121, 377 and 592) and 2 restriction sites in the vector (at nucleotides 472 and 2166) was used to distinguish between the difference in inserts of different sizes. As a result, a total of 5 fragments of sizes 215 bp, 256 bp, 412 bp, 1267 bp and 1895 bp would be expected from the digestion of a perfectly correct full length clone (Fig. 2.5). Only 2 clones from Dox IV+, Dox IV- and 8226 each showed all the 5 expected fragments. One clone from each
was sequenced in completion and no mutation was found in Dox IV which might account for the differential NF-Y activity. Most of the clones had lost the restriction site at nt 121 in NF-YA. This could be resulted from the alternative splicing of exon B as described previously (Li, et al., 1992a). Sequencing one such clone from Dox IV+, Dox IV- and 8226 each with hNF-YA primer confirmed that exon B was spliced out. Most of the clones which released the 215 bp fragment between nucleotides 377 and 592 show either of 2 bands of distinctly different sizes. The smaller band could be resulting from alternative splicing between nucleotides 546 and 565 as was also shown previously (Li, et al., 1992a; Li, et al., 1992b). Once again one such clone from Dox IV+, Dox IV- and 8226 each were sequenced with primer hNF-YA 254 to confirm the alternative splicing. Two clones from Dox IV+ had lost 2 restriction sites: at nucleotides 121 and 377. However, the predicted band of ~900 bp appeared at ~650 bp. Thus, Bam HI was used to confirm that the insert was NF-YA. Of the 2 expected bands of 1.064 kb and 2.981 kb, the 2.981 kb band was seen at the proper size but the 1.064 kb band was smaller by ~200 bp. Since this difference in size corresponded to the difference in size seen in Bgl I digestion, it was likely that there was a large deletion/alternative splicing around nucleotide 377. This region was sequenced with primer hNF-YA 254 and a large deletion between nucleotides 309 and 442 i.e. a deletion of 44 amino acids was found. This region corresponds to exon D of NF-YA (Li, et al., 1992a) and could be spliced out as a result of facultative use of exon D. The relative distribution of the different forms of NF-YA between Dox IV+, Dox IV- and 8226 is shown in Table 2.1. As can be seen, there was
no statistically significant difference in the pattern of NF-YA mRNA distribution between Dox IV+, Dox IV- and 8226.

DISCUSSION

The cell line Dox IV (now Dox IV+) has been used in earlier studies to determine the mechanism of resistance against doxorubicin. In the absence of MDR1 phenotype and topoisomerase II mutations, doxorubicin resistance has been associated with decreased topoisomerase II activity and expression (Danks, et al., 1993; Futscher, et al., 1996; Wang, et al., 1997). In order to determine if the resistance was an effect of presence of drug in the medium, a cell line of Dox IV was maintained without doxorubicin and verapamil (Dox IV-). This cell line was also found to be equally i.e. 2-fold resistant to doxorubicin. Thus, the presence of doxorubicin was not required to maintain drug resistance in Dox IV cells. The drug resistant phenotype was stable and did not require continuous drug exposure.

Since decreased topoisomerase II expression was assumed to result from reduced activity of the trans-acting factor NF-Y and independent of endogenous promoter sequence (Wang, et al., 1997) it was to be determined if ectopic expression of NF-Y in the Dox IV cell line would increase NF-Y activity and in turn reverse its drug resistant phenotype.
In this chapter, I have cloned the three subunits of the ubiquitous transcription factor NF-Y: NF-YA, NF-YB and NF-YC. The cDNAs for the three NF-Y sub-units were isolated from HeLa cells and sequenced in completion to ensure that they match the published sequences. As expected, NF-YB and NF-YC were consistent with the sequences in the GenBank database. However, several different forms of NF-YA were identified; while some of which showed variations in the regions of amino acids 25-55 or 182-189 as previously reported (Li, et al., 1992a; Li, et al., 1992b), others represent novel isoforms of NF-YA (Table 2.1).

Li, et al. (1992a) have reported that variations in the region between amino acids 25 and 55 are due to facultative use of exon B and the difference in amino acids 182-189 results from alternative splice acceptor sites, at positions 183 or 189. In addition to the isoforms described by Li, et al. (1992a), two other variants of NF-YA were also identified. These isoforms, which contained altered sequences in the regions between amino acids 22-28 and 103-148 respectively, have not been reported previously. The published sequence shows that the human NF-YA contains a repeat of 5 glutamines (amino acids 23-27) while the mouse gene has only 4 glutamines (Li, et al., 1992b). However, the NF-YA cDNAs isolated from WI-38 (a human fibroblast cell line with limited life span in culture) showed a mixed population of clones carrying either 4 or 5 glutamines. Two of two clones from HeLa and 3 of 13 clones from WI-38 carried only 4 glutamines. Since several forms of NF-YA were identified in human cells, the possibility of decreased NF-Y activity in the doxorubicin-resistant DoxIV due to a change in the abundance
between different NF-YA isoforms was investigated. During this investigation the other form of NF-YA with variation between amino acids 103 and 148 was identified in Dox 1V+ cells (2 of 13 Dox 1V+ clones). This region corresponds to exon D of NF-YA (Li, et al., 1992a) and could be spliced out as a result of facultative use of exon D. Studies of the NF-YA cDNA clones isolated from RPMI 8226 and Dox1V cells have thus far failed to demonstrate that there is a statistically significant difference in the pattern of NF-YA isoforms distribution between these three cell lines. This finding strongly suggested that decreased NF-Y activity in Dox1V likely involves mechanism(s) other than altered abundance between different NF-YA isoforms.
Figure 2.1 Cytotoxicity Assay: Sensitivity of the parent cell line RPMI 8226 and its drug resistant variants, Dox IV+ and Dox IV-, to doxorubicin was determined by the percent of viable cells after treatment with different concentrations of doxorubicin. Cells not treated with any doxorubicin were used as controls. The number of viable cells in the controls was set to 100%. 
Figure 2.2 Nucleotide and protein sequence of hNF-YA (accession # A20548). The primers hNF-YA and αhNF-YA are double underlined. Beyond the indicated sequence they carry additional nucleotides on the 5' or 3' end, respectively, which carry the Hind III and Xho I restriction site. The primers hNF-YA 254 and hNF-YA 547 are underlined. The primers are assigned numbers according to the position of their 3' end.
Figure 2.2 (continued)
Figure 2.2 (continued)

gct gaa ggg cac acc atc gtc tat caa cca gtt aat gca gat ggc 531
    A E G Q T I V Y Q P V N A D G 177

gga ctt ccc gtc tgg tag cag ata gtt ggt caa tta cgt cta ccc 576
    T I L Q Q V T V P V S G M I T 192

gtg gaa gtc gtt cca tgg cag gga caa agt ccg tac tag tga
    I P A A S L A G A Q I V Q T G 207

gcc aat acc acc aac acc acc agt ggg caa ggg gac acc act gtc act gtc
   A N T N T S S G Q G T V T V 222

ggg cag att cca gca ggc atg tgg gca gga gca cag att gtt caa aca gga
   T L P V A G N V N S G M V 237

tgg taa gac gtc tgg gtt caa tgg taa cgg tta gtc cgg cgc cgc cgc cgc
   A K L E A E G K I P K E R R K 297

cgg tta gat gtt ctc ctt gtt cgc ctt cgc ttt ctt ctc cct cct ctt
   Y L H E S R H R H A M A R K R 312

gtt gaa ggt gga cgg ttc ttc ttc cca aag gaa aag gat agt ccc
   G E G G R F F S P K E K D S P 327

(continued)
Figure 2.2 (continued)

cat atg cag gat cca aac caa gcc gat gaa gaa gca atg aca cag 1026
qta tac gtc cta ggt ttg gtt cgg cta ctt ctt cgt tac tqt gtc
H M Q D P N Q A D E E A M T Q 342
atc atc cga gtt tcc taa ccc cac gcc atg tga tgg agc cga tea 1071
tag tag gct cac agg att ggg gtt cgg tac act acc tcg act agt
I I R V S ->
Figure 2.3 Nucleotide and protein sequence of hNF-YB (accession # A20553). The primers hNF-YB and αhNF-YB are double underlined. Beyond the indicated sequence they carry additional nucleotides on the 5' or 3' end, respectively, which carry the Hind III and Xho I restriction site. The primer hNF-YB 198 is underlined. The primer is assigned a number according to the position of its 3' end.
Figure 2.3 (continued)
Figure 2.3 (continued)

CAA CAG ATT TCT GGT GTG CAG CAA ATT CAG TTT TCA TGA
GTT GTG TAA AGA CCA CAG GTG GTT TAA GTG AAA AGT ACT
Q Q I S G V Q Q I Q F S *
Figure 2.4 Nucleotide and protein sequence of hNF-YC (accession # U78774). The primers hNF-YC and ωhNF-YC are double underlined. Beyond the indicated sequence they carry additional nucleotides on the 5' or 3' end, respectively, which carry the Hind III and Xho I restriction site. The primers hNF-YC 210, hNF-YC 504 and hNF-YC 769 are underlined. The primers are assigned numbers according to the position of their 3' end. The primer hNF-YC 769 is in the antisense orientation.
Figure 2.4 (continued)
Figure 2.4 (continued)

gca cag cct cag cag ggc cag acc aca cct gtg aca atg cag gtt 585
  A Q P Q Q G T T P V T M Q V

gga gaq ggt cag cag cgg gtc gtc gtt cag cgg gtc gtc gtt cag 630
  G E Q Q Q V Q I V Q A Q P Q G

caa gcc cag ccc cag gtc gtc gtc cta cag gtc ggc gtc gtt 210
  Q A Q Q A Q S G T G Q T M Q V

tag cag cag att cag cag gca gqa ggt cag cag cag cag ggg 720
  M Q Q I I T N T G E I Q I P

gtc cag cgg gca cag ggg cag gtt gtc gtt gtc gtc gtt gtt cag 765
  V Q L N A G Q V Q Y I R L A Q

cct gta tca ggc act cca gtt gtc cag gga cag atc cag cag aca 810
  P V S G T Q V V Q G I Q T L

gtc atc act cag gtt gta gtc gtc cag cag cag cag cag cag cag 855
  V T N A Q Q I I T Q T E V Q Q G

cag cag cag ttc aac cag cag cag cag gtc cag cag cag cag cag 900
  Q Q F S Q F T D G Q Q L Y Q

tag cag cag gtc cag ggg cag gcc gtc cag gcc cag ccc cct tcg 945
  I Q Q V T M P A G Q D L A Q P

tag ttc cag cag ccc cag cag cag ggg cag gcc ccc ccc ccc ccc 990
  M F I Q S A N Q P S D G Q A P

cag gtt act gqa gac tga

tgc cag tgg cct cag act

Q V T G D *>

52
Figure 2.5 Restriction map of Bgl I digestion: The grey shaded region indicates the NF-YA fragment in the pBluescript vector (thin circle). The thick lines indicate the positions of Bgl I digestion on NF-YA as well as the vector. The numbers on the inside indicate the nucleotide position carrying the Bgl I restriction site. The sizes of the fragment expected from Bgl I digestion of the construct are denoted on the outside.
Table 2.1 Distribution of the different isoforms of NF-YA between the parent cell line RPMI 8226 and its drug resistant variants, Dox IV+ and Dox IV-.
CHAPTER 3

STUDY OF ALTERATIONS IN NF-Y ACTIVITY AND PROTEIN

INTRODUCTION

Earlier studies on the Dox 1V cell line have suggested that resistance to doxorubicin is associated with concomitant decrease in topoisomerase II levels and activity (Wang, et al., 1997). Since the resistance of Dox 1V- cells to doxorubicin is comparable to the resistance of Dox 1V+ cells it is logical to think that the changes observed in Dox 1V (Dox 1V+) should also be mirrored in the Dox 1V- cells. Thus, a topoisomerase II activity assay was performed to compare the amount of topoisomerase II activity in the Dox 1V+ and Dox 1V- cell lines with reference to the topoisomerase II activity in the parent 8226 cell line. The results obtained showed that drug resistant phenotype of Dox 1V- cells was also due to a decrease in topoisomerase II activity.

Reduced levels of topoisomerase II in Dox 1V cells (now Dox 1V+) were associated with apparently reduced NF-Y activity in Dox 1V cells compared to the parent 8226 cells.
NF-Y is a ubiquitous multimeric eukaryotic transcription factor, also referred to as CPI and CBF (Nakshatri, et al., 1996; Chodosh, et al., 1988; Hatamochi, et al., 1988). The DNA binding activity of the NF-Y complex resides in the specific association of its three nonidentical subunits, NF-YA, NF-YB and NF-YC (Nakshatri, et al., 1996; Sinha, et al., 1995; McNabb, et al., 1995; Maity, et al., 1992). Since reduced activity of NF-Y in nuclear extracts has been shown to be associated with resistance to doxorubicin in Dox IV cells (Wang, et al., 1997) it was important to find whether all the three subunits or only one of the three subunits has been reduced in the Dox IV cells compared to the parent 8226 cells. Because of the requirement of all the three subunits for its DNA binding activity a decrease in any one subunit will destroy the stoichiometry between them, thus affecting their function. Thus, Western blots for the three subunits and electrophoretic mobility shift assay (EMSA) to measure the relative DNA binding activity of NF-Y in the three cell lines were performed.

METHODS AND MATERIALS

*Total cellular extract*

$2 \times 10^6$ proliferating cells were suspended in $50 \mu$l 5% SDS / 0.375 M Tris-HCl (pH 6.8) and boiled at 100 °C for 10 mins. The clear lysate was harvested after centrifugation at maximum speed. Protein concentration in the lysate was determined using the Micro Protein BCA kit (Pierce, Rockford, IL).
Nuclear extracts

Nuclear extracts were prepared as described (Dignam, et al., 1983) with slight modifications. Cell pellets were suspended in 5X packed cell volume (PCV) buffer A (10 mM HEPES, pH 7.9 at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) and put on ice for 5 mins. The homogenate was centrifuged at 1000 rpm for 10 mins. The pellet was then suspended in 5X PCV buffer A + 0.2% Nonidet P-40 and allowed to stand on ice for 8 mins. After centrifugation at 11,000 rpm for 10 mins at 4 °C the pellet was suspended in 3X PCV buffer C (20 mM HEPES, pH 7.9 at 4 °C, 25% glycerol, 0.5 M KCl, 15 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT) and sonicated for 1.5 mins at 4 °C. The homogenate was mixed at 4 °C for 30 mins and centrifuged at 13,000 rpm for 25 mins at 4 °C. The supernatant obtained was diluted 5X with buffer D (20 mM HEPES, pH 7.9 at 4 °C, 18.75% glycerol, 0.2 mM EDTA, 0.5 mM DTT). All the buffers contained the protease inhibitors pepstatin, leupeptin and pefabloc. Protein concentration in the nuclear extracts was determined using the Micro Protein BCA kit (Pierce, Rockford, IL).

Topoisomerase II activity assay

Topoisomerase II catalytic activity was determined by the ATP-dependent decatenation of kinetoplast DNA isolated from *Crithidia fasciculata* (Sullivan, et al., 1989). Decatenation reactions were performed by incubating increasing quantity (0.025 μg – 0.2 μg) of nuclear protein (from Dox LV+, Dox LV- and 8226) with the standard reaction mixture for the decatenation assay which contained 150 ng kDNA (topoGEN), 50 mM Tris-HCl (pH 7.5), 85 mM KCl, 20 mM MgCl₂, 1 mM ATP, 0.5 mM DTT and 30 μg/ml
bovine serum albumin (BSA) per 20 µL. The reactions were incubated at 37 °C for 30 mins and terminated by quenching on ice. The positive control contained 2 µL of purified human topoisomerase II (a generous gift from Dr. M.T. Muller of topoGEN, Inc). The reaction products were fractionated by agarose gel electrophoresis (0.8%) in 1X TBE in the presence of 0.5 µg/ml ethidium bromide. To quantify the amount of decatenated DNA, photographic negatives of the ethidium-bromide-stained agarose gels were densitometrically scanned using the Kodak Digital Science ID Program.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from Dox 1V+, Dox 1V- and 8226 cells harvested at mid-log phase. The oligonucleotide (CCAAT box #3) 5'-TCGATCCCTAACCTGATTGGTTTATTCAAAC-3' was used since it was found to have maximum NF-Y binding activity (Wang, et al., 1997). The oligonucleotide was converted to double stranded DNA by annealing to the complementary strands. The double stranded oligo (10 ng) was end labeled with 32P-γ-ATP by T4 polynucleotide kinase (Amersham Life Sciences) reaction. The reaction was incubated at 37 °C followed by enzyme inactivation at 68 °C for 10 mins. The probe was then purified using sepharose G-50 and the amount of 32P incorporation was determined by scintillation counting on the Beckman LS6500 Multi-purpose Scintillation Counter. The reactions of DNA-protein complex formation were performed by incubating the probe, nuclear protein (5 µg), poly dI:dC, reaction buffer (20 mM HEPES, 0.1 M KCl, 0.2 mM EDTA, 2 mM DTT, 1 mg/ml BSA) and 20% glycerol (to a final concentration of 5%) at R.T. for 20 mins. The reactions were
quenched by placing on ice. The DNA-protein complexes were fractionated by gel electrophoresis and detected by autoradiography. The resulting X-ray films were photographed using a Kodak Digital Science DC40 camera. The photograph was scanned by the Kodak Digital Science 1D program for densitometry to estimate the relative levels of the required DNA-protein complex in each cell line. EMSA were carried out on three independent nuclear extracts.

*Western blot analysis*

Total cellular extracts or nuclear extracts from Dox IV+, Dox IV- and 8226 were electrophoresed on 10% polyacrylamide gels and electroblotted onto nitrocellulose membranes. NF-YA, NF-YB and NF-YC were detected using specific rabbit antibodies anti-NF-YA, anti-NF-YB and mouse antibody anti-NF-YC, respectively. The immunoblot signals were visualized by enhanced chemiluminescence (ECL) kit (Amersham). The resulting X-ray films were photographed using a Kodak Digital Science DC40 camera. The photograph was scanned by the Kodak Digital Science 1D program for densitometry to estimate the relative levels of NF-YA, NF-YB and NF-YC in each cell line. Immunoblots were carried out on three independent cellular extracts.
RESULTS

Topoisomerase II activity is reduced in nuclear extracts of both the drug resistant cell lines

To determine the difference in topoisomerase II activity between the parent cell line 8226 and the two drug resistant cell lines, Dox IV+ and Dox IV-, nuclear proteins were extracted from the cells harvested at mid-log phase. Topoisomerase II activity was measured by decatenation of kDNA (Fig. 3.1). Though topoisomerase II activity was observed in all three cell lines, multiple experiments consistently showed less topoisomerase II activity in extracts of both the drug resistant cell lines, Dox IV+ and Dox IV-, compared with the parent 8226 cell line. Densitometric analysis of 50% topoisomerase II activity with respect to the positive control showed a reduction of 48.15 ± 2.92% and 35.38 ± 5.04% for Dox IV+ and Dox IV-, respectively, with reference to the parent 8226 cell line. This difference between Dox IV+ and Dox IV- may be slightly significant (P=0.06). However, this difference could be due to differences in the extractability of the enzyme and the semi-quantitative nature of the assay. Alternatively, it could be accounting for the slight difference in resistance between Dox IV+ and Dox IV- cells.

Levels of NF-Y subunits remain unchanged in total cellular extracts of Dox IV and 8226.

Western analysis was performed to determine the relative levels of the three NF-Y subunits: NF-YA, NF-YB and NF-YC in Dox IV+, Dox IV- and 8226 cells. Total
cellular extracts prepared with 5% SDS/0.375 M Tris pH 6.8 were subjected to SDS-PAGE. The protein transferred to nitrocellulose were detected with antibodies specific for NF-YA, NF-YB and NF-YC. Protein loading was standardized with PCNA (data not shown). Previous work in our laboratory (Wang, et al., 1997) had suggested that reduced topoisomerase II activity and expression was a result of reduced activity of the transcription factor NF-Y in nuclear extracts of Dox IV cells. As a result it would be expected that there will be a reduction in the amount of NF-Y protein in Dox IV cells compared to 8226 cells. However, the Western blots show all the three NF-Y subunits to be equal in the 3 cell lines (Fig. 3.2). Thus, it needs to be verified that NF-Y activity is indeed reduced in the drug resistant cell line Dox IV as opposed to the parent 8226 cell line.

Reduction in NF-Y activity only seen in Dox IV+ cells

Prior work in our laboratory (Wang, et al., 1997) tested the interaction of the topoisomerase II CCAAT boxes with nuclear proteins isolated from the RPMI 8226 and Dox IV cells and obtained evidence that CCAAT binding factor CP-I/NF-Y interacted with the human topoisomerase II CCAAT elements. The proximal region of the topoisomerase II promoter has 5 CCAAT boxes designated as #1-5 in the order of 5' to 3'. Of these elements, CCAAT box #3 showed the highest level of complex formation with NF-Y. Thus, CCAAT box #3 was used as a probe in EMSA to test for the difference in NF-Y binding activity between Dox IV+, Dox IV- and 8226 (Fig. 3.3a). The depletion of complex formation by self-competition confirmed the specificity of the interaction.
Complex formation of the topoisomerase II CCAAT boxes is $65.66 \pm 1.38\%$ in Dox IV+ extracts with reference to the parent 8226 cells (Fig 3.3b). However, complex formation in Dox IV- is $94.43 \pm 3.35\%$ with respect to the parent 8226 cell line (Fig. 3.3b). Thus, there is no significant difference in NF-Y activity between Dox IV- and 8226 cells ($P=0.1$). Reproducible results were obtained from three different experiments using different preparations of nuclear extracts from these cell lines.

Though a 2- to 3-fold decrease in topoisomerase II complex formation in Dox IV cells concomitant with its drug resistance phenotype was demonstrated by Wang, et al. (1997), the NF-Y binding activity in this study does not correlate as well with the drug resistance of Dox IV+ and Dox IV- cells. While both Dox IV+ and Dox IV- are equally resistant to doxorubicin compared to the parent 8226 cell line, NF-Y activity was reduced ~1.5-fold only in Dox IV+. However, the activity in Dox IV- extracts was comparable to that in the parent extracts. Thus, it is possible that there may be a difference in nuclear localization of the three subunits in Dox IV+ cells. The differences in NF-Y in Dox IV+ relative to the parental 8226 and drug resistant Dox IV- cells may also result from the continuous exposure of the Dox IV+ cells to the drug, and not a contributor to actual drug resistance.

**Varied levels of NF-Y subunits in nuclear extracts of Dox IV and 8226**

Though the amounts of the NF-Y subunits were equal in total cellular extracts of Dox IV+, Dox IV- and 8226 (Fig. 3.2), the NF-Y activity was found to be reduced in
Dox 1V+ nuclear extracts compared to nuclear extracts from Dox 1V- and 8226 (Fig. 3.3). Thus, to determine the NF-Y subunit levels in nuclear extracts of these cells a Western blot was performed. The nuclear extract proteins were separated on 10% SDS gel and blotted onto nitrocellulose paper. The subunit proteins were detected with the specific antibodies anti-NF-YA, anti-NF-YB and anti-NF-YC. The nuclear extracts show varied levels of the 3 subunits (Fig. 3.4a). A densitometric analysis performed with the Kodak Digital Science 1D program is shown (Fig. 3.4b). This analysis revealed a 1.5 ± 0.06 fold increase in NF-YA in Dox 1V+ nuclear extracts compared to both Dox 1V- and 8226, a 4.1 ± 0.28 fold decrease in extractable NF-YB in both Dox 1V+ and Dox 1V- compared to 8226 and a 1.8 ± 0.09 fold decrease in extractable NF-YC in Dox 1V+ compared to both Dox 1V- and 8226. Since NF-YC is absolutely required for the binding of the NF-Y trimeric complex to the DNA (Maity, et al., 1992) and since only NF-YC levels are in agreement with the NF-Y activity in nuclear extracts it may be the limiting factor in the formation of the trimeric complex. However, since there is a big decrease in the level of NF-YB it could also be a possible factor in determining the activity of the trimeric complex.

**DISCUSSION**

In this study, I developed a cell line from the drug resistant Dox 1V+ cell line. This new cell line was maintained without the presence of doxorubicin and verapamil (Dox 1V-).
In the last chapter I reported that the level of resistance in Dox 1V- was comparable to that in Dox 1V+. Previous studies in our laboratory had demonstrated that the decrease in topoisomerase II expression and activity correlated with the drug resistant phenotype of Dox 1V+ and that this reduced expression was associated with reduced NF-Y activity (Wang, et al., 1997). Thus, in this chapter I studied the correlation of drug resistance in Dox 1V- with the activity of topoisomerase II extractable from nuclei. Decatenation assays for topoisomerase II activity demonstrated that the decrease in extractable topoisomerase II activity correlated roughly with the drug resistant phenotype of Dox 1V- cells, as in the Dox 1V+ cells assuming a linear relationship between the topoisomerase II level and resistance.

To take the analysis a step further it was to be determined which of the three NF-Y subunits was responsible for the reduced DNA binding activity of NF-Y. Western blot analysis with total cellular extracts from the drug resistant cell lines Dox 1V+ and Dox 1V- and the parental 8226 cell line showed equal levels of the three subunits in all three lines. Thus, the next question was whether the difference in NF-Y activity between the drug resistant and parent cell lines was responsible for down-regulating topoisomerase II expression. Electrophoretic mobility shift assay with a DNA probe corresponding to CCAAT box #3 of the topoisomerase II promoter showed a slight reduction in NF-Y activity only in the Dox 1V+ cells, whereas the activity in the Dox 1V- cell line was similar to that in the parent 8226 cell line.
Since there was a decrease in NF-Y activity in the Dox IV+ cells but no changes in the levels of the subunits in whole cell extracts, nuclear extracts from the three cell lines were subjected to Western analysis for the three NF-Y subunits. A 1.5-fold increase in NF-YA is not in accordance with the decreased NF-Y activity observed in Dox IV+ cells. However, a ~4-fold decrease in NF-YB or a ~1.8-fold decrease in the amount of NF-YC protein could reflect the reduced NF-Y activity seen in the nuclear extracts of Dox IV+ cells. The changes observed in NF-Y protein and activity with respect to drug resistance are summarized in Table 3.1. These data suggest that the reduction in NF-Y activity is a result of the presence of doxorubicin rather than the cause of drug resistance in Dox IV cells. Doxorubicin, a DNA intercalator, could somehow be interfering with the binding of NF-Y to DNA in Dox IV+ cells. Thus, there may be other factors responsible for the stable drug resistant phenotype in Dox IV cells.
Figure 3.1 Decatenation assay of RPMI 8226, Dox IV+ and Dox IV-. (a) Topoisomerase II catalytic activity was measured by decatenation of kDNA using nuclear extracts (0.025-0.2 μg nuclear protein) from 8226, Dox IV+ and Dox IV- cells. The upper and lower arrowheads indicate the catenated kDNA (substrate, remained in the wells) and the decatenated DNA (products), respectively. M: molecular size marker, Hind III digests of λ DNA, +: positive control, purified human topoisomerase II and -: negative control, without purified enzyme or nuclear extract. (b) Densitometric analysis of the topoisomerase II activity. The error bars indicate ±SD (n=3)
Figure 3.1

% topoisomerase activity

---

M
+-

Dox IV+
Dox IV-
8226

0.2
0.1
0.05
0.025
0.2
0.1
0.05
0.025
0.2
0.1
0.05
0.025
Figure 3.2 Western blot analysis of the three NF-Y subunits in total cellular extracts of Dox 1V+, Dox 1V- and 8226. Protein loading was standardized using PCNA (data not shown)
Figure 3.3 Electrophoretic mobility shift assay for binding activity at CCAAT box #3 of human topoisomerase IIα. (a) $^{32}$P-labeled human topoisomerase II CCAAT element was incubated with nuclear extracts from Dox 1V+, Dox 1V- and 8226 cells. For self-competition (Dox 1V+/c, Dox 1V-/c and 8226/c), 50-fold molar excess of unlabeled probe was included in the reaction. Arrow head indicates the protein-DNA complex. (b) Densitometric analysis of the protein-DNA complex. Error bars indicate ±SD (n=3).
Figure 3.4 Western blot analysis of the three NF-Y subunits in nuclear extracts from Dox 1V+, Dox 1V- and 8226 cells. Protein loading was standardized using PCNA (data not shown). (b) Densitometric analysis of the NF-Y subunits. Error bars represent ±SD
Table 3.1 Summary of changes in NF-Y in nuclear extracts of Dox IV+ and Dox IV- with respect to drug resistance.

<table>
<thead>
<tr>
<th></th>
<th>8226</th>
<th>Dox IV+</th>
<th>Dox IV-</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-YA</td>
<td>--</td>
<td>$\uparrow 1.5 \pm 0.06$</td>
<td>--</td>
</tr>
<tr>
<td>NF-YB</td>
<td>--</td>
<td>$\downarrow 4.4 \pm 0.04$</td>
<td>$\downarrow 3.75 \pm 0.18$</td>
</tr>
<tr>
<td>NF-YC</td>
<td>--</td>
<td>$\downarrow 1.8 \pm 0.09$</td>
<td>--</td>
</tr>
<tr>
<td>NF-Y activity</td>
<td>--</td>
<td>$\downarrow 1.52 \pm 0.04$</td>
<td>--</td>
</tr>
<tr>
<td>Doxorubicin resistance</td>
<td>--</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
CHAPTER 4

ANALYSIS OF CIS- AND TRANS-ACTING FACTORS IN REGULATION OF TOPOISOMERASE IIα TRANSCRIPTION

INTRODUCTION

Doxorubicin resistance in Dox IV+ and Dox IV- cells has been found to be associated with a corresponding decrease in extractable nuclear topoisomerase II activity (Chapter 3). This decrease in extractable nuclear topoisomerase II activity was found to be associated with reduced activity of NF-Y in Dox IV+ cells (Wang, et al., 1997 & Chapter 3). However, a decrease in extractable NF-Y was not found to be correlated with reduced nuclear topoisomerase II activity in Dox IV- cells. Since both cell lines, Dox IV+ and Dox IV-, were similarly resistant to doxorubicin there must to be another factor controlling the expression of topoisomerase II in these cells. A number of potential transcription-factor-binding sites exist in the 630 bp region upstream of the translation start codon. This includes, in addition to others, a myb binding site and two GC boxes (putative Sp1-binding site). The myb binding site located between -12 and -17 bp nearly overlaps the transcription initiation site. One GC box (-46 to -52) is close to the transcription initiation site and the other is located between -551 and -559 bp upstream of
the five CCAAT boxes. Due to the close proximity of these two control elements, they could be playing a role in regulating transcription of topoisomerase IIα.

The regulation of eukaryotic gene expression is a complicated process involving the interaction of a large number of transacting factors with specific cis-regulatory elements. In this chapter I have studied the activity of transacting factors i.e; myb and Sp1 that could possibly regulate transcription of topoisomerase IIα in Dox IV cells. DNA methylation and histone acetylation act in cis to modulate protein-DNA interactions.

Since alteration of the DNA methylation status of CpG islands in the 5' sequence of genes has been demonstrated to change their expression (Jones, 1999), I examined DNA methylation as a potential molecular mechanism for regulating topoisomerase IIα gene transcription. The relative amount of topoisomerases (I & II) and their activities has been found to be enhanced after a protracted treatment of Chinese hamster ovary (CHO) cells with 5-azaacytidine (5-azaC), a cytidine analog containing a nitrogen atom at the 5' position instead of a carbon (Pinero, et al., 1999). It functions as a hypomethylating agent either by incorporation into DNA during replication (Takahashi-Hyodo, et al., 1999) or by trapping the DNA methyltransferase enzyme in a covalent complex with the DNA (Robertson, et al., 2000). In addition to its effect on topoisomerase II expression, methylation affects topoisomerase II activity by changing the chromatin structure. The toxicity of topoisomerase inhibitors is dependent on the progression of replication fork through the cleaved complexes (Larsen, et al., 1998). The change in chromatin structure
following DNA hypomethylation with 5-azaC might increase the number of replication forks enhancing the probability of a collision between a blocked cleavable complex and a replication fork. Thus, enhanced sensitivity to topoisomerase inhibitors has been observed in synchronous CHO cells pre-treated with 5-azaC (Lopez-Baena, et al., 1998).

In contrast, simultaneous treatment of CHO cells with 5-azaC and ellipticine (EPC), also an intercalating topoisomerase II inhibitor, reduced the frequency of chromosomal aberrations (CA) (Takahashi-Hyodo, et al., 1999). The authors have suggested that 5-azaC incorporation into DNA may change the topoisomerase II cleavage sites, protecting the DNA from the induction of damage. Alternatively, the hypomethylation induced by the incorporation of 5-azaC into DNA may change the chromatin structure, thus facilitating the access to DNA repair enzymes. Another possibility is that 5-azaC can reactivate methylated genes involved in the repair of DNA double-strand breaks induced by topoisomerase II inhibitors (Takahashi-Hyodo, et al., 1999). These observations imply that hypomethylation may enhance or reduce the cytotoxicity of topoisomerase II poisons.

It was interesting therefore, to check the effect of hypomethylation, by treatment with 5-azaC, on the sensitivity of Dox IV cells to doxorubicin. This would also throw light on the involvement, if any, of methylation in doxorubicin resistance.
Histone acetylation is emerging as a major regulatory mechanism thought to modulate gene expression by altering the accessibility of transcription factors to DNA. The histone acetylation status is regulated by the equilibrium of histone acetyl-transferase activity (HAT) and the histone deacetylase activity (HDAC) (Dressel, et al., 2000). Trichostatin A (TSA), a specific histone deacetylase inhibitor, induces histone hyperacetylation and modulates the expression of some mammalian genes (Sowa et al., 1999; Sowa, et al., 1997). TSA has been found to induce the expression of p21/WIF1/Cipl protein. Use of several mutant p21/WIF1/Cipl promoter fragments to study the responsiveness to TSA has indicated that TSA activates the p21/WIF1/Cipl promoter through Sp1 sites (Sowa, et al., 1999; Sowa, et al., 1997). Examination of changes of acetylated isoforms of histone H4 upon TSA treatment demonstrated that activation of the p21/WIF1/Cipl promoter through hyperacetylation of chromatin at Sp1 sites coincides with induced hyperacetylation of histone H4 (Sowa, et al., 1997).

Since the topoisomerase IIα promoter also has two Sp1 regulatory elements, it was interesting to check if TSA treatment would alter the expression of topoisomerase IIα and hence sensitivity of Dox IV cells to doxorubicin.
MATERIALS AND METHODS

Nuclear extracts

Nuclear extracts were prepared as described (Dignam, et al., 1983) with slight modifications. Cell pellets were suspended in 5X packed cell volume (PCV) buffer A (10 mM HEPES, pH 7.9 at 4 °C, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT) and put on ice for 5 mins. The homogenate was centrifuged at 1000 rpm for 10 mins. The pellet was then suspended in 5X PCV buffer A + 0.2% Nonidet P-40 and allowed to stand on ice for 8 mins. After centrifugation at 11,000 rpm for 10 mins at 4 °C, the pellet was suspended in 3X PCV buffer C (20 mM HEPES, pH 7.9 at 4 °C, 25% glycerol, 0.5 M KCl, 15 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT) and sonicated for 1.5 mins at 4 °C. The homogenate was mixed at 4 °C for 30 mins and centrifuged at 13,000 rpm for 25 mins at 4 °C. The supernatant obtained was diluted 5X with buffer D (20 mM HEPES, pH 7.9 at 4 °C, 18.75% glycerol, 0.2 mM EDTA, 0.5 mM DTT). All the buffers contained the protease inhibitors pepstatin, leupeptin and pefabloc. Protein concentration in the nuclear extracts was determined using the Micro Protein BCA kit (Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from Dox 1V+, Dox 1V- and 8226 cells harvested at mid-log phase. The oligonucleotides used were:

- GC box: 5’-CTAGCTGATTTGGGGCGGGCTCGGG-3’
- Myb-binding site: 5’-CTAGCCCATCCTAACCAGCTCGG-3’
The oligonucleotides were converted to double stranded DNA by annealing to their respective complementary strands. Double stranded oligos (10 ng) were end labeled with $^{32}$P-$\gamma$-ATP by T4 polynucleotide kinase (Amersham Life Sciences) reaction. The reaction was incubated at 37 °C followed by enzyme inactivation at 68 °C for 10 mins. The probe was then purified using sepharose G-50 and the amount of $^{32}$P incorporation was determined by scintillation counting. The reactions of DNA-protein complex formation were performed by incubating the probe, nuclear protein (5 μg), poly dl:dC, reaction buffer (20 mM HEPES, 0.1 M KCl, 0.2 mM EDTA, 2 mM DTT, 1 mg/ml BSA) and 20% glycerol (to a final concentration of 5%) at R.T. for 20 mins. The reactions were quenched by placing on ice. The DNA-protein complexes were fractionated by gel electrophoresis and detected by autoradiography. Quantification of the required complex was done using the Kodak Biomax 1D Program.

**Cell lysate**

6 X $10^6$ cells from proliferating cultures of Dox 1V+, Dox 1V- and 8226 cells were boiled in 30 μl 1X SDS-PAGE loading buffer for 10 mins. The clear supernatant was separated from the pellet after brief centrifugation (~ 10 secs).

**Western blot analysis**

Total cellular extracts or nuclear extracts from Dox 1V+, Dox 1V- and 8226 were electrophoresed on 10% polyacrylamide gels and electroblotted onto nitrocellulose membranes. Myb and Spl were detected using specific antibodies mouse anti-myb, clone 77.
1-1 (Upstate Biotechnology) and rabbit anti-Sp1 (Santa Cruz Biotechnology, Inc), respectively. The immunoblot signals were visualized using an enhanced chemiluminescence (ECL) kit (Amersham). The resulting X-ray films were photographed using a Kodak Digital Science DC40 camera. The photograph was scanned by the Kodak Digital Science 1D program for densitometry to estimate the relative levels of myb and Sp1 in each cell line. Immunoblots were carried out on three independent cellular extracts.

Cytotoxicity assay with 5-azaC/TSA pre-treatment

30 ml of 1 X 10⁵ cells were treated with 1X10⁻⁸ M TSA or 1X10⁻⁷ M 5-azaC for 24 hrs. After 24 hrs the cells were pelleted by centrifugation at 1000 rpm and washed three times with 1X PBS. They were then suspended in fresh RPMI 1640 at a density of 1.5 X 10⁵ cells/ml. Cell suspension (1 ml) was transferred to several wells in a twelve well plate. The cells were then treated with increasing concentrations of doxorubicin (1 X 10⁻⁸ M to 128 X 10⁻⁸ M) for 2 hrs. After 2 hrs incubation at 37°C the cells were washed three times with 1X PBS, suspended in 1.5 ml fresh RPMI 1640 and incubated at 37°C for 72 hrs. A viable count was taken after 72 hrs incubation. As a control, cells not treated with either 5-azaC or TSA were used for treatment with increasing doxorubicin concentrations. The effect of methylation or acetylation in Dox IV cells was compared with the controls.
RESULTS

Reduction in myb binding activity correlates with drug resistance in Dox IV

To determine whether the activity of transcription factor myb is reduced, thus affecting the transcription of topoisomerase II, an EMSA was performed. The myb binding element of the topoisomerase II promoter, located at the transcription start site, was used as a probe. DNA-protein complexes formed by the interaction between the probe and nuclear proteins were fractionated by gel electrophoresis and detected by autoradiography. The specificity of the interaction was confirmed by elimination of the complex in the presence of excess self-competitor. Though the signal was fuzzy and difficult to separate from a non-specific signal (Fig. 4.1a) it was clear that myb binding activity was reduced in Dox IV+ and Dox IV- nuclear extracts compared to the parent 8226 nuclear extracts. Densitometric analysis done with Kodak Digital Science 1D program (Fig. 4.1b) shows that the myb-binding activity in the drug resistant cell lines was reduced approximately 2 fold compared to the parent cell line. These results need further analysis by super shift assay to confirm the DNA-protein complex to be due to the binding of myb. However, due to technical difficulties of protein being extremely unstable (see below) further experimentation was not possible.

Myb protein expressed at very low levels in Dox IV and 8226

The basis for reduced myb binding activity was next investigated. Since activity may be regulated at the protein level or by post translational modification it was of interest to
determine if the protein level is similarly reduced in Dox IV+ and Dox IV-. Cellular extracts prepared from Dox IV+, Dox IV- and 8226 cells harvested at mid-log phase were used for Western blot analysis. When probed with anti-myb antibody no signal was detected in any of the three cell lines (data not shown). However, the positive control provided with the antibody showed a band corresponding to myb implying that the antibody was functional. No signal could be obtained even upon repeated trying. Thus, to overcome the possibility that myb may be degraded during protein extraction, a cell lysate prepared by boiling cells in SDS-PAGE loading buffer was used for Western analysis. There was still no signal except in the positive control. Since the positive control provided was Jurkat cell lysate, Jurkat cells were used to prepare extracts along with the Dox IV+, Dox IV- and 8226 cells in order to check for any experimental errors. Western blots with the new lysates (along with Jurkat cell lysate) show that the amount of myb protein in the three cell lines under study, i.e., Dox IV+, Dox IV- and 8226, is very low compared to the amount in Jurkat cells (Fig. 4.2). Also, the protein appears to be extremely unstable, as seen in Western analysis (Fig. 4.2).

*Activity at GC-box also reduced in agreement with drug resistance.*

The third promoter element which could possibly regulate topoisomerase II transcription is the GC-box. The GC-box is a binding site for the Sp family of transcription factors i.e. Sp1 and Sp3. In order to determine the binding activity at the GC-box an EMSA was performed using a GC-box probe with nuclear extracts from Dox IV+, Dox IV- and 8226. Inclusion of self-competition reactions confirmed the specificity of the interaction
between the probe and the Sp family of proteins. Once again it can be seen that Sp1 activity has been reduced in nuclear extracts of both drug resistant cell lines, Dox IV+ and Dox IV-, compared with the parental 8226 line (Fig. 4.3a). Densitometric analysis show that the decrease in Sp1 activity in Dox IV+ and Dox IV- is ~2.5 fold compared to activity in 8226 (Fig. 4.3b).

Sp1 protein levels equally reduced in Dox IV+ and Dox IV-

The observed decrease in Sp1 activity could be due to decreased Sp1 protein or a difference in post-translational modification. Thus, the difference in protein levels between the drug resistant cell lines, Dox IV+ and Dox IV-, and the parent cell line, 8226, were determined by Western blot. Sp1 protein levels were reduced in nuclear and total cellular extracts of the drug resistant cell lines compared to the parent cell line (Fig. 4.4a). However, there is excessive degradation of the protein preventing accurate quantitation. According to the technical datasheet from Santa Cruz Biotech, K562 extracts had been used to demonstrate the activity of the antibody. Thus, nuclear extract of K562 was prepared along with 8226 to check for any technical errors which could result in degradation of Sp1. Though there was some amount of degradation of Sp1 in K562 extracts it was relatively low compared to the degradation in 8226 nuclear extracts (data not shown). To further reduce the protein degradation cells boiled in SDS-PAGE loading buffer were directly loaded onto SDS gels for Western analysis. (Fig. 4.4b). Using cell lysates prepared as described above, Sp1 was found to be reduced in both the drug resistant cell lines as opposed to the parental cell line (Fig. 4.4c). This data was
consistent with the drug resistant phenotype of Dox IV+ and Dox IV- cell lines making Sp1 a likely candidate for regulating topoisomerase II transcription.

**Effect of histone acetylation and DNA methylation on topoisomerase IIα transcription**

In order to check for the effect of acetylation on topoisomerase IIα transcription, the Dox IV+, Dox IV- and 8226 cells were initially treated with $1 \times 10^{-8}$ M trichostatin A (TSA) followed by exposure to different concentrations of doxorubicin. TSA being a histone deacetylase inhibitor would be expected to induce hyperacetylation and increased gene expression. If decreased acetylation was the cause of reduced topoisomerase IIα in drug resistant Dox IV cells, treatment with TSA would induce hyperacetylation resulting in enhanced expression of topoisomerase IIα and a concomitant increase in drug sensitivity. In my experiment with treatment of Dox IV+, Dox IV- and 8226 cells with TSA, there was no significant difference in drug sensitivity of either Dox IV+ cells (Fig. 4.5) or Dox IV- cells (data not shown).

The effect of methylation on drug resistance in Dox IV via regulation of topoisomerase IIα expression was also studied. The cells were treated with $1 \times 10^{-7}$ M 5-azaC prior to treatment with different concentrations of doxorubicin. If methylation was responsible for the drug resistance in Dox IV cells, treatment with the hypomethylating agent might be expected to increase topoisomerase IIα transcription and activity, thereby reversing the drug resistant phenotype of Dox IV cells. In my
experiment however, no significant change in drug sensitivity was observed in either Dox
1V+ (Fig 4.6) or Dox 1V- cells (data not shown).

While these experiments don’t prove roles for acetylation or methylation, they don’t rule
out the possibility of their involvement in regulating topoisomerase IIα transcription. For
a dramatic change in resistance, a particular site would need to be altered in most of the
cells in the population. However, in the experiments performed, TSA and 5-azaC may be
causing random changes throughout the genome and the critical site; i.e., the
topoisoraserase IIα control region may be affected in only a few percentage of cells in the
population.

DISCUSSION

Doxorubicin requires the presence of the target enzyme, topoisomerase II, for its
cytotoxic effect. Reduction in topoisomerase II protein confers drug resistance to such
topoisoraserase II poisons. Generally a decrease in transcription of the topoisomerase IIα
gene is found to be involved in its downregulation. Control of transcription of the gene
for topoisomerase IIα therefore, plays a pivotal role in cancer chemotherapy. A good
understanding of the mechanisms controlling transcription of topoisomerase IIα would
enhance our understanding of the mechanism of drug resistance in cells selected for
doxorubicin resistance in the absence of the MDR phenotype.
Earlier studies on the Dox IV cell line selected for resistance to doxorubicin in the presence of verapamil to prevent the emergence of P-gp mediated resistance had reported the decreased activity of ubiquitous transcription factor, NF-Y, to be responsible for transcriptional downregulation of topoisomerase IIα (Wang, et al., 1997). However, my studies on the DNA binding activity and protein levels of NF-Y (Chapter 3) demonstrated that reduced activity of NF-Y was the result of doxorubicin treatment rather than the true cause of drug resistance in Dox IV cells. This suggests that the mechanism of transcriptional control of topoisomerase IIα gene in drug resistant Dox IV cells is much more complex. In addition to the inverted CCAAT boxes (ICB's) in the topoisomerase IIα promoter, there is a consensus myb binding site and two GC boxes (putative sites for Spl binding) which could be involved in regulation of gene transcription relative to the drug resistant phenotype.

In this chapter I studied the regulation of the topoisomerase IIα gene by myb and Spl. Myb-binding activity in nuclear extracts, as determined by EMSA, was found to be reduced comparably in both the drug resistant cell lines, Dox IV+ and Dox IV-, compared to the parent cell line 8226. However, the identity of the binding factor could not be confirmed by supershift assays since the protein was extremely unstable. Western analysis also did not reveal the presence of quantifiable amount of protein. This high degree of instability could possibly be the cause of reduced protein levels and hence reduced DNA binding activity of myb in Dox IV cells. Myb is a short lived transcription factor and is rapidly degraded by the 26S proteasome pathway (Bies, et al., 1997; Bies, et
al., 2000; Feikova, et al., 2000). As indicated in Chapter 6 there may be higher proteasome activity in both the drug resistant cell lines resulting in greater degradation of myb in these cells compared to the parent cells and thus affect transcription of topoisomerase IIα. Alternatively, the protein may be degraded during extraction. Thus, a protease inhibitor cocktail can be used to check whether there is any difference in extracted protein. Also, co-extraction of Jurkat cells with the cells under study can throw light on the degradation during extraction, if any. Phosphorylation of myb can also alter its function in transcription activation. Serine 528 in the negative regulatory domain of c-myb is phosphorylated and substitution of serine 528 to alanine (S528A) results in increased ability of myb to activate transcription. The DNA binding and multimerization activities of c-myb appear to be unaffected by the S528A substitution, suggesting that phosphorylation may mediate its effect on transactivating activity of c-myb by regulating interactions with other proteins (Miglarese, et al., 1996). Thus, increased phosphorylation of c-myb in the Dox IV cells could be responsible for reduced transcriptional activity and thus, the drug resistant phenotype, due to low levels of topoisomerase IIα.

Activity at the Sp1 consensus site was studied with EMSA. There was an equal reduction in activity in both drug resistant cell lines, Dox IV+ and Dox IV−, compared to the parent 8226 cell line. Consistent with the reduced activity, levels of Sp1 protein were also found to be reduced in accordance with the drug resistant phenotype of Dox IV+ and Dox IV−. This observation makes Sp1 the most likely candidate for regulating topoisomerase IIα expression.
Studies with deacetylase inhibitor TSA and cytidine analog 5-azaC did not reveal a significant effect on the drug sensitivities of Dox IV cells. Thus, acetylation and methylation may not be contributing to the regulation of topoisomerase IIα in these cells.
Figure 4.1 Electrophoretic mobility shift assay of human myb-binding site with nuclear extracts from 8226, Dox 1V+ and Dox 1V-. (a) $^{32}$P-labeled human myb-binding element was incubated with 5 µg nuclear extract from 8226, Dox 1V+ and Dox 1V-. For self competition (Dox 1V+/c, Dox 1V-/c and 8226/c), a 50-fold molar excess of unlabeled probe was incubated in the reaction. The protein-DNA complex formed is indicated with the arrow head. (b) Densitometric analysis of the protein-DNA complex was accomplished using the Kodak Digital Science Imaging and the relative intensities plotted.
Figure 4.2 Western blot analysis of myb protein: Proteins in the cell lysates were separated on 10% SDS-PAGE, blotted onto nitrocellulose and probed with anti-myb antibody. The western analysis shows that myb protein was expressed at extremely low levels in the cell lines under study.
Figure 4.3 Electrophoretic mobility shift assay of human GC-box site with nuclear extracts from 8226, Dox 1V+ and Dox 1V-. (a) $^{32}$P-labeled human GC-box was incubated with 5 μg nuclear extract from 8226, Dox 1V+ and Dox 1V-. For self competition (Dox 1V+/c, Dox 1V-/c and 8226/c), a 50-fold molar excess of unlabeled probe was incubated in the reaction. The protein-DNA complex formed is indicated with the arrow head. (b) Densitometric analysis of the protein-DNA complex was accomplished using the Kodak Digital Science Imaging and the relative intensities plotted.
Figure 4.4 Western analysis of Sp1. (a) Nuclear and cellular extracts from 8226, Dox IV+ and Dox IV- analysed by Western blot for Sp1 shows excessive degradation of protein hindering quantitation of the signal (b) Comparison of degradation of Sp1 in cell lysates of 8226 and K562 (c) Relative levels of Sp1 in cell lysates of Dox IV+, Dox IV- and 8226.
Figure 4.5 Effect of TSA on the sensitivity of Dox IV+ to doxorubicin. Error bars indicate ±SD (n=2)
Figure 4.6 Effect of 5-azaC on the sensitivity of Dox IV+ to doxorubicin. Error bars indicate ±SD (n=2)
CHAPTER 5

IS TOPOISOMERASE IIα THE SOLE CAUSE OF DRUG RESISTANCE IN DOX IV?

INTRODUCTION

The topoisomerase II activity assay used to determine the relative activity of topoisomerase II in Dox IV+, Dox IV- and 8226 cells (Chapter 3) is a semi-quantitative assay and reflects the topoisomerase II activity only in the nuclear extractable protein. Also, if there was a 50% reduction in topoisomerase IIα, there would be a much higher degree of drug resistance in the Dox IV cells than the observed 2-fold resistance. There is also a possibility that the low level of resistance seen in the Dox IV cells was due to changes in topoisomerase IIβ. Thus, there are several unanswered questions about the relationship between the observed reduced levels of topoisomerase IIα in nuclear extracts and the observed 2-fold resistance of Dox IV cells.

To address this issue an in vivo assay for cellular topoisomerase II-DNA crosslinks was performed. If there was an actual 50% reduction in topoisomerase IIα protein in Dox IV
cells, as demonstrated by Wang, et al. (1997), the amount of DNA-protein crosslinking would be expected to be reduced by an equivalent amount when compared with the parent 8226 cells.

METHODS AND MATERIALS

Filter Assay for protein-DNA crosslinks

This assay was performed in collaboration with Edith Yamasaki (The Ohio State University) as per Gao, et al. (1999). Dox IV+, Dox IV- and 8226 cells were labeled with $^3$H-dT (6 µl of 1.0 mCi/ml $^3$H-dT (Amersham) was added to ~1.2 X 10^6 cells in 6 ml 5% FCS/RPMI 1640) for 40 hrs. After labeling, the cells were aliquoted into 6 microfuge tubes (~1 ml cells/tube) and pelleted by centrifuging at 150Xg for 10 mins. The supernatant was removed and pellets were resuspended in 200 µl serum free RPMI 1640. DMSO (2 µl) was added to 3 replicates of each cell line and 2 µl 10 mM VM-26 (Bristol Myers) was added to the other 3 replicates (final conc. 100 µM) and incubated at 37°C for 15 mins. The reaction was stopped by the addition of 200 µl 2X HIRT lysis buffer (20 mM Tris-HCl, pH 7.4, 20 mM EDTA, pH 7.4 and 1.2% SDS). The HIRT buffer contains SDS, which denatures topoisomerase trapped in topoisomerase-DNA cleavage complexes by topoisomerase poisons and thus renders the covalent topoisomerase-DNA crosslinks irreversible. The cell suspension was transferred to microfuge tubes containing a small stainless steel nut. The tube was capped securely and the DNA was sheared by
vortexing at top speed for 1 min. The lysate was then heated at 65°C for 10 mins to ensure denaturation and removal of noncovalently attached proteins from the DNA. After cooling to room temperature, aliquots of the lysate were assayed with the GF/C filter assay for the percentage of labeled DNA that is crosslinked to protein. 30 µl aliquot of the HIRT extracted supernatants were mixed with 1 ml 4.0 M GuHCl buffer (guanidinium chloride) and filtered through GuHCl pre-wetted glass fiber filters followed by washing with 2.75 ml GuHCl buffer and finally with 5 ml ice cold 95% Et-Oh. The same procedure was repeated with 0.4 M GuHCl. The dried filters were counted with 5 ml Ready Gel.

In 4.0 M GuHCl (DNA binding conditions), all nucleic acids bind to the filter. The radioactivity retained on the filter under DNA binding conditions gives the value for total labeled DNA in the aliquot and the radioactivity retained on the filter under protein binding condition (0.4 M GuHCl) gives the value of the DNA crosslinked to protein. Thus, the ratio of the radioactivity retained on GF/C filters in 0.4 M GuHCl to the radioactivity retained on filters in 4.0 M GuHCl gives the fraction of labeled DNA that is crosslinked to protein.
RESULTS

Reduced VM-26 induced topoisomerase II-DNA crosslinks in Dox IV

Crosslinking with doxorubicin (0.2 M – 1.0 M) induced only up to ~7% DNA crosslinking which was not much higher than that observed with the negative control done with DMSO (~4.5%) (data not shown). This is because doxorubicin is a very weak topoisomerase II poison. Thus, a strong isoyme-nonspecific topoisomerase II poison, VM-26, was used to analyze the difference in DNA-protein crosslinking between Dox IV+, Dox IV- and 8226. The percent crosslinking observed was 79.4 ± 0.46%, 69.2 ± 1.04% and 72.5 ± 3.03% for 8226, Dox IV+ and Dox IV- respectively (Fig. 5.1). A statistically significant difference was observed in crosslinking between 8226 and Dox IV+ (P=0.002) and between 8226 and Dox IV- (P=0.004) with no statistically significant difference between Dox IV+ and Dox IV-. However, the reduction of crosslinking in Dox IV cells when compared to the parent 8226 cells is not as high as expected with a 50% reduction in topoisomerase II protein as suggested by assays on nuclear extracts.

DISCUSSION

In order to confirm the hypothesis that reduction in topoisomerase IIα expression was the cause of drug resistance in Dox IV cells, a topoisomerase poisoning assay was
performed. This assay did not demonstrate a big difference in DNA-topoisomerase cross linking between the parent 8226 cells and its drug resistant variants Dox 1V+ and Dox 1V-. The slight (~9%) reduction in topoisomerase observed in this assay is possibly contributing to the low level of resistance in Dox 1V cells. However, this assay does not distinguish between topoisomerase IIα or topoisomerase IIβ. Therefore, the reduced topoisomerase activity could be a result of either of the two isoforms. It is possible that reduction in topoisomerase II is not the only cause of drug resistance in Dox 1V cells.
Figure 5.1 Topoisomerase II-DNA crosslinks in 8226, Dox 1V+ and Dox 1V-. Error bars indicate ±SD (n=3).
CHAPTER 6

GLOBAL CHANGES IN PROTEIN EXPRESSION BETWEEN RPMI 8226 AND ITS DRUG RESISTANT VARIANT, DOX IV

INTRODUCTION

Acquired resistance to anticancer drugs is multifactorial in which several mechanisms are active simultaneously. Numerous mechanisms of resistance have been studied, most of which have been described earlier in the introduction chapter of the thesis. However, there may be other unknown mechanisms which may contribute to drug resistance in tumor cells. The most common type of resistance seen in treatment with anti-cancer drugs is the multidrug resistance phenotype. The cell lines Dox IV+ and Dox IV- used in this study were selected against the MDR phenotype by incorporation of verapamil in the selection strategy (Chapter 2). The main mechanism of resistance for topoisomerase II poisons is the down-regulation of topoisomerase IIα. This was thought to be the case in the cell lines under study; i.e., Dox IV+ and Dox IV-. However, as seen in Chapter 5 by the topoisomerase poisoning assay, reduced expression and activity of topoisomerase II was not the only cause of the observed resistance in Dox IV cells. Thus, to determine the
mechanism(s) of resistance, the proteomics approach of analyzing global protein expression can be used i.e. a phenotypic characterization of cancer cell lines and their chemoresistant counterparts using two-dimensional electrophoresis and mass spectrometry.

In order to study the difference in global gene expression patterns between the parent 8226 cell line and its drug resistant counterparts, total cellular extracts from these cell lines were subject to two-dimensional electrophoresis and the 2D protein patterns were observed to check for any obvious change in expression. In all, eight protein spots were found to be differently expressed in parent and drug resistant cells. Of these, four were increased or expressed in the drug resistant cells while the remaining four were decreased in the resistant cells. These protein spots were then analyzed by MALDI-MS to identify the proteins. These proteins and their possible roles in drug resistance have been analyzed.

METHODS AND MATERIALS

*Cellular protein extraction*

2 X 10^6 cells from Dox 1V+, Dox 1V- and 8226 cultures harvested at mid-log phase were washed with PBS and suspended in 40 μl osmotic lysis buffer (10 mM Tris pH 7.4, 0.3% SDS, 5 mM MgCl_2, 50 μg/ml RNase A from bovine pancreas type IIIA, Sigma R5125,
100 μg/ml DNase I, Type II from bovine pancreas Sigma D4527) and mixed by vortexing. The lysate was incubated on ice until the viscosity disappeared, and the protein concentration was determined by Kendrick Labs, Inc (Madison, WI) using the Pierce BCA total protein assay. Samples were then diluted to 5 mg/ml in SDS Boiling Buffer and heated in a boiling water bath for 5 mins before loading.

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis was performed according to the method of O'Farrell (1975) by Kendrick Labs, Inc (Madison, WI) as follows: isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm using 2.0% pH 3.5-10 ampholines (Amersham Pharmacia Biotech, Piscataway, NJ) for 9600 volt-hrs. One mg of an IEF internal standard, tropomyosin was added to each sample. This protein migrates as a doublet with lower polypeptide spot of MW 33,000 and pI 5.2; arrowheads on the stained gels mark its position.

After equilibration for 10 mins in buffer “0” (10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 hrs at 12.5 mA/gel. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as Mol. Wt. standards to the agarose that sealed the tube gel to the slab gel: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000) and lysozyme (14,000). These standards appear as
bands across the Coomassie Brilliant Blue R-250-stained 10% acrylamide slab gel. The Coomassie blue-stained gel were dried between sheets of cellophane with the acid edge to the left.

*In-gel digestion of proteins*

This procedure followed by MALDI-MS was performed by the Protein Chemistry Core Facility, Columbia University. Gel spots were transferred to clean tubes and water added to completely hydrate the gels. Gel spots were prepared for digestion by washing twice with 200 μl 0.05 M Tris, pH 8.5/50% acetonitrile for 20 min with shaking. After removing the washes, the gel pieces were dried for 30 min in a Speed-Vac concentrator. Gels were digested by adding 0.05 μg modified trypsin (sequencing grade, Roche Molecular Biochemicals) in 13-15 μl 0.025 M Tris, pH 8.5. The tubes were placed in a heating block at 32° C and left overnight. Peptides were extracted with 2 X 50 μl 50% acetonitrile/2% TFA and the combined extracts were dried and resuspended in matrix solution.

*MALDI-MS Analysis*

Matrix solution was prepared by making a 10 mg/mL solution of 4-hydroxy-α-cyanocinnamic acid in 50% acetonitrile/ 0.1% TFA and adding two internal standards, angiotensin and bovine insulin, to the matrix solution. The dried digest was dissolved in 3 μl matrix/standard solution and 0.7 μl was spotted onto the sample plate. When the spot was completely dried, it was washed twice with water. MALDI mass spectrometric
(MALDI-MS) analysis was performed on the digest using a PerSeptive Voyager DE-RP mass spectrometer in the linear mode.

Peptide Mass Search

Peptide masses were entered into search programs to search the NCBI and/or GenPept databases for a protein match. Programs used were:

ProFound at http://129.85.19.192/profound-bin/WebProFound.exe and

MS-Fit at http://prospector.ucsf.edu.

RESULTS

Comparision of 2D gel electrophoresis patterns

The investigations of proteins associated with drug resistance were aimed at elucidating protein changes between cells that were sensitive to doxorubicin (parent cell line 8226), those that had been selected for resistance to doxorubicin (Dox 1V+) and the drug resistant cell line maintained without the selection pressure (Dox 1V-). To account for experimental variations, two gels were run for each cell line. Numerous spots were detected in each of the gels in the separating area of the 3.5-10 pH range and molecular mass range of 14-220 kDa (Figs. 6.1a, 6.1b, 6.1c). Protein spot positions and intensities were visually compared between patterns from the three cell lines on analytical gels. This comparison yielded information about spots related to development of chemoresistance
(increase or decrease of spots). Eight protein spots were found to be significantly different in intensity (spots marked #1—8 on Figs. 6.1a, 6.1b, 6.1c). Thus, they were analyzed by excision of the gel spots, in-gel tryptic digestion and subsequent mass analysis of the eluted peptide mixture (Brockstedt, et al., 1998). This was performed commercially by Columbia University, Protein Chemistry Core Facility, New York, NY. Protein spots #1—4 were either unique to or significantly darker in both Dox IV+ and Dox IV- compared to the parent 8226 cell line. These proteins might be upregulated or stabilized due to the treatment with doxorubicin and could be candidates responsible for the drug resistant phenotype. Spots #5—8 were either unique to or darker in parent cell line 8226 when compared to both drug resistant cell lines, Dox IV+ and Dox IV-. These proteins could be responsible for making the cells sensitive to the drug and their downregulation in the Dox IV cells could make them resistant to the drug. These proteins and their functional role in imparting resistance to doxorubicin in the cell lines under study will be discussed below.

Spot #1: Similar to heterogenous nuclear ribonucleoprotein (hnRNP) A2/B1
Spot #1 Identified as similar to heterogenous nuclear ribonucleoprotein A2/B1 (Fig. 6.2), was expressed at higher levels in Dox IV+ compared to 8226 and Dox IV- cells. The alternative splicing of the second mini exon of the A2/B1 gene produces A2 and the less abundant B1 (Kamma, et al., 2001a; Kamma, et al., 2001b; Kamma, et al., 1999). hnRNP A2/B1 are nuclear RNA binding proteins involved in pre-mRNA processing. Although the biological reason is not clear, overexpression of hnRNP A2/B1, especially of B1 is a
useful marker for detection of cancers in early stages (Kamma, et al., 2001a; Kamma, et al., 2001b; Satoh, et al., 2000; Zhou, et al., 1996). hnRNP A2/B1 have been reported to be functional ssDNA-binding proteins that co-mediate telomerase access and contribute to telomerase replication. In addition, binding of hnRNP A2/B1 to telomeric repeats stabilizes them against nuclease digestion (Kamma, et al., 2001a). Also, topoisomerase II has cleavage sites in the telomeric repeats. Thus, binding of hnRNP A2/B1 may protect these sites from topoisomerase II cleavage. This function of hnRNP A2/B1 in Dox IV cells could contribute to their drug resistant phenotype. The low abundance of this protein in the Dox IV- cells (where doxorubicin resistance is not significantly different from that of Dox IV+ cells) suggests that it may be an effect of the presence of doxorubicin. However, the cytotoxicity assay used to determine the sensitivity of the cells to doxorubicin (Chapter 2) is not precise enough to show that resistance in Dox IV+ and Dox IV- cells are identical. In fact, Dox IV+ cells are slightly more resistant than Dox IV- cells and this may be contributed by higher levels of hnRNP A2/B1. As a DNA intercalator it is possible that doxorubicin interferes with telomere function or replication. This data suggests that telomeres may be an undiscovered cytotoxic target of doxorubicin.

**Spot #2, 3: UDP-N-acetylhexosamine pyrophosphorylase**

Spots 2 & 3 were both identified as UDP-N-acetylhexosamine pyrophosphorylase (Fig. 6.3). They could represent different forms of the enzyme due to post-translational modification. The modification would result in difference in charge on the protein and
hence the difference in their mobilities on the 2D gel. Both forms of the enzyme were found to be elevated in both Dox 1V+ and Dox 1V- cells compared to the parent 8226 cells. Since both Dox 1V+ and Dox 1V- are significantly resistant to doxorubicin, UDP-N-acetylhexasamine pyrophosphorylase is well correlated to doxorubicin resistance in these cells. This enzyme catalyzes the synthesis of both UDP-N-acetylgalactosamine (UDP-GlcNAc) and UDP-N-acetylgalactosamine (UDP-GalNAc) from UTP and the appropriate N-acetylhexasamine-1-phosphate (HexNAc-1-P) (Szumilo, et al., 1998; Wang-Gilliam, et al., 1998). The presence of a 17-amino acid peptide near the carboxyl terminus (aa 454 – 469) modifies the specificity of the pyrophosphorylase from synthesis of UDP-GalNAc to synthesis of UDP-GlcNAc (Wang-Gilliam, et al., 1998). No peptides in this region have been identified by the mass spectra. Thus, we cannot predict which of the two enzymes was present in the spots. The action of this enzyme provides UDP-N-acetylhexasamine donor substrates for O- HexNAc transferase which catalyzes the glycosylation of proteins at serine and threonine residues (Boehmelt, et al., 2000; Yang, et al., 2001). Thus, the availability of UDP-GlcNAc would correlate with glycosylation levels of proteins. It has been shown that transcriptional levels of some genes are also dependent on the levels of UDP-GlcNAc (Du, et al., 2000; Yang, et al., 2001). This is based on the observation that many transcription factors are modified by O-GlcNAc, most often in their transcriptional activation domain (Comer, at al., 1999; Courey, et al., 1989; Jackson, et al., 1988; Yang, et al., 2001). It has been proposed that glycosylation inhibits otherwise strong hydrophobic interactions that occur between transcription factors (Jackson, et al., 1988). On similar lines, the transcription factor Sp1 which
contains two glutamine-rich activation domains, each associated with a serine/threonine-rich region bears the O-GlcNAc modification (Jackson, et al., 1988; Yang, et al., 2001). This O-GlcNAcylation of Sp1 represses Sp1-mediated transcription (Yang, et al., 2001). Sp1 is a ubiquitous transcription factor that plays an important role in controlling the transcription of TATA-less housekeeping genes. One such gene of interest to this study is topoisomerase IIα. Thus, increased levels of UDP-GlcNAc synthesis in the Dox IV cells could result in reduced transcriptional activity of Sp1. This reduced activity of Sp1 would translate into reduced expression of topoisomerase IIα conferring drug resistance to Dox IV cells. In agreement with this hypothesis, the Sp1 and topoisomerase II activities have indeed been found to be reduced in nuclear extracts of Dox IV cells compared to the parent 8226 cells (Chapter 3 & Chapter 4). Additionally, both topoisomerase II poisoning (Chapter 5) and topoisomerase IIα in nuclear extracts (Wang, et al., 1997) were reduced. Finally, whole cell lysates also indicate reduced Sp1 protein.

Spot #4: Aldose Reductase

Spot #4 was identified as aldose reductase (Fig. 6.4). Aldose reductase, officially called aldehyde reductase, was elevated in both the drug resistant cell lines Dox IV+ and Dox IV- compared to the parent 8226 cell line. This aldo-keto reductase can reduce a broad spectrum of substrates with NAD(P)H as donor. A study with hydrogen peroxide treatment of rat vascular smooth muscle cells (A7r5) showed that aldose reductase mRNA was induced during oxidative stress (Spycher, et al., 1997). Anthracyclines have been previously shown to induce oxidative stress (Chenais, et al., 2000; Nowak, et al.,
Thus, it is highly likely that the oxidative stress as a result of doxorubicin treatment induced higher expression of aldose reductase in both Dox IV+ and Dox IV- when compared with the 8226 cell line. Increased expression of aldose reductase mRNA was found in a resistant descendant of the human stomach carcinoma cell line EPG85-257 selected for resistance against daunorubicin (Ax, et al., 2000). The aldose reductase was responsible for reduction of daunorubicin to daunorubicinol. Similarly, doxorubicin is also subject to C13 carbonyl reduction to doxorubicinol (Ahmed, et al., 1978; Ax, et al., 2000). The 13-hydroxy metabolites of anthracyclines, such as doxorubicinol and daunorubicinol, are significantly less potent than the parent drug in terms of inhibiting tumor cell growth in vitro (Ax, et al., 2000; Beran, et al., 1979; Kuffel, et al., 1992). Thus, detoxification of doxorubicin by aldose reductase could be responsible for drug resistance in Dox IV+ and Dox IV- cells.

Spot #5: Protein disulfide isomerase and UV excision repair protein RAD23 homolog

Spot #5 was found to contain both proteins, protein disulfide isomerase (PDI) and the human homolog of the Saccharomyces cerevisiae nucleotide excision repair (NER) gene product RAD23 (hHR23) (Fig. 6.5). These proteins were found to be down regulated in both the drug resistant cell lines Dox IV+ and Dox IV- compared to the parental 8226 cell line.
Protein disulfide isomerase (PDI) is a multifunctional cytoplasmic enzyme involved in post-translational modifications of secreted proteins (Freedman, 1989; Tager, et al., 1997; Sipes, et al., 1990). PDI has been shown to catalyze the formation of native disulfide bonds in nascent proteins and facilitate the folding and assembly of a wide range of disulfide-bonded proteins (Creighton, et al., 1980; Freedman, et al, 1995; Tager, et al., 1997; Sipes, et al., 1990). Besides the formation of disulfide bonds, PDI may also play a role in the transfer of oligosaccharide chains in glycosylation reactions of newly synthesized proteins (Geetha-Habib, et al., 1988; Sipes, et al, 1990) and in modification of proline residues to hydroxyproline (Pihlajaniemi, et al., 1987; Sipes, et al., 1990). It is speculated that PDI may be involved in maintaining an unfolded, open conformation of nascent polypeptides in the endoplasmic reticulum so that the appropriate co- and post-translational modifications can occur (Freedman, 1989; Sipes, et al., 1990). Inhibitors/effectors of PDI increase the density of thiol groups by inhibiting SH consumption via the sulfhydryl disulfide interchange reactions catalyzed by the PDI (Tager, et al., 1997).

Glutathione (GSH), the major cellular thiol is found to produce resistance to a wide range of cytotoxic drugs including alkylating agents, cisplatin, and anthracyclines in both *in vitro* and *in vivo* studies (Barranco, et al., 1990; Hosking, et al., 1990; Hromas, et al., 1987; Lee, et al., 1988; Lee, et al., 1989; Russo, et al., 1986; Tager, et al., 1997). Certain studies have even found lack of effect on adriamycin sensitivity and increased sensitivity to adriamycin associated with increased thiol levels (Barranco, et al., 1988; Barranco, et
Thus, the resistance to adriamycin due to the presence of thiols is cell- or cancer-specific.

Several mechanisms can be proposed by which PDI downregulation results in a drug resistance phenotype of Dox IV cells: (1) One of the proposed mechanisms of action of adriamycin is via the formation of free radicals which damage DNA. A downregulation of protein disulfide isomerase in the drug resistant cells would result in an increase in cellular thiol which in turn would quench the free radicals generated by adriamycin thus reducing its cytotoxicity. (2) An increase in GSH levels in drug resistant cell lines would result in the formation of adriamycin-GSH conjugate. A study of the accumulation of adriamycin, daunomycin and their glutathione (GSH)-conjugates, obtained by anaerobic reaction of the parent anthracyclines with reduced GSH, in drug sensitive and multidrug resistant cell lines using confocal microscopy demonstrated that conjugation of anthracycline antitumor drugs with GSH prevents their access to the nucleus and decreases their cytotoxicity (Serafino, et al., 1998). However, this type of conjugation, observed in several families of drugs, has never been documented satisfactorily for anthracyclines. Adriamycin-resistant human breast cancer MCF-7/DOX cells (showing a 14-fold increase of glutathione S-transferase activity relative to the sensitive MCF-7 cells) when treated with adriamycin in the presence of verapamil and scrutinized for any production of GSH-adriamycin conjugates (by HPLC analysis of cell content and culture broth) unequivocally show that no GSH conjugates are present either inside the cell or in the culture broth. The only anthracycline present in the cell after 24 hrs of incubation was
>98% pure adriamycin (Gaudiano, et al., 2000). Though the formation of GSH-adriamycin conjugates have not been found to form in vivo, they could possibly be forming in the drug resistant Dox 1V cells and be the basis of their drug resistant phenotype. (3) Since PDI is also known to play a role in glycosylation, a decrease in glycosylation levels of certain proteins in Dox 1V cells due to downregulation of PDI could be contributing to its drug resistant phenotype. One such protein of interest to this study is Spl. It has been shown that hypoglycosylated Spl is subject to rapid degradation by the proteasome pathway (Han, et al., 1997). Thus, a decrease in Spl would effectively mean downregulation of topoisomerase IIα which is the target of doxorubicin.

**Rad23** is a nucleotide-excision repair protein with a previously unknown biochemical function (Ortolan, et al., 2000). Besides its role in nucleotide excision repair, Rad23 is found to inhibit multi-ubiquitin (Ub) chain formation and the degradation of proteolytic substrates. The stability of proteolytic substrates is governed by the competing action of multi-Ub chain-promoting and chain-inhibiting factors (Ortolan, et al., 2000).

Regulation of protein expression can be achieved by polyubiquitination which targets them for degradation by the 26S proteasome (Fig. 6.6) (Lommel, et al., 2000; Tanaka, et al., 1998). Ubiquitin is an evolutionarily highly conserved 76 amino acid polypeptide that is abundant in all eukaryotic cells. Ubiquitin is activated by ubiquitin-activating enzyme (Uba/E1) and is transferred to ubiquitin-conjugating enzymes (Ubc/E2) and ubiquitin protein ligases (E3) through a series of transesterification reactions. Then it is ultimately
attached to lysine residues in target proteins through the formation of isopeptide bonds (Lommel, et al., 2000; Tanaka, et al., 1998; Hofmann, et al., 1999). The consecutive addition of ubiquitin moieties to the substrate by ubiquitin chain assembly factors (E4) generates a polyubiquitin chain which targets the protein to the 26S proteasome (Lomme, et al., 2000; Koegl, et al., 1999).

The 26S proteasome consists of two different subunits—the 19S regulatory subunit and the 20S catalytic core subunit (Lommel, et al., 2000; Varshavshy, 1997). The 20S subunit is a barrel-shaped protein complex with the catalytic sites on the interior surface. Astride each end of the 20S core is a 19S regulatory subunit, also called PA700 which contains 700kDa proteasome activators (Lommel, et al., 2000). The multi-ubiquitin side chain both targets and tethers substrates to the S5 subunit of the PA700. ATP-dependent unfolding of the substrate allows its translocation through the 13A° entrance of the 20S catalytic channel. This translocation may be accompanied by partial disassembly of the ubiquitin chains. Peptidases on the inner surface of 20S degrade the substrate, releasing ubiquitinated peptides. Ubiquitin is then recycled by the action of deubiquitinating enzymes on these fragments.

Rad23/hHR23 is related to ubiquitin but differs in terms of primary sequence and three-dimensional structure. It is able to bind the so-called ubiquitin binding subunit (S5) of the 19S regulator of the 26S proteasome (Hiyama, et al., 1999). It has been recently demonstrated that hHR23 acts as a competitor for lysozyme degradation in reticulocyte
extracts without its own degradation (Hiyama, et al., 1999). Thus, the Ub-like domain of the hHR23 protein is very competitive with multi-ubiquitin chain and acts as a negative regulator of proteolysis via specific interaction with S5 (Hiyama, et al., 1999; Lommel, et al., 2000; Ortolan, et al., 2000).

Of the several proteins that are degraded via the ubiquitin-proteasome pathway, those of specific interest to this investigation are the topoisomerase IIα, Sp1 and c-myb. Degradation of all the three proteins, topoisomerase IIα (Salmena, et al., 2001), Sp1 (Han, et al., 1997; Su, et al., 1999) and c-myb (Bies, et al., 1997; Bies, et al., 2000; Feikova, et al., 2000) has been shown to occur via the ubiquitin-proteasome pathway. Downregulation of hHR23 in the drug resistant cell lines translates to decreased competition with ubiquitinated topoisomerase IIα, Sp1 and c-myb. Thus, there would be increased degradation of these proteins in the drug resistant cell lines Dox 1V+ and Dox 1V- compared to the parent 8226 cell line. Consistent with this hypothesis all the three proteins have been found to be lowered in the drug resistant cell lines Dox 1V+ and Dox 1V- compared to the parent 8226 cell line. Also, preliminary experiments with pulse labeling of proteins indicated that Sp1 was synthesized at similar rates in all the three cell lines (data not shown). Therefore, decreased levels would result from increased degradation in the drug resistant cells. Decreased levels of Sp1 and c-myb would contribute to decreased transcription of topoisomerase IIα. In addition to decreased expression, increased degradation of topoisomerase IIα would result in the drug resistant phenotype of Dox 1V+ and Dox 1V- cells.
Spot #6: Heterogenous nuclear ribonucleoprotein (hnRNP) H and Hypothetical protein FLJ10830

Spot #6 was identified as Heterogenous nuclear ribonucleoprotein (hnRNP) H and hypothetical protein FLJ10830 (Fig. 6.7). hnRNP H is a factor involved in regulating pre-mRNA splicing. It is capable of binding to exonic splicing silencer (ESS) to inactivate splicing (Chen, et al., 1999) or to intronic splicing enhancer sequence to activate splicing (Chou, et al., 1999). The function of the hypothetical protein is not known. Therefore, not enough is known about these proteins to implicate them in drug resistance to doxorubicin. However, there may be some novel mechanism of resistance involving these proteins that has not yet been identified.

Spot #7: Rho GDP dissociation inhibitor (GDI) beta

Spot #7 was identified as rho GDP dissociation inhibitor (GDI) beta (Fig. 6.8). Rho GDI is a general regulator that forms a complex with the GDP-bound inactive form of the Rho family small G proteins (including: RhoA, B, C, Rac1, 2, Cdc42) and inhibits their activation (Sinha, et al., 1999; Takahashi, et al., 1997; Takai, et al., 1995). This protein was found to be downregulated in both the drug resistant cell lines compared to the parent 8226 cell line.

The main substrate for Rho-GDI is Rho. Rho undergoes interconversion between active (GTP-bound) and inactive (GDP-bound) forms which is regulated by: GDP/GTP exchange protein (GEP), GDP dissociation inhibitor (GDI), and GTPase activating

Overexpression of Rho-GDI has been reported in the mitoxantrone (a topoisomerase II inhibitor) resistant fibrosarcoma cell line (Sinha, et al., 1999). It was postulated that since GDI forms complexes with Rho, Rac1 or Cdc42 thus inactivating them, an increase in this protein would block the apoptotic signal pathway mediated by Ras and c-Jun kinase. However, in the present investigation Rho-GDI was found to be downregulated in the resistant cell lines rather than being upregulated as expected from this postulation. It has been shown that the 28 kDa mature form of Rho-GDI underwent proteolytic cleavage to give a 5 kDa and 23 kDa fragment concomitantly with DNA digestion in cells induced to undergo apoptosis (Krieser, et al., 1999). Cleavage of Rho-GDI was found to occur simultaneously with the activation of caspase-3 but preceded DNA fragmentation and the morphological changes associated with apoptotic cell death (Essmann, et al., 2000; Pan, et al., 2001). Inhibitor experiments with Z-DEVD-fmk, a selective irreversible inhibitor of caspase-3/CPP32 (in which Z stands for benzyloxy carbonyl and fmk for fluoromethyl...
ketone) and microsequencing of the Rho-GDI fragment revealed that the cleavage occurred at the caspase-3 cleavage site. Thus, Rho-GDI is regulated by the proteolytic activity of caspase-3 which cleaves it at amino acid 19 (Essmann, et al., 2000). Cleavage of Rho-GDI would relieve the block from Rho-GTPase and induce apoptosis. Also, the 23 kDa fragment translocates into the nucleus and induces apoptosis by activating the Jun N-terminal kinase (Fig. 6.9).

Analysis of the MALDI-MS data shows that the tryptic peptide of amino acids 5-21 was identified in the MS suggesting that the protein in the spot was not cleaved by caspase 3. Based on these findings it can be postulated that there is reduced amount of caspase 3 substrate in the Dox IV cells which would result in reduced levels of the 23 kDa product. Lower level of the cleaved protein could thus result in the drug resistant phenotype of Dox IV+ and Dox IV- due to reduced apoptosis in these cells via the Jun N-terminal kinase. On the other hand this would also mean that the Rho protein would be relieved from the GDP-bound state and be able to induce apoptosis. However, there may be some compensatory mechanism present in the Dox IV cells which could be reducing the apoptotic effect of Rho protein.

Spot #8: Unnamed protein product Genpept accession # 12405531

Spot #8 was identified as an unnamed protein (accession # 12405531) (Fig. 6.10). The function of this protein is still unknown. Therefore, it is not possible to analyze its effect on doxorubicin resistance.
DISCUSSION

Our study of the parent 8226 cell line and its drug resistant counterparts Dox IV+ and Dox IV- using two-dimensional electrophoresis has shed light on nine proteins that may be involved in the development of drug resistance.

Heterogenous nuclear ribonucleoprotein (hnRNP) A2/B1 is an RNA binding protein involved in pre-mRNA processing. Also, due to reasons unknown it has been found to be elevated in certain cancers, especially lung cancer and has been used as a marker for diagnosis of cancer far in advance of clinical presentation (Kamma, et al., 2001a; Kamma, et al., 2001b; Satoh, et al., 2000; Zhou, et al., 1996). In addition to its role in pre-mRNA processing it also has functions in ssDNA binding and is able to bind telomeric sequences. Binding of hnRNP A2/B1 to telomeric sequences protects it from nuclease digestion and its helicase function promotes telomerase activity. Its roles in promoting telomerase function may be the key to maintaining telomeres in cancer cells (Kamma, et al., 2001a). Due its overexpression in Dox IV+ cells, these cells are better able to maintain telomeres and prevent DNA degradation in response to apoptotic signals compared to the Dox IV- and 8226 cells. Also, the presence of this protein could protect the telomeres from topoisomerase II which has cleavage sites in telomeres thereby resulting in reduced sensitivity to topoisomerase II poisons. However, since this effect is only dependent on the presence of selection pressure it cannot be implicated to be a true cause of resistance to doxorubicin or it could contribute to the slightly higher degree of
resistance of the Dox IV+ cells (as seen by the cytotoxicity assay in Chapter 2) compared to the resistance of Dox IV- cells, though the difference was not found to be statistically significant.

UDP-N-acetylhexosamine pyrophosphorylase is the enzyme involved in the interconversions of HexNAc-1-P and UDP-HexNAc (Szumilo, et al., 1996; Wang-Gilliam, et al., 1998). UDP-HexNAc is the substrate for O-HexNAc transferase which catalyzes the O-glycosylation of proteins at serine and threonine residues (Boehmelt, et al., 2000; Yang, et al., 2001). A number of transcription factors are glycosylated, usually in their activation domains, affecting their transcription activity (Comer, et al., 1999; Jackson, et al., 1988; Yang, et al., 2001). Thus, in the present study it can be postulated that UDP-N-acetylhexosamine pyrophosphorylase indirectly confers drug resistance to the Dox IV cells by glycosylating the transcription factor Sp1. Glycosylation of Sp1 would inhibit its ability to transcribe topoisomerase IIα gene resulting in lower levels of topoisomerase IIα.

Aldose reductase is an oxidoreductase catalyzing the reversible reaction of aldose to alditol using NAD(P)H as donor (Ax, et al., 2000). It has been found to reduce anthracyclines (daunorubicin and doxorubicin) to their less potent daunorubicinol and doxorubicinol forms (Ahmed, et al., 1978; Ax, et al., 2000). Thus, the overexpression of aldose reductase in Dox IV cells could be responsible for the drug resistant phenotype by detoxifying the drug.
Protein disulfide isomerase is a multifunctional protein with important physiological functions (Sipes, et al., 1990). This enzyme plays a crucial role in regulating cellular thiol levels. Thus, a decrease in PDI has been shown to result in increased thiols in the cells (Tager, et al., 1997). An increase in GSH, the major cellular thiol, has been found to be associated with resistance to several anti-tumor agents including Adriamycin (Barranco, et al., 1990; Hosking, et al., 1990; Hromas, et al., 1987; Lee, et al., 1988; Lee, et al., 1989; Russo, et al., 1986; Tager, et al., 1997). Thus, increased thiols in Dox IV cells as a result of PDI downregulation could be the cause of its drug resistant phenotype. In addition to the proposed action of thiols in drug resistance, PDI can affect the levels of Spl via its role in glycosylation. Since hypoglycosylated Spl is rapidly degraded by proteasomes (Han, et al., 1997) downregulation of PDI in the drug resistant Dox IV cells would result in the downregulation of the drug target, topoisomerase IIα.

Human homolog of RAD23 (hHR23) is an evolutionarily conserved protein which has a regulatory role in nucleotide-excision repair (Batty, et al., 2000; Schauber, et al., 1998). It has also been found to have ubiquitin like domains. Therefore, hHR23 may be associated with ubiquitin metabolic pathways outside the context of the core NER machinery (Hiyama, et al., 1999). It has been found to bind to S5 subunit of the 19S regulatory subunit of the preteasome and competitively inhibit the degradation of polyubiquitinylated proteins (Hiyama, et al., 1999; Lommel, et al., 2000; Ortolan, et al., 2000). Since topoisomerase IIα, Spl and c-myb are known to degrade via the ubiquitin-proteasome pathway (Bies, et al., 1997; Bies, et al., 2000; Feikova, et al., 2000; Han, et
al., 1997; Salmena, et al., 2001; Su, et al., 1999), less competition from hHR23 in the Dox IV cells would result in higher degradation of these proteins in the drug resistant cell lines. Reduced transcription and increased degradation of topoisomerase IIα in Dox IV cells results in its drug resistant phenotype.

Heterogenous nuclear ribonucleoprotein H is a member of the hnRNP family of proteins involved in regulation of alternative splicing by binding to either the exonic splicing silencer or enhancer (Chen, et al., 1999; Chou, et al., 1999). However, so far it does not seem to have any function related to drug resistance in cancer cells.

Rho GDP dissociation inhibitor (GDI) beta is a negative regulator of the Rho family of small G proteins found abundantly in hematopoetic cells. It regulates the GDP/GTP exchange reaction by inhibiting the dissociation of GDP from and subsequent binding of GTP to Rho (Ohga, 1992; Sinha, et al., 1999; Takahashi, et al., 1997; Takaî, et al., 1995). Rho proteins are involved in a variety of cellular processes including apoptosis. Thus, a block in its activation by Rho-GDI affects cell death by apoptosis. GDI is a substrate for caspase 3 which cleaves it into a 5 kDa and 23 kDa protein. The 23 kDa protein is found to activate apoptosis via the c-Jun pathway. Since, the action of an anti-cancer drug ultimately depends on cell death, reduced apoptosis via c-Jun pathway would result in resistance of the cells to the drug.
The remaining two proteins identified i.e. hypothetical protein FLJ10830 and unnamed protein product Genpept accession # 12405531 do not have any known function and therefore cannot be assessed for their role in drug resistance.

Summarizing these results we can say that of the nine proteins identified in this study, aldose reductase (Ax, et al., 2000; Lee, et al., 2001), protein disulfide isomerase (Chaudhuri, et al., 1992; Hasegawa, et al., 1995; Sipes, et al., 1990; Tager, et al., 1997) and Rho-GDI (Sinha, et al., 1999) have already been implicated in drug resistance. Others, hnRNP A2/B1, UDP-N-acetylhexosamine pyrophosphorylase, and hHR23 can be implicated in drug resistance based on their functions.
Figure 6.1 2-DE pattern of total cell extract from Dox IV+ (a), Dox IV- (b) and RPMI 8226 (c). Proteins were detected by Coomassie staining. The black arrowhead indicates an internal standard, tropomyosin, pI 5.2 and molecular weight 32,700. The molecular weight standard lines are due to myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000) and lysozyme (14,000) which were added to the sealing agarose. The pH gradient was measured using a surface pH electrode for 3 blank IEF tube gels. Protein spots which were found to be significantly altered between the parent and drug resistant cell lines are indicated by arrows and numbered 1—8.
Figure 6.1
Figure 6.1 (continued)
Figure 6.1 (continued)
Figure 6.2  Protein identification of spot #1. (a) MALDI-MS of the tryptic digest of spot #1. 15 peaks matched peptides of the protein hnRNP A2/B1. (b) protein sequence of hnRNP A2/B1. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence.
Figure 6.2

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Figure 6.2 (continued)

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(b)

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Figure 6.3 Protein identification of spots #2 & #3. (a) MALDI-MS of the tryptic digest of spot #2. (b) MALDI-MS of the tryptic digest of spot #3. 24 peaks matched peptides of the protein UDP-N-acetylhexosamine pyrophosphorylase. (c) Protein sequence of UDP-N-acetylhexosamine pyrophosphorylase. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence.
Figure 6.3

(a)

Figure 6.3
Figure 6.3 (continued)


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Figure 6.4 Protein identification of spot #4. (a) MALDI-MS of the tryptic digest of spot #4. 12 peaks matched peptides of the protein aldose reductase. (b) protein sequence of aldose reductase. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence.
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Figure 6.5 Protein identification of spot #5. (a) MALDI-MS of the tryptic digest of spot #5. 21 peaks matched peptides of the protein disulfide isomerase and 8 peaks matched the protein human homolog of RAD23 (hHR23). (b) Protein sequence of protein disulfide isomerase. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence. (c) Protein sequence of hHR23. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence.
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Figure 6.6 26S proteasome assembly and formation and degradation of ubiquitylated protein substrates (adapted from http://www.proteasome.com).
Figure 6.7  Protein identification of spot #6. (a) MALDI-MS of the tryptic digest of spot #6. 8 peaks matched peptides of hnRNP H and 21 peaks matched the hypothetical protein FLJ10830. (b) Protein sequence of hnRNP H. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence. (c) Protein sequence of hypothetical protein FLJ10830. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence.
Figure 6.7

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Figure 6.7 (continued)

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(c)
Figure 6.8  Protein identification of spot #7. (a) MALDI-MS of the tryptic digest of spot #7. 8 peaks matched peptides of the protein rho GDP dissociation inhibitor (GDI) beta. (b) protein sequence of GDI. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence.
Figure 6.8
Figure 6.8 (continued)

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(b)
Caspase 3 cleaves GDI into a 5 kDa and a 23 kDa protein. The 23 kDa protein can activate apoptosis via the c-Jun pathway. Cleavage of GDI in turn releases the block on Rho which can be converted to the active GTP bound form and induce membrane and cytoskeleton changes leading to apoptosis.
Figure 6.10  Protein identification of spot #8. (a) MALDI-MS of the tryptic digest of spot #8. 6 peaks matched peptides of an unnamed protein (accession # 12405531). (b) protein sequence of the unnamed protein. The peptides that matched the peaks in MALDI-MS and the corresponding m/z (av) are listed in the table following the sequence.
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(b)
CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

Developments in cancer treatment, although definitive, have so far not resulted in a large increase in survival. Drug resistance to cancer chemotherapy is one of the causes of failure of treatment in several cancer types. Among the anticancer drugs, the DNA topoisomerase inhibitors represent an important group of agents. Several in vitro studies have shown a relation between drug sensitivity and expression of topoisomerase. Similarly, the cell lines under study Dox 1V+ and Dox 1V-, doxorubicin resistant variants of human multiple myeloma RPMI/8226 were thought to be expressing reduced levels of topoisomerase II in the absence of MDR-1 phenotype and topoisomerase II mutations. In a previous study this reduction in topoisomerase expression was suggested to result from reduced activity of the ubiquitous transcription factor NF-Y which has binding sites on the topoisomerase II promoter. Thus, the hypothesis was that if NF-Y was expressed at reduced levels in Dox 1V cells, its ectopic expression would increase its activity, thereby increasing the expression of topoisomerase II which would in turn alter the sensitivity of the Dox 1V cells to doxorubicin.
To this effect, I cloned the three NF-Y subunits: NF-YA, NF-YB and NF-YC (Chapter 2). In the process I found that there are several isoforms of NF-YA, some of which were previously identified while some were novel isoforms. Thus, I investigated the possibility of altered distribution of the various isoforms of NF-YA between the Dox IV and 8226 cells as the cause of reduced NF-Y activity. However, there was an equal distribution of these isoforms in the parent and drug resistant cell lines. I also maintained a drug resistant cell line without the presence of the drug (Dox IV-) to demonstrate that the drug resistant phenotype in Dox IV was stable.

In chapter 3 I investigated whether the topoisomerase II activity had been reduced in the drug resistant cell lines Dox IV+ and Dox IV- as had been demonstrated by earlier investigators. Since there was a decrease in the extractable topoisomerase II activity in both the drug resistant cell lines, I went on to study the activity of the transcription factor NF-Y. In EMSA (electrophoretic mobility shift assay) the activity of NF-Y was found to be only reduced in the Dox IV+ cells but not in the Dox IV- cells. This suggests that the reduction in activity of NF-Y could be a result of presence of the drug rather than the cause of drug resistance.

As a result I went on to investigate the roles of additional transcription factors myb and Spl which also have binding sites on the topoisomerase IIα promoter (Chapter 4). The EMSA and Western blots for myb were inconclusive because the protein was highly unstable. The EMSA with Spl binding element demonstrated a decrease in activity in
both the drug resistant cell lines. Western analysis with whole cell lysates detected lower levels of Spl in both Dox IV+ and Dox IV- cell lines compared to the parent 8226 cell line. Molecular size and preliminary experiments with anti-Sp3 antibody (which detected protein different from that detected by anti-Spl) confirmed the identity of the protein as Spl. In this chapter I also studied the effect of acetylation and methylation. Acetylation and methylation can be involved in the regulation of gene expression and relative to this study it could affect the expression of topoisomerase IIα, transacting factors involved in transcription of topoisomerase IIα, components of the apoptotic pathway, etc. Any of these alterations could affect the sensitivity of Dox IV cells to doxorubicin. However, treatment with TSA, a histone deacetylase inhibitor or 5-azaC, a cytidine analog and demethylating agent did not have any significant effect on the sensitivity of Dox IV cells to doxorubicin implying that probably acetylation and methylation are not involved in the regulation of these factors or that there was no difference in the acetylation and methylation status of Dox IV cells and the parental 8226 cells.

The reduced topoisomerase II activity seen in Chapter 3 was measured by the decatenation assay which is semi-quantitative and measures the extractable topoisomerase II. The difference seen between the drug resistant Dox IV cells and the parent 8226 cells could be due to a difference in nuclear extractability. Therefore, to investigate whether topoisomerase II expression was indeed reduced and this reduction contributed to drug resistace in Dox IV cells a topoisomerase poisoning assay was performed (Chapter 5). This assay did not demonstrate more than ~9% reduction in
topoisomerase poisoning in Dox IV cells when compared to the poisoning in the parent 8226 cells. Thus, the reduction in topoisomerase activity was nowhere close to ~50% that would be expected in the case 50% reduction in topoisomerase expression demonstrated in nuclear extracts by earlier researchers. Also, the level of drug resistance observed in Dox IV cells (2-fold) is very low for a 50% reduction in topoisomerase expression. The low level of decrease in topoisomerase poisoning explains the low level of resistance in Dox IV cells. However, this assay does not distinguish between the two isoforms of topoisomerase. Also, reduced topoisomerase II is apparently not the only cause of the drug resistant phenotype in Dox IV cells, contrary to earlier belief.

Future studies undertaken in this field need to verify whether there is a reduction in levels of topoisomerase IIβ in the Dox IV cells. This can be achieved by performing a Western blot on whole cell extracts with a topoisomerase IIβ-specific antibody. However, this method would not be able to detect an inactivating or resistance-producing mutation. An in vivo complex of topoisomerase (IVCT) assay (Yoon, et al., 1998) can determine the proportion of the two isoforms of topoisomerase II crosslinked to DNA. Thus, it would be an accurate measurement of the levels of active topoisomerase IIα and topoisomerase IIβ in the cells as well as their contribution towards drug resistant phenotype of Dox IV cells.

It was also necessary to investigate other possible mechanism(s) of resistance in the Dox IV cells. The proteomics approach of investigating global changes in gene
expression between the parent 8226 cells and its drug resistant variants Dox IV was adopted (Chapter 6). This 2D electrophoresis approach determined eight protein spots that were significantly altered in intensity between the parent and its drug resistant counterparts. Of these, four were increased or expressed in the drug resistant Dox IV cells while the other four were decreased. A decrease in a particular protein may not necessarily mean that its expression is altered. It is possible that it may have undergone post-translational modification which could alter its position on the 2D gel. This may cause the spot to disappear under another spot and if that spot if big e.g. actin or ribosomal protein then the difference may be difficult to visualize. Thus, specific antibodies will be required to determine whether these proteins were down-regulated or modified. However, for practical purposes the terms up-regulated and down-regulated will be used for explanation of their roles in drug resistance. These spots were further analyzed by MALDI-MS to determine the identity of the proteins. In all nine proteins were identified. Spot #2 and #3 were both identified as the same protein while two proteins each were identified in spot #5 and #6. Of these aldose reductase (spot #4), protein disulfide isomerase (spot #5) and Rho GDI (spot #7) have been implicated in drug resistance in earlier studies. Two other proteins UDP-N-acetylhexosamine pyrophosphorylase (spot #2 and #3) and hHR23 (spot #5) can be implicated in resistance to doxorubicin in Dox IV cells based on their functions in regulating cellular gene expression, especially Sp1, a major factor involved in regulation of topoisomerase IIα in these cells. The protein hnRNP A2/B1 (spot #1) was downregulated only in the Dox IV+ cells. Thus, it could either be the cause of the presence of drug or it could contribute to
the small percentage of higher drug resistance in Dox IV+ (as determined by the
cytotoxicity assay in Chapter 2, though the difference in drug resistance between Dox
IV+ and Dox IV- was not statistically significant) compared to Dox IV-. The rest of the
proteins, hnRNP H (spot #6) and an unnamed protein (spot #8, function unknown) could
not be implicated in resistance of Dox IV cells. These observations are summarized as
follows:

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<td>Detoxification of drug via reduction</td>
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<tr>
<td></td>
<td>PDI</td>
<td></td>
<td>Increase in thiols &amp; thiols detoxify by conjugation</td>
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<tr>
<td>Target</td>
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<td>Increased glycosylation of Sp1, therefore reduced activity in transcribing topoisomerase II</td>
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<tr>
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<td>PDI</td>
<td></td>
<td>Decreased glycosylation of Sp1, therefore increased proteosome degradation</td>
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<tr>
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<td>hHR23</td>
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<td>Reduced protection from proteasome degradation</td>
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<tr>
<td></td>
<td>Rho GDI beta</td>
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<td>Reduced apoptosis</td>
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Future research needs to be undertaken to exactly determine whether these proteins
contribute to drug resistance in Dox IV cells and what is their mode of action. Some
directions that could be taken are suggested as follows:
hnRNP A2/B1 has been proposed to protect telomeres and therefore may be inhibiting apoptosis in Dox IV cells. The effect of mutant hnRNP A2/B1 or a deletion of hnRNP A2/B1 can be used to study its effect on apoptosis and drug sensitivity.

UDP-N-acetylhexosamine pyrophosphorylase and PDI could increase glycosylation of Sp1, the transcription factor implicated in regulation of topoisomerase II expression in Dox IV cells. However, UDP-N-acetylhexosamine pyrophosphorylase is upregulated in Dox IV cells while PDI is downregulated. Therefore, Sp1 could be either hypo- or hyper-glycosylated in the Dox IV cells. Hyperglycosylation results in reduced activity (Yang, et al., 2001) which could translate to reduced expression of topoisomerase II (the drug target) while hypoglycosylation increases its degradation (Han, et al., 1997), which could once again translate to reduced expression of topoisomerase II. Thus, MALDI-TOFMS and HPLC analysis can be used to verify whether Sp1 is hypo- or hyper-glycosylated in the Dox IV cells compared to the parent 8226 cells. However, glycosylation of Sp1 occurs at several different sites which serve different functions. It is possible that glycosylations at these sites are differentially regulated. Thus, crude measures of total Sp1 glycosylation may not necessarily correlate with specific functional changes (Han, et al., 1997). A pulse chase experiment can also be performed to determine whether there is increased degradation of Sp1 in the drug resistant cells.

Aldose reductase is able to detoxify doxorubicin by reducing it to doxorubicinol, a less potent form of the drug. This enzyme is upregulated in the drug resistant Dox IV cells.
Thus, treatment of the cells with an aldose reductase inhibitor e.g. epalrestat, fidarestat, zenarestat, sorbinil, etc. could be used to prevent detoxification of doxorubicin and test its effect on sensitivity of Dox IV cells to doxorubicin.

In addition to its minor role in glycosylation, PDI regulates the levels of cellular thiols. A decrease of this enzyme in the Dox IV cells could be increasing the concentration of cellular thiols which are capable of detoxifying drugs by conjugation. This conjugation prevents the access of the drug to the nucleus and hence inhibits its action. Though conjugates of GSH-doxorubicin have not yet been detected in vivo, it could possibly be present in the drug resistant Dox IV cells. This could be determined by HPLC analysis of the cell extracts. The localization of the drug can also be determined. Since GSH conjugates are thought to be excluded from the nucleus, doxorubicin would be detected in the cytoplasm of drug resistant cells if GSH was detoxifying it.

hHR23 has ubiquitin like domains and is found to competitively inhibit the binding of ubiquitinated proteins to the S5a regulatory subunit of the 26S proteasome, thereby preventing their degradation. Thus, a decrease of this protein in the Dox IV cells would result in increased degradation of proteins via the ubiquitin-proteasome pathway. These proteins could include Sp1, c-myb and topoisomerase IIα. The effect of this protein on drug resistance can be studied by transfection to increase the expression in Dox IV cells and determining the change in sensitivity to doxorubicin.
Thus, the identification of these proteins that have different expression in drug resistant Dox IV cells and the parent 8226 cells has opened up a whole new world of possibilities. However, there is one major limitation in studying the factors responsible for drug resistance. Since drug resistance could be multifactorial with several mechanisms acting simultaneously, it would be difficult to observe small changes in sensitivity by alteration of a single factor. Thus, experiments where direct detection of the effect of the factor is possible would yield better results than the determination of drug sensitivity in response to such an alteration. Another problem is that doxorubicin is multifunctional. As a DNA intercalator it can interfere with helicases, DNA and RNA polymerases, telomerase, etc. Also, the generation of free radicals is thought to be a significant anticancer mechanism. Therefore, it is difficult to analyze multiple changes in terms of such low levels of resistance.
LIST OF REFERENCES


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