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The Nuclear Export of DNA Topoisomerase IIα in Hematological Myeloma Cell Lines as a Function of Drug Sensitivity: Clinical Implications and a Theoretical Approach for Overcoming the Observed Drug Resistance

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The Nuclear Export of DNA Topoisomerase IIα in Hematological Myeloma Cell Lines as a Function of Drug Sensitivity: Clinical Implications and a Theoretical Approach for Overcoming the Observed Drug Resistance

by

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Note to Reader

Note to Reader: The original of this document contains color that is necessary for understanding the data. The original dissertation is on file with the USF library in Tampa, FL.

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ABSTRACT

The focus of this investigation is about DNA topoisomerases, the molecular targets of clinically important chemotherapy, and mechanisms of drug resistance in human myeloma and leukemia cell lines. The ultimate goal of this investigation was to identify mechanism(s) of drug resistance to anticancer agents so that a strategy to overcome drug resistance could be conceived. We established an *in vitro* cell model by using human leukemia and myeloma cell lines to investigate possible mechanisms of drug resistance that are observed in confluent cells. Plateau cell densities demonstrated *de novo* drug resistance to commonly used chemotherapeutic agents that was independent of altered drug transport. We established that cellular drug resistance in these cells is a function of topo IIα subcellular localization and further demonstrate that topo IIα translocates to the cytoplasm in a cell-density dependent manner. We provide experimental data that supports the nuclear export of topo IIα as the most likely event contributing to drug resistance to topoisomerase II inhibitors, which occurs when transformed cells transition from log to plateau cell density. We provided a plausible nuclear export pathway for topo IIα, by identifying two

Leptomycin B sensitive nuclear export signals, which are homologous to the binding sites recognized by the nuclear export receptor, exportin-1. Thus, topo IIα is likely to be exported from the nucleus at plateau cell densities when exportin-1 binds topo IIα. We confirmed that the nuclear export signals identified in topo IIα are functional when expressed in human myeloma cells transfected with an epitope-tagged topo IIα gene. Furthermore we demonstrate that the nuclear export signals can be abolished by site-directed mutagenesis of specific amino acids residues found in the nuclear export signal. Our data may have clinical relevance because plasma cells obtained from bone marrow aspirates of patients with multiple myeloma contain a cytoplasmic distribution of topo IIα. The potential implications of a functioning nuclear enzyme located in the cytoplasm of cells and theoretical mechanisms for overcoming the observed drug resistance are considered.

Chapter One

Introduction

1.1 Cancer Trends and Statistics.

Cancer is the second leading cause of death in the United States, exceeded only by heart disease (American Cancer Society, 2005). Increased life expectancy, environmental factors, genetics, and socio-economic issues can all contribute to the emergence of cancer around the world (Knudson, 2002; Doll, 1996). According to the American Cancer Society (ACS), approximately 1,368,030 new cancer cases will have been diagnosed in the year 2004 and 563,700 Americans are expected to have died of cancer, more than 1500 people per day. The ACS anticipates the trend in further cancer cases to continue for the year 2005. The National Institutes of Health also estimate that medically related cancer costs will exceed the initial 2004 projection of 200 billion dollars (National Institute of Health, 2005). Preventive measures have been insufficient at reducing the incidence of cancer, thus driving numerous medical researchers to seek new drugs and drug combinations for more effective cancer treatment. The sum of these investigations has lead to the development of new treatment strategies, promising anti-cancer agents, and improved quality of life for many cancer patients. Consider, for instance, the recent advancements in the clinical management of multiple myeloma, a mostly incurable hematologic malignancy (Morgan and Davies, 2005; Rajkumar et al.,

2002). For example, the administration of melphalan plus prednisone has been the conventional chemotherapy given for the treatment of multiple myeloma (Rajkuman et al., 2002). The overall response rate with this regimen is approximately 50%, but the complete response rate is less than 10% and the median survival is approximately 3 years (Rajkumar et al., 2002). High-dose therapy followed by autologous stem cell transplantation is a newer treatment strategy for multiple myeloma with improved response rates. Response rates with this strategy exceed 75% to 90% and complete response rates range from 20% to 40% (Rajkuman et al., 2002). Despite these improvements however, multiple myeloma remains to be a mostly incurable plasma cell malignancy with a median survival of 3-4 years (Terpos et al., 2005). Even when conventional therapy or high-dose chemotherapy is administered with autologous stem cell transplantation, myeloma patients continue to relapse (Rajkuman et al., 2002). These findings can be attributed in large part to unresponsive or drug resistant cellular phenotypes at the time of diagnosis or relapse (Yang et al., 2003). In these instances, a detailed understanding of the mechanisms of drug action and a rationale for their effective application is required before effective cancer therapy can be delivered. Thus, medical and scientific investigators have combined efforts to analyze the molecular basis of cellular drug resistance in the laboratory to improve clinical outcome, often referred to as translational or "bench-to-bedside" medicine (Horousseau et al., 2004; Goessel, 2003; Fishman and Sullivan, 2001). A group of enzymes that regulate DNA topology, called DNA topoisomerases (topos), are at the center of many of the translational studies that are addressing cellular drug resistance in hematological malignancies.

DNA topoisomerases are a key subject in cancer research because these proteins are the molecular targets of some of the most commonly used chemotherapy agents in the treatment of human cancers. Topo targeted agents have been used in the effective treatment of leukemias, lymphomas, multiple myeloma, breast cancer, and other malignancies. However, their clinical effectiveness is often limited by acquired (after drug exposure) or intrinsic cellular drug (before drug exposure) resistance, especially in the treatment of multiple myeloma (Valkov and Sullivan, 1997). Thus, researchers are investigating acquired and intrinsic mechanisms of drug resistance at the molecular level, to circumvent drug resistance observed in myeloma patients.

The focus of this investigation is about DNA topoisomerases, their roles as molecular targets of chemotherapy, and mechanisms of cellular drug resistance to topoisomerase targeting agents. The ultimate goal of this investigation was to identify mechanism(s) of drug resistance so that a strategy to overcome drug resistance could be conceived. We established an *in vitro* cell model that demonstrated *de novo* drug resistance to commonly used chemotherapeutic agents. We determined that the observed drug resistance was specific to topoisomerase targeting agents. We provide experimental data that supports the nuclear export of topo IIα as the most likely event contributing to drug resistance in our cell model. We established a plausible nuclear export pathway for topo IIα by identifying two Leptomycin B sensitive nuclear export signals, and then demonstrate that these signals are functional in human myeloma cells transfected with a FLAG tagged topo IIα gene. Hematological cell lines are used throughout this discussion as a cell model for elucidating the role that topoisomerases impart on intrinsic cellular drug resistance to anti-cancer agents.

1.2 Discussion Points.

To appreciate the mechanisms of cellular drug resistance to topo targeting cytotoxic agents, several concepts are reviewed. First, the fundamental concepts for the molecular basis of cancer pathogenesis are covered in chapter two followed by a review of human DNA topoisomerases, including but not limited to, their structure, function, and mechanisms of catalysis. Chapters four and five depicts the physical biochemistry of commonly used anti-cancer agents with particular attention to cytotoxic agents that target DNA topoisomerase I and II. These chapters detail how specific anti-cancer agents exert their cytotoxic effects, a subject that is necessary to realize how cells evade drug cytotoxicity. Chapters six and seven cover our current understanding of *in vitro* and *in vivo* mechanisms of *de novo* cellular drug resistance related to multiple myeloma. These chapters illustrate many of the challenges that clinicians must overcome for therapy to become more effective. The mechanisms of nuclear-cytoplasmic transport of proteins are discussed in chapter eight because the subcellular distribution of proteins between the nuclear and cytoplasmic compartments has recently been established as another mechanism of regulating protein function. Finally, the nuclear-cytoplasmic transport of topo is revealed as a novel mechanism of cellular drug resistance in human myeloma cell lines in chapters eleven through thirteen. The significance of these findings in the clinical environment is assessed and future projections are deliberated.

Chapter Two

Molecular Basis of Cancer Pathogenesis

2.1 What is Cancer?

Cancer is not a single disease but rather a group of diseases characterized by the proliferation of cells that bypass regulatory checkpoints of the cell-cycle and/or escape induction of apoptosis (reviewed in Bocchetta and Carbone, 2004; Bertram, 2000). In general, the proteins responsible for maintaining DNA and the cell cycle can be divided into six categories that include: (1) cytokines/growth factors; (2) growth factor receptors; (3) intracellular signaling molecules; (4) transcription factors; (5) cell-cycle/apoptotic regulatory proteins; and (6) proteins that regulate DNA topology and metabolism (i.e., DNA topoisomerases). There is a high degree of cross-talk between proteins of the same class and between different classes of proteins, resulting in a network of highly synchronized events often referred to as a signal transduction cascade. Malignant cells usually acquire DNA mutations in one or more of these cell cycle or DNA regulatory proteins, which gives them growth or proliferative advantages over other cells. Since proteins function as part of a coordinated network with other proteins, even a single gene mutation can result in the propagation of additional gene mutations (i.e., if a gene mutation occurs in a DNA repair protein) or proliferative advantages (i.e., if a gene mutation occurs in a growth factor receptor). These genetic advantages disrupt the

balance between cell proliferation and cell death. For example, the retinoblastoma protein (pRb) is encoded by the RB tumor suppressor gene and regulates the cell cycle by arresting DNA replication when DNA damage is present (Sellers, 1997). Mutations in the RB gene occur in 30 to 40% of all human cancers, thus permitting cells with DNA damage to divide endlessly (Merck, 2004). In addition to genetic mutations, tumor cells can also influence their microenvironment to favor their survival. For example, tumor cells secrete or promote the secretion of cytokines and growth factors to stimulate angiogenesis (Sivridis et al., 2003). The new blood vessels supply nutrients and additional cytokines that continue to activate cell signaling pathways that further tumor sustenance. To better illustrate some of the points described above, let us take a closer look at one type of cancer, multiple myeloma.

2.2 Cancer Biology of Multiple Myeloma:

Multiple myeloma is a type of B-cell neoplasm characterized by the slow proliferation of well-differentiated plasma cells in the bone marrow (Bhawna, 2003; Ho et al., 2002). Although mature plasma cells are the predominant type of cell found in multiple myeloma, pre-B, myeloid and T-cells are coexpressed with the mature plasma cell (Barlogie, 1989). The population of malignant cells is collectively referred to as myeloma cells.

Multiple myeloma tumor progression is a multistep process that can be described to occur in three distinct phases: immortalization, unchecked cell proliferation, and dissemination of the malignant plasma cells (Pratt, 2002; Shain et al., 2000). The disease may first present as a monoclonal gammopathy of undetermined significance (MGUS), a

clinical term used to describe a single plasma cell that has formed a limited number of clones (Tricot, 2002; Dalton et al., 2001). The precise cellular origin is not well understood, but is believed to be an immature B-cell that has been repeatedly exposed to antigen in the germinal centers of the lymphoid tissue. The disease advances when the transformed cells clonally expand and grow in the bone marrow (intramedullary myeloma) (Van Riet et al., 1998). During this time, the disease is usually chemosensitive and may enter a dormant phase (Pratt, 2002). The myeloma cells receive growth and anti-apoptotic signals that facilitate their survival through interactions with the bone marrow microenvironment (Dalton, 2003; Shain, et al., 2000). For example, expression of the adhesion receptors, very late antigen-4 (VLA-4) and very late antigen-5 (VLA-5) (Hata et al., 1993), permits myeloma cells to bind fibronectin, whereas the expression of the β2 integrin lymphocyte function associated antigen-1 allows for cell-cell aggregation (Asosingh et al., 2003; Kawano et al., 1991). Such interactions stimulate the secretion of various cytokines from myeloma and stromal cells in the bone marrow microenvironment (Klein et al., 2003). IL-6, the main plasma cell activating factor, stimulates cell proliferation via the Ras/Mitogen-activated kinase (Ras/MAPK) signaling cascade (Kawano et al., 1988; Ogata et al., 1997). IL-6 also induces myeloma cell migration and angiogenesis via vascular epithelial growth factor (VEGF) induction (Dankbar et al., 2000). Advanced disease is distinguished by independence from growth factors in the bone marrow microenvironment and proliferation to extramedullary sites (extramedullary myeloma) (Pratt, 2002). Throughout this time, advanced myeloma is characterized by resistance to apoptosis and chemotoxic agents (Dalton, 2003). Understanding the bone marrow microenvironment in multiple myeloma disease pathogenesis has had a role in

the development of new cytotoxic agents. For example, bortezomib is the first proteasome inhibitor to be evaluated in clinical trials for the treatment of hematological malignancies (Adams, 2004; Adams and Kauffman, 2004).

2.3 Conclusions.

In summary, cancer is a multifaceted disease that is propagated by multiple DNA mutations that are acquired via hereditary and natural means. The gene mutations can result in altered cellular homeostasis and confer growth and proliferative advantages. In turn, the collection of proteins that are involved in regulating the cell cycle and DNA replication/topology are the frequent targets of anti-cancer drugs (Damiens, 2000). Thus, understanding the proteins involved in the intracellular cell signaling pathways, DNA replication/topology, and cell cycle regulation are the cornerstones of cancer research.

The focus of this investigation is about DNA topoisomerases, enzymes that regulate DNA topology. We focus on the role of DNA topoisomerases as molecular targets of anti-cancer agents and mechanisms of drug resistance. Although the complete family of human DNA topoisomerases is reviewed, the focus of this work centers around type II enzymes, topo IIα and topo IIβ, the current targets of cancer chemotherapy.

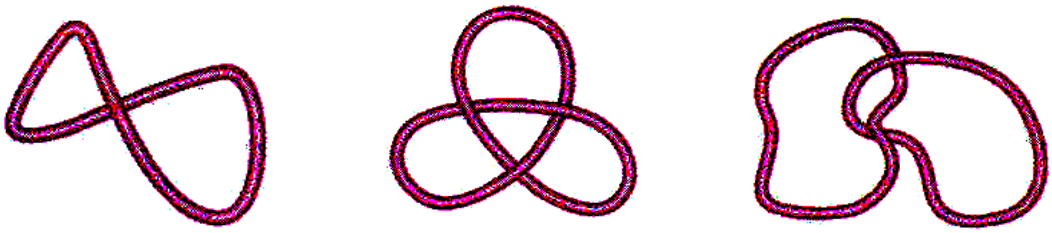
Chapter Three

Biochemistry of DNA Topoisomerases

3.1 General Introduction to the DNA Topoisomerase Family of Enzymes.

The human genome consists of approximately 3.2 billion base pairs and would be about 2 meters in length, if stretched out from end to end (Porter et al., 2004). Nevertheless, DNA squeezes into the nucleus, which is approximately 6 μM in diameter (Porter et al., 2004). For example, the smallest human chromosome is 14 mm long but is compacted to just 2 μM in length (Ball, 2003; Greeley, 1978). Thus, DNA exists in a tightly compacted and dense form. Conversely, DNA must also allow itself to become accessible to numerous transcription factors and regulatory proteins to allow gene transcription and DNA replication to occur (Khorasanizadeh, 2004). The ability of DNA strands to separate and/or unwind, however, is limited by the torsional constraints of a double helix. DNA topoisomerases are a family of nuclear enzymes that regulate the topological state and relieve the torsional stress of the DNA that arise during gene transcription, DNA replication, and cell division (Champoux, 2001; Wang, 1996). DNA can become tangled into knots, supercoiled, and form interlocked circles that must be resolved by topo action (Figure 1) (Pulleyblank, 1997). To regulate DNA topology, all topo enzymes introduce single- or double strand breaks in DNA to allow another strand of DNA or duplex DNA to pass through the newly generated gap (Champoux, 2001). Topo activity has been shown to be important during DNA replication, transcription,

genetic recombination, sister chromatid segregation, and chromosome condensation and decondensation. For example, the human type I and II topoisomerases relax positive supercoils that are generated in front of the moving replication fork. This is significant because RNA polymerase generates tension in the DNA that must be relieved for gene transcription to occur. Topo II also has an essential role in the proper execution of DNA replication and cell division. Newly synthesized DNA strands must be completely unlinked from the parent strand and every replicated chromosome must be disentangled from each other to allow proper segregation of the chromosomes into daughter cells.



a. Supercoiled b. Knotted c. Catenated DNA

Figure 1. Topological Configurations in DNA that are Resolved by Topo. (a) Twisting of DNA about its axis results in either positive or negatively supercoiled DNA, (b) DNA knots, and (c) catenated DNA is when two or more circles of DNA interlock. Although the family of topoisomerase enzymes are required for DNA replication, recombination, transcription and chromosome segregation, each type of enzyme makes different contributions to each process. For example, human topo I and II are able to relax supercoiled DNA, but only topo II is proficient knotting/unknotting and catenation/decatenation reactions. This figure was reproduced from reference Pulleyblank, 1997.

"Molecular Houdini's" and enzymes with a "double-edged sword" are two terms that are used to describe DNA topoisomerases because the integrity of DNA is susceptible to topo cleavage activity (Osheroff, 1998; Anderson and Berger, 1994). To ensure the integrity of DNA, all DNA breaks generated by topo are accompanied by the

formation of a covalent phosphotyrosine bond that tethers topo to the broken strand of DNA while DNA strand passage occurs (Tse et al., 1980). The covalent topo-DNA intermediate, called a "cleavage complex" or a "cleavable complex", is transient and freely reversible (Roca and Wang, 1992). Some anticancer agents exploit the DNA cleavage activity of topo to turn these enzymes into molecular toxins (Nitiss and Wang, 1996). Thus, there has been considerable interest in topo because they have become the molecular targets of some clinically important anticancer agents.

In summary, DNA topoisomerase enzyme activity is essential for maintaining the topology of DNA during many DNA processes, including DNA replication, transcription, and chromosome segregation. DNA topoisomerases have also emerged as important molecular targets of anti-cancer agents, especially in hematological malignancies (Wang, 1987; Zijlstra et al., 1990). The next topic to be covered describes the nomenclature and classification system for the family of DNA topoisomerases. This will be followed by a complete description of each type of human topoisomerases.

3.2 Classification of Mammalian DNA Topoisomerase.

To date, six different DNA topoisomerases have been identified in humans and mice (referred to as topo I, topo II, and topo III, etc) and are classified as either type I or II enzymes (Champoux, 2001; Wang, 1996). The human type I, type II, and type III topo enzymes have been distinguished by differences in their gene locus, molecular weights, catalytic requirements, mechanism of catalysis, and biological roles (table 1) (Champoux, 2001; Wang, 1996). Type I enzymes include topo III, topo I, and mitochondrial DNA topo I (topo Imt). All type I enzymes are active as monomers and catalyze transient

single strand DNA breaks without ATP. The type I enzymes can be further distinguished as IA or IB enzymes based on more specific differences in their reaction mechanisms. For example, the type IA enzymes covalently bind to the 5'-end of the cleaved DNA strand while type IB enzymes bind to the 3'-end of the cleaved DNA strand.

In contrast to type I enzymes, type II enzymes form dimers and couple double-strand DNA breaks with ATP hydrolysis. Type II enzymes have two known isoforms referred to as topo IIα and topo IIβ. Several monoclonal and polyclonal antibodies have been used to detect the different enzymes by Western blot and immunohistochemistry. Monoclonal antibodies are available for human topo I (Beidler and Cheng, 1995), whereas several monoclonal (Kellner et al., 1997; Negri et al., 1993) and polyclonal antibodies are available for human topo IIα (Sullivan et al., 1993) and topo IIβ (Austin et al., 1995). One monoclonal antibody has been used to detect topo IIIα expression by immunohistochemistry (Lodge, 2000).

A review covering human type I and type II enzymes ensues. The type I enzymes (i.e., topo Imt, topo I, and topo III) are discussed first, followed by a discussion of the type II enzymes (topo IIα and topo IIβ). The type II enzymes are the focus of the work presented here and thus, are discussed in greater detail than the type I enzymes.

Table 1. Characteristics of Human DNA Topoisomerases I, II, and III*

	Topo I	Topo Imt**	Topo IIα	Topo IIβ	Topo IIIα	Topo IIIβ
Type	IB	IB	IIA	IIA	IA	IA
Chromosome	20q12-13.2	8q24.3	17q21-22	3q24	17p11.2-12	22q11
Molecular Weight (kDa)	91	60-70	170	180	~110	~96
mRNA (kb)	4.2	nd[a]	6.2	6.5	4.0-7.2	2.8-3.8
DNA strand cleavage	single	nd[a]	double	double	single	single
Covalent Intermediate	3'	nd[a]	5'	5'	5'	5'
ATP dependence	no	no	yes	yes	no	no
Cell cycle dependence	no	nd[a]	yes, maximal in G2/M	no	nd[a]	nd[a]
Knockout mouse	early embryonic lethal	nd[a]	presumed lethal	perinatal death	early embryonic lethal	shortened mean life span
Active site	Tyr-723	predicted Tyr-559	Tyr-805	Tyr-821	predicted Tyr-337	predicted Tyr-336
Location	nucleolus nucleoplasm	mitochondria	nucleoplasm nuclear matrix	nucleoplasm nucleolus	nucleus[d] mitochondria[d]	isoform I: nucleoplasm[ee] isoform II: cytoplasm[ee]
Intracellular Targeting Signal(s)	1. basic NLS[b] 2. acidic NLS[c] 3. nucleolar[c]	1. mitochondrial*	1. bipartite NLS[f] 2. LMB sensitive NES[g] (*in vitro/in vivo*)	1. bipartite NLS[f] 2. LMB sensitive NES[h], (*in vitro, only*)	1. mitochondrial[d], (*in vitro/in vivo*)	nd[a]
Inhibitors	topotecan, karenitecin, 9-nitrocamptothecin, camptothecin, CPT-11	CPT sensitive; potential molecular target	VP-16, VM-26, mitoxantrone, m-AMSA, doxorubicin, merbarone, daunomycin, ICRF-193, ICRF-187, ICRF-159, and XK469 (beta specific)		potential molecular target	potential molecular target

3.3 Type IB: Human Mitochondrial DNA Topoisomerase I (Topo Imt).

There are approximately 1000 mitochondria in every mammalian cell (Zhang et al., 2004a). Each human mitochondrion contains 5-10 copies of closed circular mitochondrial DNA (mtDNA) that has16, 569 base pairs (Moraes, 2001; Anderson, 1981; Bibb, 1981). Human mtDNA encodes 22 tRNAs, 13mRNAs, 12S and 16S rRNA (Anderson, 1981). DNA topo enzyme activity resolves the topological constraints of DNA within the mitochondria (Wang et al., 2002). The human *TOP1mt* gene is located on chromosome 8q24.3 and encodes for a 601 amino acid polypeptide that is highly homologous to DNA topo I (Zhang et al., 2001). The protein was identified as mitochondrial DNA topo I (topo Imt) because it contains a mitochondrial targeting sequence and lacks a nuclear localization signal (Zhang et al., 2001). Topo Imt also has different biochemical properties from those of the nuclear enzyme, DNA topo I (Zhang et al, 2001). A MegaBlast search of all National Library of Medicine databases was conducted and indicated that only vertebrates contain both mitochondrial and nuclear topo I gene (Zhang et al., 2004a). These results raise the question of how non-vertebrates perform mitochondrial DNA metabolic functions. It is possible that one gene encodes two polypeptides that are targeted either to the nucleus or to the mitochondria (Zhange et al., 2004a). Current investigations are underway to elucidate gene mutations in mitochondrial topo I that could potentiate human disease. Thus, topo Imt could evolve as a new molecular target in the future. Unless otherwise noted, this discussion will focus on nuclear DNA topo I, the current molecular target of anticancer drugs.

3.4 Type IB: Human DNA Topoisomerase I (Topo I).

Eukaryotic topo I was first described in mammalian cells in 1972 (Champoux and Dulbecco, 1972). Although topo I gene knockouts are lethal in mouse models, yeast topo I gene knockouts are viable (Champoux, 2001). The gene for human DNA topo I was cloned in 1988 and the complete amino acid sequence of topo I protein is shown in Appendix A on page 270 of this manuscript (Juan et al., 1988). The structure topo I includes four functional domains (Figure 2): a highly charged N-terminal domain, a conserved core domain and an essential C-terminal domain (Redinbo et al., 1999; Stewart et al, 1996). The C-terminal domain contains the active site tyrosine residue (Tyr^{723}) (Eng et al., 1989) and is essential for DNA binding and relaxation *in vitro,* whereas the

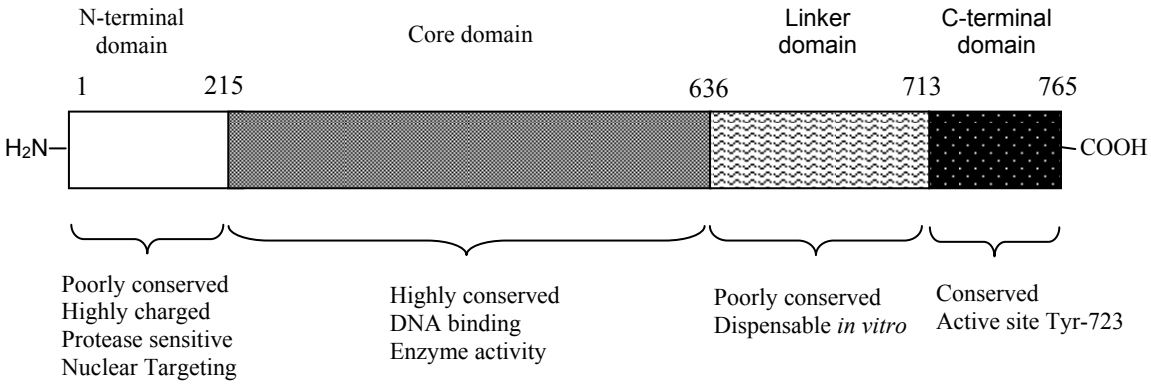


Figure 2. Structural Domains of Human DNA Topoisomerase I. Topo I has four domains: an N-terminal domain (▭) (amino acids 1-215), a core domain (▩) (amino acids 215-636), a linker domain (▨) (amino acids 636-713), and a C-terminal domain (■) (amino acids 713-765). The most significant properties are described below each domain. Figure reproduced from reference (Champoux, 2001).

amino terminal region may have an important role in regulating the cellular localization of topo I because it contains the nuclear localization signal (NLS) and nucleolin binding site. Amino acids 150-156 have been shown to be sufficient for nuclear localization

(D'Arpa et al, 1988), but a second NLS has been identified within amino acids 117-146 (Mo et al., 2000). Topo I shows a stable expression throughout the cell cycle (Heck et al., 1988). Topo I is post-translationally modified by phosphorylation (D'Arpa and Liu, 1995) and ubiquitination (Desai et al., 1997), which may be important for regulating its activity, localization or degradation. Topo I has an important role in transcription, rRNA synthesis, DNA replication and transcription initiation and is the specific target for camptothecin (CPT) and its derivatives (topotecan and CPT-11) (D'Arpa, 1990).

DNA topo I catalyzes transient single strand DNA breaks to relax DNA supercoils (reviewed in Wang, 2002). Topo I is capable of relaxing both negatively and positively supercoiled DNA without the presence of a cofactor, but metal cations such as Mg^{2+} stimulate topo I activity (Liu and Miller, 1981). Figure 3 illustrates the catalytic cycle of topo I. To cleave DNA, topo I catalyzes the transesterfication of the active site tyrosine (Tyr-723) to form a 3'-phosphotyrosine covalent enzyme DNA intermediate (D'Arpa et al., 1988). The 3'-phosphotyrosine bond is formed when the O-4 oxygen of tyrosine at position 723 (Tyr723) completes a nucleophilic attack on the scissile phosphate in the DNA backbone (D'Arpa e al., 1988). The formation of the 3'-phosphotryosine bond is significant because it forms a "proteinaceous" bridge between topo I and DNA, and thus ensures the integrity of DNA. The covalent topo-DNA intermediate is also referred to as a "cleavage complex" (Pommier, 2003) or "cleavable complex" (Tewey et al., 1984).

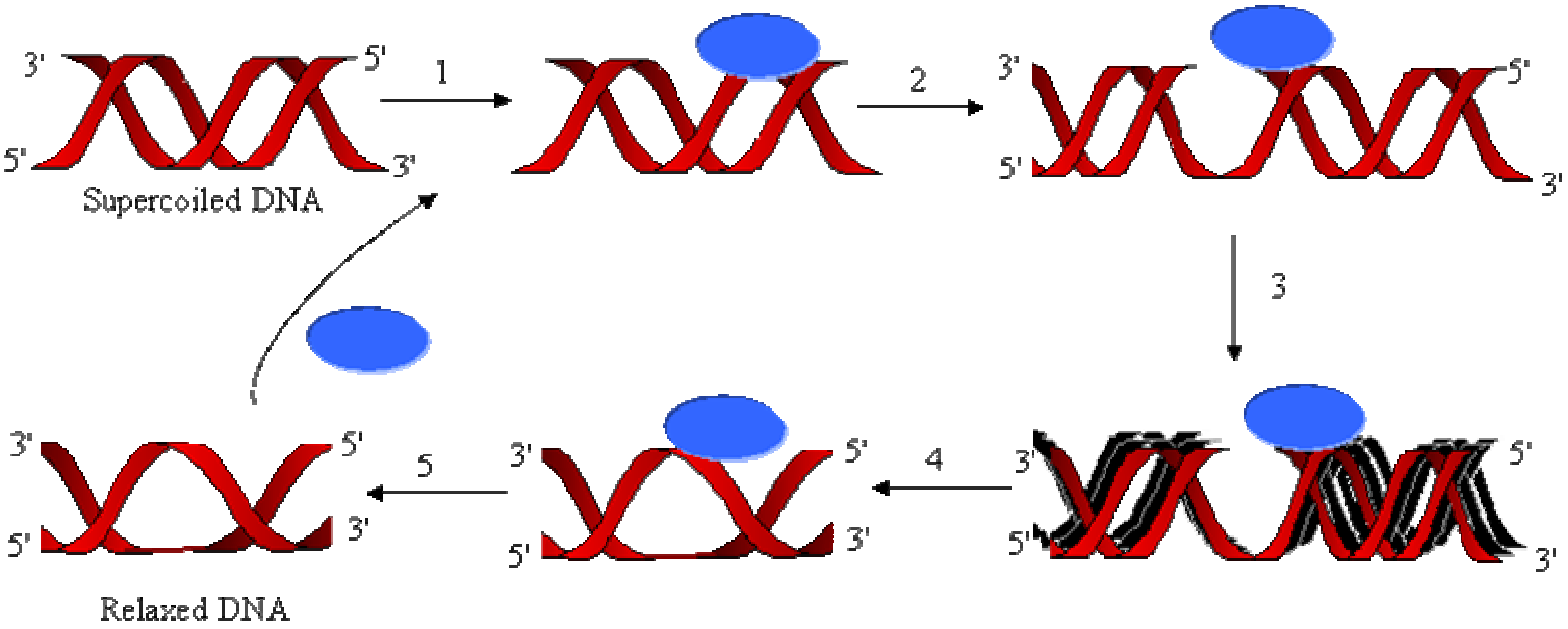


Figure 3. The Catalytic Cycle of DNA Topoisomerase I. (1) topo I (shown as a blue oval) binds supercoiled DNA (2) topo I cleaves a single strand DNA break (3) DNA unwinds (4) topo I catalyzes DNA religation (5) enzyme turnover.

After DNA unwinding, the reaction reverses when the 5' OH of the cleaved DNA strand reattacks the phosphotyrosine residue in a secondtransesterfication reaction. Under normal cellular conditions, topo I cleavage complexes are transient and usually undetectable. The steady-state concentration of the covalent 3' phosphotyrosine intermediate is kept low because the rate of DNA religation is faster than the rate of cleavage (Pommier et al., 2003). However, topo I cleavage complexes are also produced by other endogenous and exogenous factors, such as UV-induced base modifications, DNA oxidation by oxygen free radicals, mismatch repair deficiencies, DNA alkylating agents, and some anticancer agents (Pommier., 2003; Pourquier and Pommier, 2001). Such topo I cleavage complexes can result in DNA damage and cell death, if left unrepaired. The objective of some cancer chemotherapy, however, is to capture topo I cleavage complexes by administering a drug (ie, a DNA topo I inhibitor) that traps topo I on DNA in a cleaved form (Nitiss and Wang, 1996).

3.5 Type IA: DNA Topoisomerase III.

The gene for eukaryotic DNA topo III was discovered in 1989 (Wallish et al., 1989) and later identified in humans on chromosome 17p11.2-12 (Hanai et al., 1996). There are two topo III homologues that are expressed in mammalian cells, topo IIIα and topo IIIβ, and each of these has isoforms generated by alternative mRNA splicing (Seki et al., 1998). Human topo IIIα has a putative mitochondrial targeting sequence and localizes to both the nucleolus and mitochondria (Wang et al, 2002). In contrast, human DNA topo IIIβ is found only in the nucleus (Kobayashi and Hanai, 2001). Both topo IIIα and IIIβ can relax negatively supercoiled DNA, but the biological functions of the

different topo III enzymes have not been determined (Goulaouic et al., 1999). Yeast studies demonstrate that *Top3* gene mutants grow very slowly, display hyperrecombination and have defects in meiotic recombination (Gangloff et al., 1999). Furthermore, targeted disruption of the mouse topo IIIα gene shows that topo IIIα expression is essential in early embryogenesis (Li and Wang, 1998). Topo III gene expression occurs in multiple somatic tissues and is differentially regulated during different developmental stages, which is associated with binding of the upstream stimulatory factor-2 (USF-2) to an E-box element located in the topo III gene promoter (Han et al., 2001). The YY1 transcription factor may also have a role as a cell type specific topo III transcriptional activator (Park et al., 2001).

Both topo IIIα and IIIβ interact with the RecQ/SGS1 family of DNA helicases, which has been shown to occur in yeast and *Escherichia coli* (Kim et al., 1998). It has been suggested that RecQ helicases may function to recruit two molecules of topo III to DNA structures during genetic recombination to facilitate the cleavage and passage of double strand DNA (Park et al., 2001). Human topo IIIα is shown to associate with the Bloom syndrome protein (BLM), another member of the RecQ family of DNA helicases, in somatic and meiotic human cells (Shimamoto et al., 2000). A mutation in the *BLM* gene is associated with Bloom's syndrome, a rare genetic disorder that is associated with hyperrecombination between sister chromatids and increased propensity for cancer (Ellis et al., 1995). An association of human topo IIIα with BLM could be significant in regulating the frequency and fidelity of recombination events by either disrupting nascent joint molecules or by regulating the migration of Holiday structures (Harmon et al, 1999). Thus, topo III may have a role as a tumor suppressor gene, but additional investigations

are necessary to further elucidate its role in recombination and suppression of cancer. Furthermore, defining the physiological roles of topo III enzymes could lead to the development of new anti-cancer agents that specifically target these enzymes.

3.6 Conclusions to Topoisomerase I.

In summary, type I enzymes cleave single strand DNA in an ATP independent manner. Topo Imt and topo III are two of the most recent type I enzymes to be identified in humans and these enzymes could be the future molecular targets of anti-cancer drugs. However, topo I is the current molecular target of chemotherapeutic agents. In chapter 4, we will learn more about how drugs, called camptothecins, target topo I and exert their cytotoxic effects in cancer cells. Before that, however, a discussion of the type II enzymes must be covered because these too are the molecular targets of commonly used anti-cancer agents.

3.7 General Introduction to the Type II Enzymes.

In the preceding section, we reviewed the type I topoisomerases that are expressed in humans. Now, we will turn our attention to topo II, a type II enzyme. In humans, topo II has two isoforms, topo IIα and topo IIβ that share many similar properties. Although many difference in structure, tissue distribution, and gene expression have been described for topo IIα and topo IIβ , differences in their functional roles, drug action, and relative contributions to cancer chemotherapy are less clear (Austin and Marsh, 1998). Topo IIα has been the most extensively investigated isoform, whereas the role of topo IIβ is drug resistance is not well understood (Beck et al., 1999).

Immediately following in section 3.8, is a description of common principles and biochemical properties that are generally applied to both topo IIα and topo IIβ. Then, in section 3.9, the features that distinguish the two isoforms are given.

3.8 Type IIA: Similarities between Topo IIα and Topo IIβ.

The type II enzyme, called DNA gyrase, was isolated from *Escherichia coli* and was the first type II topoisomerase to be characterized (Gellert et al., 1976). Bacterial DNA gyrase is unique because it can introduce negative supercoils in DNA or convert positive supercoils into negative supercoils (Wang, 1996). DNA gyrase is the target for clinically important quinolone antimicrobial agents, such as Ciproflaxin (Hooper et al., 1993). However, DNA gyrase should not be confused with mammalian topo II enzymes, the current molecular targets of anticancer agents.

In contrast to bacterial DNA gyrase, eukaryotic topo II is unable to generate supercoils *in vitro* (Liu et al., 1983). Topo II activity is required to separate replicated chromosomes prior to cell division, and unlike topo I and topo III, is able to decatenate intertwined double strand DNA molecules (Marini et al., 1980). All type II topoisomerases form dimers (Champoux, 2001) and each topo II monomer can be divided into three domains (Jensen et al., 1996), an N-terminal domain that contains the ATP-binding region (Bjerbaek et al., 2000; Gardiner et al., 1998), the central domain containing the active site tyrosine residue (Okada et al., 2000) and the C-terminal domain that contains the nuclear localization sequences (Figure 4) (Mirski et al., 1997, 1999). The C-terminal domain has also been shown to be important for interactions with other proteins (Messenger et al., 2002; Kroll, 1997).

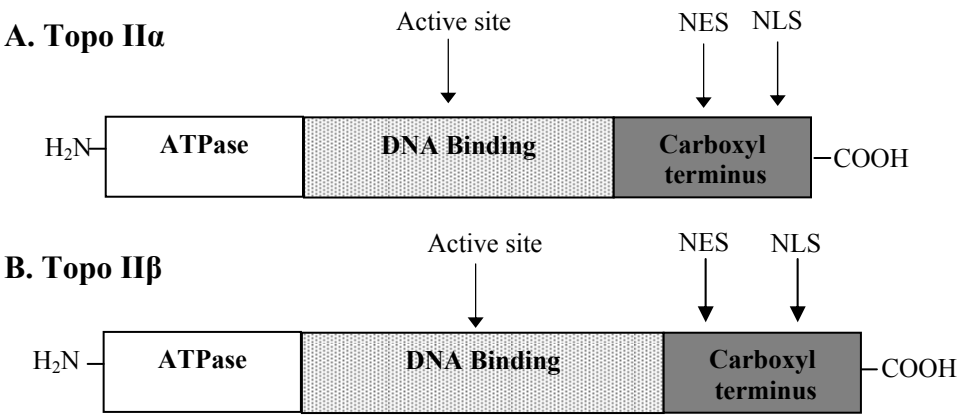


Figure 4. Structural Domains Shared by DNA Topo IIα (shown in A) and topo IIβ (shown in B). Topo IIα and topo IIβ have three domains as shown. The ATPase domain (white box) is at the amino terminus and contains the ATP binding site. The DNA domain (grey box) contains the active site Tyr residues and the carboxyl terminus (dark grey box) is important for intracellular localization because it contains the nuclear localization(NLS) and nuclear export signals (NES).

All topo II enzymes share common mechanisms for DNA-cleavage and religation reactions (Champoux, 2001; Wang, 1996). A description of the catalytic cycle of topo II is important in understanding how drugs target topo II. The catalytic cycle of topo II involves several distinct stages including: DNA binding, strand cleavage, strand passage, religation, and enzyme turnover (Figure 5) (Champoux, 2001; Wang, 1996). Topo II dimers first bind to double strand DNA, and then each monomer cleaves opposite DNA strands to generate a 4-base stagger. Topo II mediated DNA breaks occur through.

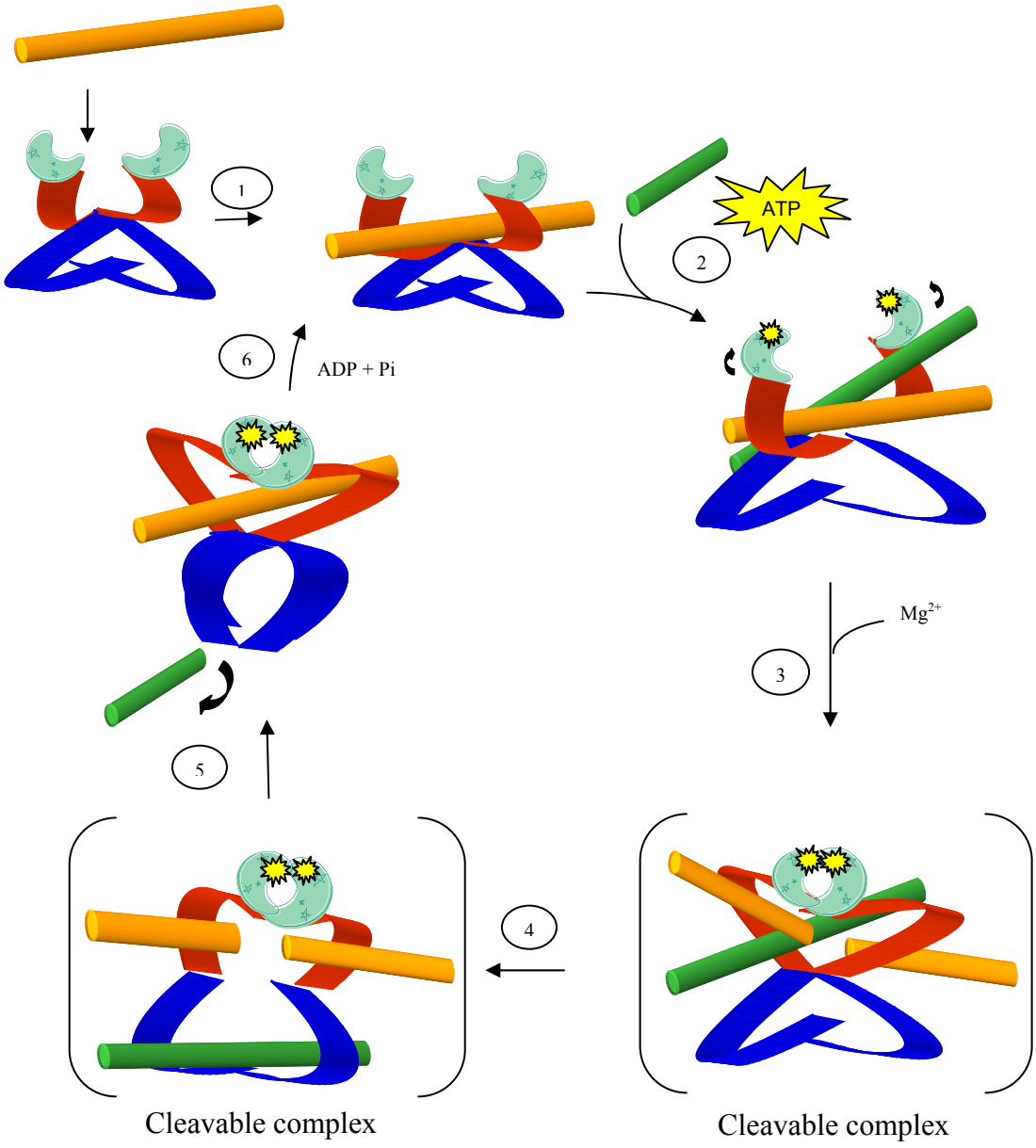


Figure 5.Catalytic Mechanism of DNA Topo II. The "two-gate model" of topo II catalysis is shown. According to this model, topo II has two lobes called A' (colored red) and B' (colored blue). The DNA strand that is cleaved is called the "gate DNA" or "G-segment". The DNA strand that is transported is called the "transport DNA" or "T-segment". (1) Topo II binds gate DNA (orange rod) and undergoes conformational changes. (2) Topo binds ATP (yellow star) and the transport DNA molecule (green rod). (3) The ATPase domains on topo II dimerize and topo II cleaves the gate DNA. The presence of a metal cation is required. (4) The transport DNA is transported through the break and into the central cavity of the B' lobe. Gate DNA is held within the A' lobe. (5) Following DNA strand passage the transport DNA is released from the enzyme through a gate formed in the B' lobe. The gate DNA is resealed. (6) ATP is hydrolyzed and ADP and inorganic phosphate (Pi) are released to complete enzyme turnover. This figure was reproduced from references Champoux, 2001 and Wang, 1996.

transesterfication reactions, in which a DNA phosphoester bond is transferred to the active site tyrosine residue in each monomer. Thus, each monomer remains covalently attached to the 5' end of DNA through a phosphotyrosine bond. A conformational change in the enzyme pulls the cleaved DNA duplex apart to create an opening that is called, "the gated or G-segment DNA". Strand passage occurs either from the same DNA molecule (relaxation, knotting, or unknotting) or different molecules (catenation/decatenation). The DNA segment that is passed through the opening is called the "transported or T-segment DNA". Enzyme turnover requires ATP hydrolysis and divalent cations (Mg^{2+}) (Osheroff, 1987). The mechanism of topo II enzyme catalysis is an important concept for understanding how topo poisons and catalytic inhibitors target topo II.

Topo II can also catenate/decatenate interlocked circles of DNA (Champoux, 2001). Topo II decatenation activity is essential at the end of DNA replication for the proper separation of daughter chromosomes. Cells lacking topo II are unable to decatenate sister chromatids or carry out chromosome condensation reactions prior to mitosis, which results in non-disjunction and chromosome breakage (Holm et al., 1989). Topo II catalytic activity is measured *in vitro* by the decatenation of kinetoplast DNA (kDNA) isolated from the mitochondria of *Crithidia fasiculata.* kDNA consists of numerous interlocked minicircles and maxicircles. Unlike the type I enzymes, topo II is able to decatenate the interlocked circles to generate free mini- and maxi- circles that can be separated from the network of kDNA by centrifugation (Sahai and Kaplan, 1986; Marini et al., 1980). Thus, the ability to decatenate kDNA is an important distinction

between topo I and topo II enzymes because it allows us to estimate topo II enzyme activity independent of topo I activity *in vitro*.

3.9 Differences between DNA Topoisomerase IIα and Topo IIβ.

Topo II has two isozymes, topo IIα and topo IIβ, with different molecular masses and gene loci (Lang et al., 1998; Drake et al., 1987) (Table 1, page 13). The presence of two different gene loci suggests that these isoforms may be differentially regulated at the level of transcription. The tissue distribution of topo IIα and topo IIβ mRNA and protein have been determined in mouse, rat, and humans (Bauman et al., 1997). Topo IIα mRNA is highest in many proliferating cells such as thymus, spleen, bone marrow, intestine, and testis. Topo IIβ mRNA is detectable in a wider range of tissue types; including those with few proliferating cells (Carpanico et al., 1992). Topo IIβ is 90 amino acids longer than topo IIα and the two isoforms are 68% homologous in amino acids (Austin and Marsh et al., 1998). The greatest variability in amino acids between topo II α and IIβ occurs in the C-terminal domain, and thus the C-terminus may impart specific differences in the functional roles of these isozymes.

Topo IIα is expressed in a cell cycle dependent manner with peaks in the S-phase and G_2/M-phase of the cell cycle and is the target of ubiquitin-mediated proteolysis (Meyer et al., 1997; Nakajima et al., 1996). Topo IIβ protein expression is more uniform throughout the cell cycle, suggesting that it may have a role as a "housekeeping gene" (Drake et al., 1989). Topo IIα is also highly expressed in many rapidly proliferating human tumors (Holden et al, 1990, Turley, et al, 1997), thus making topo IIα a frequent molecular target of anti-cancer drugs. In contrast, topo IIβ expression is constant

throughout the cell cycle with limited fluctuations in the amount or stability of the protein in either highly replicating or non-replicating cells (Woessner, 1991). Topo IIβ is emerging as a new molecular target in tumors that are refractory to cytotoxic agents that target topo IIα (Gatto and Leo, 2003).

Both topo IIα and topo IIβ are differentially phosphorylated throughout the cell cycle with maximal phosphorylation occurring at G_2/M phase (Burden et al, 1993; Burden and Sullivan, 1994). Thus, maximum topo II phosphorylation correlates with chromosome condensation and segregation. As cells transition from M phase to G_1 phase of the cell cycle, 75% of topo IIβ becomes dephosphorylated, whereas 25% of topo IIα is dephosphorylated (Wells and Hickson, 1995; Burden and Sullivan, 1994)

The kinases known to phosphorylate topo IIα *in vitro* include protein kinase C (PKC) (Wells et al., 1995), casein kinase 2 (CK2) (Ishida et al., 1996; Alghisi et al., 1994), and a proline directed kinase (Wells and Hickson, 1995). The phosphorylation of precise amino acid residues by specific kinases have not been mapped in topo IIβ.

The expression of the different topo II isoforms seems to be necessary to fulfill distinct physiological roles, but what those roles are remains unclear (Wang, 1991; Wang, 2002). Both enzymes are able to unknot DNA, relax supercoiled plasmid DNA, and catalyze ATP-dependent DNA strand passage activity *in vitro* (Austin and Marsh, 1998). Topo IIα has a significant role in cell proliferation, whereas topo IIβ is suggested to have a role in cell differentiation (Aoyama et al., 1998). Topo IIα has an essential role in chromosome condensation and segregation during mitosis (Uemura et al., 1987). Recent findings, however, suggest that topo IIβ is able to substitute for topo IIα in chromosome condensation and segregation, but that topo IIα has a crucial role in

shortening of chromosome axes (Sakaguchi and Kikuchi, 2004). Distinguishing the physiological roles of topo IIα from those of topo IIβ could lead to a better understanding of the molecular mechanisms conferring differences in drug sensitivity. A transgenic cell line lacking topo IIβ was recently established and may lead to a better understanding of the differential roles between topo IIα and IIβ (Errington et al., 1999). To date, there are no established cell lines that lack topo IIα.

I searched PubMedCentral, the U.S. National Institutes of Health free digital archive of biomedical and life sciences journal literature, and identified several proteins that have been reported to interact with topo IIα *in vitro* and/or *in vivo* (PubMedCentral, 2005). I have included a brief description of the protein partners that interact with topo IIα and speculated on the potential implication(s) of such protein-protein interactions in conferring drug resistance. The protein partners that associate with topo IIα spans a variety of protein classes with roles in various cellular processes, including chromatin remodeling, apoptosis, mRNA splicing, RNA export, and proteasome-mediated protein degradation. A complete discussion of each protein interaction identified in table 2 is beyond the scope of this investigation. Briefly however, the data presented in this investigation suggests that the nuclear export of topo IIα may be regulated by the export receptor, exportin-1. Our laboratory has also collaborated with other laboratories to investigate topo IIα interactions with HDAC (Tsai et al., 2000) and 14-3-3 proteins. For example, histone deacetylases (HDAC) modifies protein histones and has a role in regulation of gene transcription. Topo II was shown to complex with HDAC1 and HDAC2 *in vivo* (Tsai et al, 2000). Histone deacetylase activity coupled with topo II DNA cleavage has been implicated in etoposide induced apoptosis. We also investigated

a potential interaction between 14-3-3 epsilon with topo IIα in human myeloma and leukemia cell lines at different cell densities because we thought that 14-3-3 may modulate the subcellular localization of topo IIα in these cells. However, we did not observe 14-3-3- colocalization with topo IIα by confocal microscopy (data not published). The 14-3-3 protein, however, has been shown to interact with topo IIα in HL-60 and CCRF human leukemia cells (Kurz et al., 2000). This interaction was shown to abrogate topo IIα cleavable complex formation in the presence of the topo II poison, etoposide.

Although several protein-protein interactions have been described for topo IIα, specific protein-protein with topo IIβ have not been described. The only observation of a protein interaction with topo IIβ has been the observation that topo IIβ can heterodimerize with topo IIα. Although topo II usually forms homodimers, a subpopulation of α/β heterodimers has been reported to occur, but the biological significance of this interaction is not clearly established (Gromova et al., 1998; Biersack et al., 1996).

Table 2. DNA Topoisomerase IIα Protein Interactions

Name	*In vitro* or *In vivo*	Function(s) of Protein Partner	Function(s) of Topo-Protein Interaction (Observed or Predicted)	Role of Topo-Protein Interaction in Cellular Drug Sensitivity or Resistance (Observed or Predicted)	References
Topo IIβ	In vitro	DNA topology	Possible role in DNA metabolism; potential therapeutic target	Potential molecular target for new drugs that inhibit the topo IIα/β heterodimer	Biersack et al., 1996 Gromova et al., 1998
p53	In vivo In vitro	Tumor suppressor protein; regulation of G2/M transition	Interaction with topo IIα protein may be necessary for detection/repair or activation of apoptotic response following DNA damage. p53 mediated topo II gene suppression blocks cells in G2/M, allowing cells to repair damaged DNA.	p53 has been reported to have both pro- and anti-apoptotic effects depending on the cell type and cytotoxic agent investigated. For example, mouse fibroblasts expressing wild-type p53 are more sensitive to etoposide and doxorubicin, but less sensitive to UV light and alkylating agents when compared to p53 deficient mouse fibroblasts. These results are in contrast to lymphoblastoid cells that are more sensitive to UV light and alkylating agents than their p53 mutant counterparts.	Cowell et al., 2000 Bernd, K. 2003
Caspase activated DNase (CAD)	In vitro	Deoxyribonuclease involved in apoptosis and nuclear disassembly	CAD enhances topo IIα decatenation activity *in vitro*. CAD may initiate at or near topo IIα binding sites to initiate chromosomal condensation and nuclear disassembly during apoptosis.	Could be involved in apoptotic response to drug-induced DNA damage	Durrieu et al., 2000
Interleukin enhancer binding factor 2	In vivo	Transcription factor; also associated with pre-mRNA splicing complex	May be involved in regulating IL-2 gene transcription during T-cell activation	To date, there is no literature to describe ILF2-topo IIα effects on cellular drug sensitivity.	Ajuh et al., 2000 Marcoulatos et al., 1996
Peptidyl-prolyl isomerase (Pin1)	In vitro	An isomerase that interacts with several phosphoproteins during the cell cycle	Inhibits casein kinase II (CK2) phosphorylation of Thr^{1342} on topo IIα, but especially during G2/M; may regulate CK2 phosphorylation of topo during the cell cycle.	Alterations in the phosphorylation status of topo IIα could regulate topo IIα enzyme activity, protein degradation, or subcellular localization and confer alterations in drug sensitivity.	Messenger et al., 2002

Table 2 continued on next page.

Table 2 continued.

Name	*In vitro* or *In vivo*	Function(s) of Protein Partner	Function(s) of Topo-Protein Interaction (Observed or Predicted)	Role of Topo-Protein Interaction in Cellular Drug Sensitivity or Resistance (Observed or Predicted)	References
RNA helicase A (RHA)	In vivo	Unwinds double-stranded DNA and RNA; nuclear export of retroviral RNA	RHA may facilitate the access of topo IIα to chromatin and work together to maintain chromatin structure during transcription. RHA may be a transcriptional co-activator (i.e. topoIIα may activate RNA polymerase when complexed with RHA)	Sumoylation could signal RHA to interact with topoIIα to initiate transcription of specific genes in response to cellular demands (i.e. during apoptosis or when DNA damage is present)	Lee et al., 2004 Zhou et al., 2003
SUMO1/Ubc9	In vivo In vitro	An E2-type ubiquitin-conjugating enzyme involved in sumoylation (SUMO) and proteasome mediated degradation;	SUMO could regulate topo IIα degradation, subcellular localization and/or enzyme activity. Ubc9 is also necessary for the assembly of RNA helicase-A topo IIα complexes to form.	Sumoylation may alter drug sensitivity by modifying the amount, location or activity of topo IIα. Sumoylation may be a step in chromosome condensation or DNA cleavage during apoptosis. SUMO-topo IIα is observed after teniposide induced DNA damage; SUMO-topo IIβ is observed after exposure to the catalytic inhibitor ICRF-193.	Mao et al., 2000 Isik et al., 2003 Argasinska et al., 2004
Histone deacetylase-1 Histone deacetylase -2	In vivo In vitro	Chromatin remodeling	Regulation of chromatin structure and gene transcription.	The histone deacetylase inhibitor, sodium butyrate (NaB) results in a 2-fold increase in topo IIα protein and increased sensitivity to etoposide in human leukemic cells.	Tris et al., 2000 Johnson et al., 2001 Kurz et al., 2001
14-3-3- epsilon	In vivo	Adapter molecule involved in signal transduction pathways	Interaction with 14-3-3 may modulate topo IIα protein activity or subcellular localization.	14-3-3 epsilon abrogates topo IIα cleavable complex formation to etoposide as a result of reduced DNA binding activity.	Kurz et al., 2000
Exportin 1	In vivo In vitro	Protein receptor for nuclear export	Export topo IIα from the nucleus to the cytoplasm	Decreased drug sensitivity to etoposide has been associated with a subcellular distribution of topo IIα from the nucleus to the cytoplasm in human myeloma cell lines.	Engel and Turner et al., 2004 Mirski et al., 2003
c-Jun	In vivo	Transcription factor; proto-oncogene	c-Jun stimulates topo IIα decatenation activity	Etoposide induces c-jun gene expression in HL-60 and U-937 myeloid leukemia cell lines and may be an apoptotic event in etoposide-induced DNA damage	Kroll et al., 1993

3.10 Conclusions.

Thus far, cancer pathogenesis and the family of DNA topoisomerases have been reviewed. Some key points to remember are that topo I and topo II are the major nuclear enzymes that regulate DNA topology during DNA replication, transcription, and cell division. Topo I catalyzes single-strand DNA breaks in an ATP-independent manner and is not expressed in a cell-cycle dependent manner, whereas topo II enzymes cleave double-strand DNA in an ATP-dependent manner. Topo II has two isoforms, topo IIα and topo IIβ. Topo IIα protein expression is cell cycle dependent, whereas topo IIβ expression is more constant throughout the cell cycle. Topo I and topo II are the molecular targets of clinically important chemotherapeutic agents, especially in the treatment of hematological malignancies. Next, the drugs that target topo I and topo II are reviewed.

Chapter Four

DNA Topoisomerase Targeted Cytotoxic Agents

4.1 Introduction to Topo Targeting Agents.

Topo proteins are the molecular targets of commonly used anti-cancer agents (Topcu, 2001). Cytotoxic agents that target topo can be classified as poisons, catalytic inhibitors, or suppressors (Pommier et al., 1998; Andoh and Ishida, 1998). Some poisons are specific to topo II (i.e., etoposide and teniposide), whereas other poisons target topo I (i.e., topotecan and irinotecan). The poisons are further subclassified as either DNA intercalators or non-intercalators, but several drugs have multiple mechanisms of action, and thus do not fit discreetly into one specific drug category (Table 3) (Ross et al., 1984). DNA intercalators are able to bind to DNA, whereas the non-intercalators do not bind to DNA. Some drugs, such as Actinomycin D, can act as a poison against both topo I and topo II, and yet other compounds can act as a poison against one class of topo enzymes, but as a catalytic inhibitor of another class of topo enzymes (van Hille et al., 1999). The mechanism of drug action of the poisons is distinctly different from that observed with the catalytic inhibitors and suppressors. The main difference between catalytic inhibitors and suppressors is their molecular target. Catalytic inhibitors inhibit topo II activity, whereas the suppressors inhibit topo I activity. The topo I and topo II poisons are described in a separate section from the catalytic inhibitors and suppressors.

4.2 Topo I and Topo II Poisons.

To comprehend how anti-cancer agents target DNA topoisomerases, it is important to recall that, when all topo enzymes cleave DNA, they remain bound the cleaved DNA via a phosphotyrosine bond. In this manner, topo bridges the gap between the cleaved DNA strands, thus ensuring that the correct strands of DNA are resealed back together. Under normal physiological conditions, the complex between topo and the cleaved DNA strands, called a "cleavage" or "cleavable complex", is transient and freely reversible. Some anti-cancer agents, however, stabilize the form of topo that is bound to cleaved DNA and are called, topoisomerase poisons. Topo II poisons include the epipodophyllotoxins (i.e., etoposide, teniposide) and anthracyclines (i.e., doxorubicin), whereas the topo I poisons include the camptothecins (i.e., topotecan, irinotecan). The topo poisons act by increasing the steady-state concentration of covalently bound DNA-topo cleavage complexes by blocking the religation of cleaved DNA and stabilizing the DNA-drug-enzyme intermediate (Osheroff et al., 1994). However, it is not clear why cells die, since the topo II-DNA covalent complexes trapped by topo poisons are freely reversible so that removal of the drug allows the DNA to return to an undamaged state (Nitiss and Wang, 1996). It is possible that cells accumulate DNA damage to a point of no return, such that apoptotic pathways are triggered and cell death occurs even if DNA damage is repaired. It is generally believed that the drug stabilized cleavage complexes become lethal when either DNA helicases (Howard et al., 1994) or an advancing replication fork collides with the drug-topo-DNA ternary complex (Hsiang et al., 1989). Such collisions are believed to be lethal because they disrupt the proteinaceous bridge between topo and the cleaved DNA, and thus the transient single- or double- strand DNA

break that was being held together by topo becomes a permanent double-stranded break (Osheroff, 1995; Chen and Liu, 1994). The double-stranded DNA break becomes a target for gene recombination and repair pathways (Osheroff, 1998), resulting in sister chromatid exchanges, large insertions and deletions, and chromosomal translocations (Chen and Liu, 1994; Anderson and Berger, 1994; Corbet and Osheroff, 1993). Therefore, at least some of the double-strand breaks that are generated by collision with the replication fork can not be repaired by the enzyme (Nitiss and Beck, 1996; Zhang et al., 1990). So, although the covalent topo-DNA complex is transient and freely reversible, collision with the replication fork can produce DNA lesions that persist after the drug is removed (Nitiss and Beck, 1996). Though not well understood, these cellular events eventually trigger cell death pathways (Gupta et al., 1995, Pommier, 1993). One additional caveat is that generation of a lethal collision is dependent on the orientation of the cleavable complex relative to the advancing replication fork. Collision is lethal only if the cleavable complex is formed on the lagging-strand of DNA (Pourquier et al., 1999l Tsao et al., 1993).

4.3 Topo I Suppressors and Topo II Catalytic Inhibitors.

In contrast to the topo poisons, the suppressors of topo I and catalytic inhibitors of topo II do not stabilize DNA-enzyme complexes, but rather work at another step of enzyme catalysis (Pommier et al., 1998). The catalytic inhibitors include the topo II targeting agents, merbarone and the bisioxopiperazines (ICRF-193, ICRF-187).

Table 3. DNA Topoisomerase I and II Inhibitors

	Class	Drug	Poisons	Catalytic Inhibitors	DNA Intercalators	Free Radical Formation
Topo II Agents	**Epipodophyllotoxins**	etoposide (VP-16), teniposide (VM-26)	+++	-	-	+
	Isoflavinoids	genistein	++	-	-	-
	Anthracyclines	doxorubicin, daunomycin	++	+	++	+
	Actinomycins	actinomycin D	+++	-	-	-
	Anthracenediones	mitoxantrone	++	+	+	-
	Acridines	m-AMSA	+++	+	+	-
	Miscellaneous	fostriecin, merbarone, ICRF-193, ICRF-187, ICRF-159	-	+++	-	-
Topo I Agents	**Alkaloids**	topotecan, camptothecin (CPT), irinotecan (CPT-11), 9-nirocamptothecin (9-NC), karenitecin	+++	-	-	-

Topo suppressors and catalytic inhibitors may inhibit binding between DNA and topoisomerase, stabilize noncovalent DNA topoisomerase complexes, or inhibit ATP binding (Larsen et al., 2003; Burden and Osheroff, 1998). For example, catalytic inhibitor, called ICRF-193, has been shown to lock the enzyme in a closed form on DNA, preventing DNA cleavage (Roca et al., 1994, Fortune et al., 1998). Recall that topo IIα cleavage activity is described to occur in a two-gate model. The enzyme undergoes two forms, open and closed, in the absence or presence of ATP, respectively. ATP binding triggers a conformational change in topo II, resulting in topo II gate closure around DNA. ATP hydrolysis results in opening of the topo II gate. Therefore, ICRF-193 stabilizes topo II in a closed formation around DNA and prevents topo from opening again. In general, the drugs that target topo IIα come from a range of classes with differing structures and mechanism of action. Conversely, most of the antitumor agents that target topo I are poisons (refer to Table 3), and thus are the focus of this investigation.

A summary of the mechanisms of drug action of topo II targeting agents are illustrated in figure 7. In general, topoisomerase poisons can be distinguished from catalytic inhibitors by their cytotoxic profiles (Froelich-Ammon and Osheroff, 1995 Nitiss, 1994). Increased cellular content of topo protein corresponds with cellular hypersensitivity to topo poisons, but decreased drug sensitivity to catalytic inhibitors (Bjornsti et al., 1989; Ishida et al., 1995). Conversely, decreased cellular content of topo protein corresponds with decreased sensitivity to topo poisons but hypersensitivity to catalytic inhibitors (Bjornsti et al., 1989; Ishida et al., 1995; Nitiss and Wang, 1988).

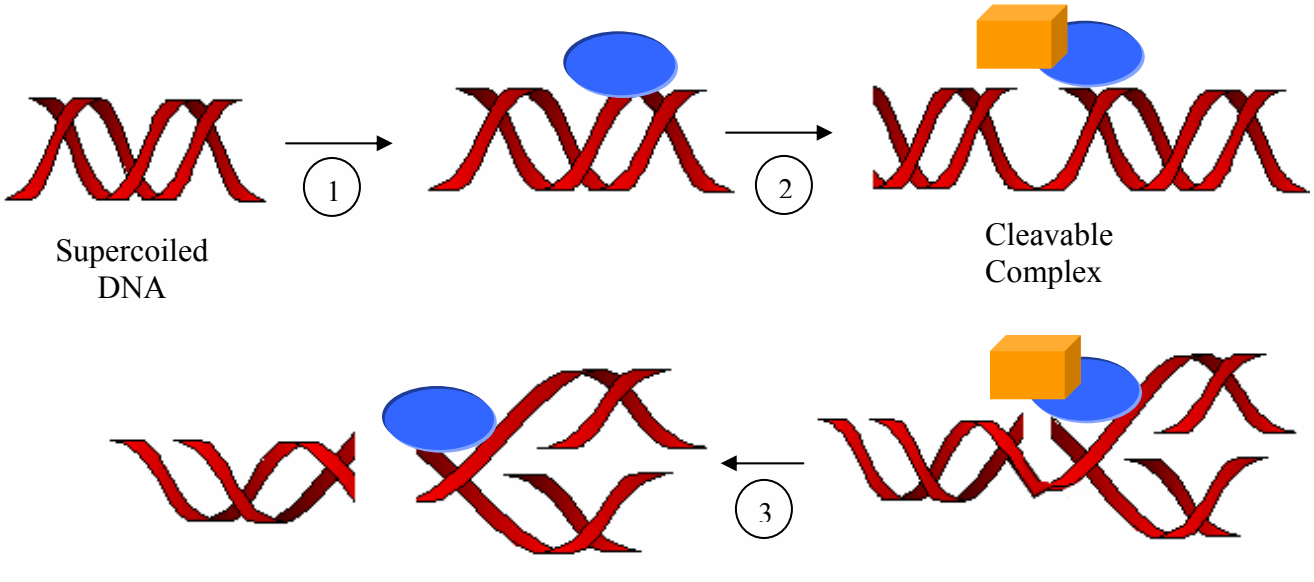


Figure 6.The Mechanism of DNA Topo I Poisons. (1) Topo I monomer binds to supercoiled DNA (2) Topo I cleaves single strand of DNA .The cleavable complex is stabilized in the presence of topo I poison (shown as orange box) (3) Advancing replication fork collides with cleavage complex, resulting in double strand DNA break.

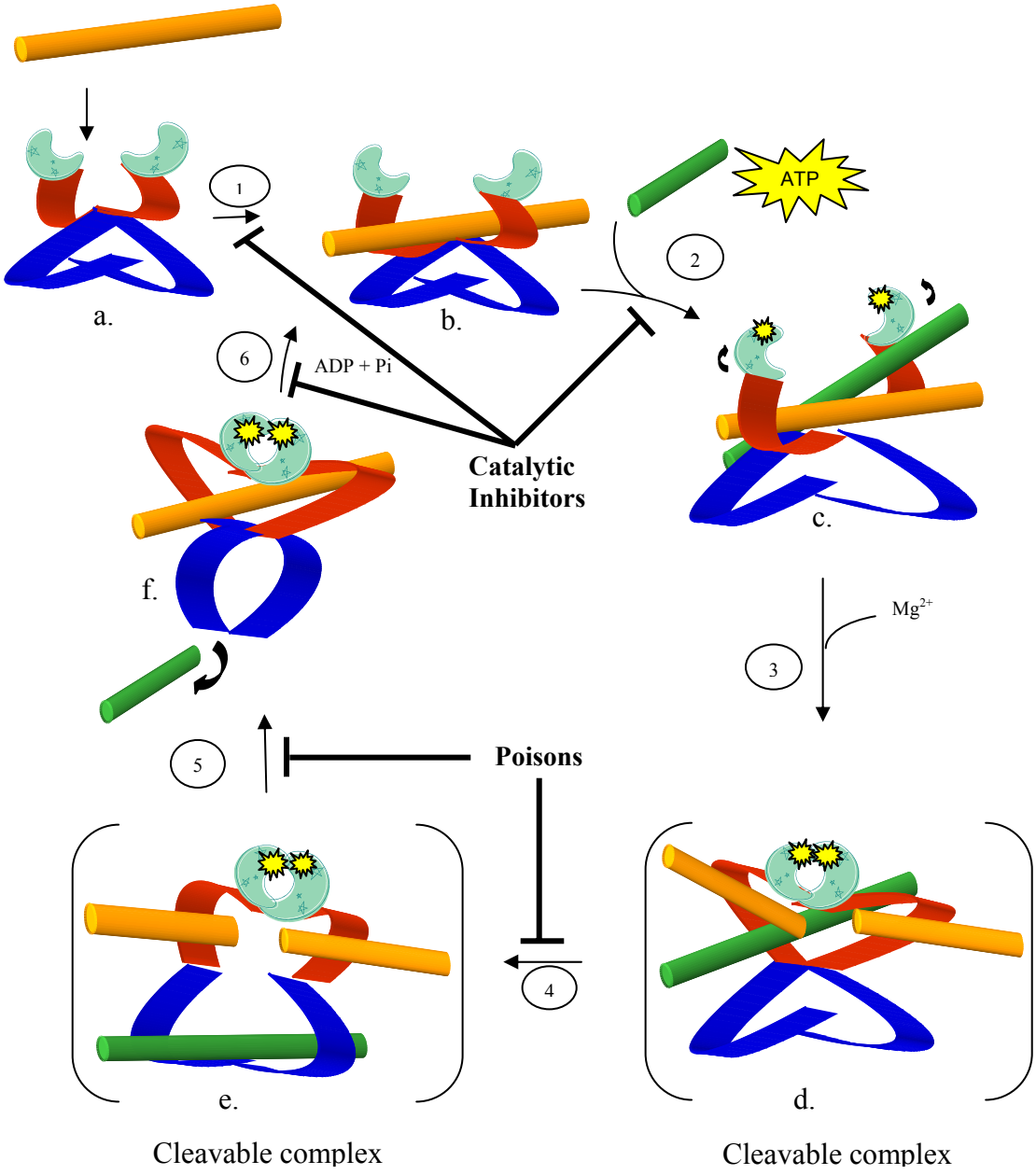


Figure 7. The Mechanism of DNA Topo II Poisons and Catalytic Inhibitors. This is the same figure illustrated on page 23, but now the steps of enzyme inhibition by topo II poisons and catalytic inhibitors are indicated. Briefly, (1) Topo II binds DNA (orange rod) (2) Topo II binds as second DNA strand, and then binds ATP (yellow star) (3) The ATPase domains on topo II dimerize, and then topo II cleaves DNA.(4) Strand passasge occrs through the gap (5) transport DNA is released, and then gate DNA is resealed. (6) ATP is hydrolyzed and ADP and inorganic phosphate (Pi) are released to complete enzyme turnover. Topo poisons act on the cleavable complex (forms d and e) and prevent reaction 4 from proceeding forward. The catalytic inhibitors act on any other step of the catalytic cycle (forms a, b, c, and f). Catalytic inhibitors prevent topo binding to DNA, ATP binding, and ATP hydrolysis. This figure was modified from references Champoux, 2001 and Wang, 1996.

4.3 DNA Topoisomerase I Targeting Agents.

Camptothecin (CPT) (Figure 8.) is a plant alkaloid isolated from *Camptotheca acuminata* (Wall et al., 1986). Initial studies showed that CPT displayed potent antitumor activity against a wide range of tumors, but the drug was discontinued because of toxic side effects (Pommier, 2005). There was a renewed interest in CPT when subsequent findings identified topo I as the main molecular target (Hsiang et al., 1985). Since CPT was the only known drug to target topo I, finding less toxic and more water soluble CPT derivatives became a subject of numerous investigations (Kollmannsberger, et al., 1999; Wani et al., 1987, 1986; Kunimotto et al., 1987). Topotecan (TPT) (Figure 9.) and irinotecan (CPT-11) are two water soluble CPT derivatives that are currently being used for the treatment of human cancers (Lackey et al., 1996; Luzzio et al., 1995). CPT-11 has been used in the treatment of colon, breast, gastric and small-cell lung cancers as well as leukemia (Rosen, 1998), whereas topotecan has been used against recurrent small cell lung cancer, ovarian cancer and endometrial cancer (Treat et al., 2004; Ahmad and Gore, 2004; Traina et al., 2004). CPT-11 must be converted to the active compound, SN-38 (7-ethyl-10-hydroxycamptothecin B-glucuronide) by carboxylesterase (Satoh et al., 1994). Thus, increased drug sensitivity to irinotecan

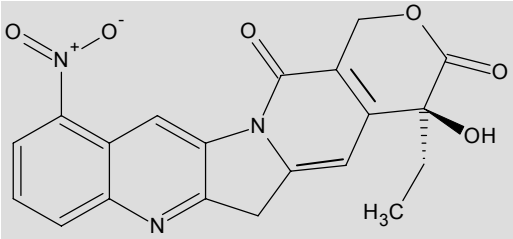


Figure 8. Camptothecin (CPT)

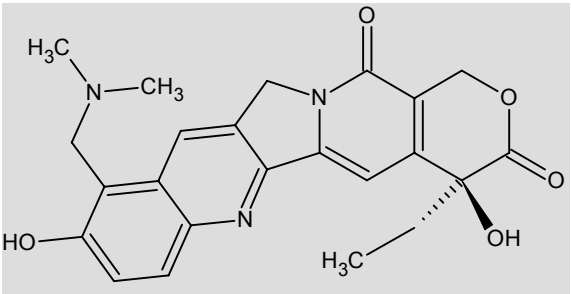


Figure 9. Topotecan (TPT)

correlates with carboxylesterase expression and activity (Wierdl et al., 2001). Cisplatin is also a substrate of the ABC drug transport protein, multidrug resistance related protein-3 (MRP3).

CPT and its derivatives interact reversibly with a DNA-enzyme complex and usually have no detectable binding to isolated topo I or DNA (Fan et al., 1998; Hsiang et al., 1985). However, the x-ray crystal structure of human topo I-DNA-topotecan ternary complex shows that topotecan can mimic a DNA base pair (Staker et al., 2002). These findings may partially explain how topotecan can intercalate in DNA in the absence of topo enzyme in some instances. In the presence of camptothecins, topo I is unable to reseal nicked DNA because the drug blocks the active site of the covalently bound enzyme (Woo et al., 2002). However, single strand DNA breaks alone are not sufficient for cell death to occur and instead must be converted into permanent DNA double strand breaks by collision with the moving replication fork (Mosseso, 2000). Conversely, it has also been reported that CPT induced DNA strand-breaks can occur in unstimulated leukocytes where little or no replication is occurring (Daza et al., 2002), and thus other cellular events besides the collision of cleavable complexes with replication forks may have a role in CPT induced DNA damage. The molecular basis of CPT induced cell death is not completely understood, since topo I cleavable complexes are readily reversible after drug removal (Pommier, 2005). It is generally accepted that collision of the moving replication fork with the topo I cleavable complexes leads to three biochemical events: formation of a double-strand DNA break, irreversible arrest of the replication fork, and formation of an irreversible topo I-linked DNA break (Tsao et al.,

1993; Hsiang et al., 1989). It is thought that one or more of these events initiates S-phase cell death and G_2 phase cell cycle arrest.

4.4 DNA Topoisomerase II Targeting Agents: Etoposide and Mitoxantrone.

In contrast to the limited number of drug classes that target topo I, topo II is the molecular target for a number of diverse drug classes, including the epipodophyllotoxins, isoflavinoids, anthracyclins, actinomycins, anthracenediones and acridines (Table 3). Etoposide (VP-16) and teniposide (VM-26) are two semi-synthetic epipodophyllotoxins. Etoposide and teniposide are derived from 4'-demethylepipodophyllin benzylidene glucoside (DEPG), a naturally occurring compound synthesized by *Podophyllum peltatum*, the North American May Apple Mandrake (Figure 10) (Imbert, 1998; Hande, 1998; Sinkule, 1984). The May Apple was first described in 1731 and can be found growing from the coasts of Northern Florida to Maine (Catersby, 1731). Native American Indians and American pioneers reportedly used the plant for medicinal purposes, such as for the treatment of skin cancer (Hande, 1998). In 1844 the first alcohol extraction of the plant was reported and the isolated resin was called podophyllin (King, 1844). For over twenty years, numerous isolation procedures were conducted, fronting an intense search for the active compound. These

Figure 10. *Podophyllum peltatum*, also known as the May Apple Mandrake. The plant shown here is bearing fruit, the richest source of podophyllotoxins. This photograph was reproduced with written permission from the Maryland Conservation Council.

investigations lead to the development of VP-16 and VM-26, which were renamed, etoposide and teniposide, respectively (Stahelin and Von Wartburg, 1989).

Unlike topo I targeting agents, topo II targeting drugs are able to kill cells at all points in the cell cycle (Dubrez et al., 1995). Topo II inhibitors such as VP-16 (Figure 11) and mitoxantrone (Alberts et al., 1980; Koeller and Eble, 1988) (Figure 12) stabilize the complex formed by topo II and the 5' cleaved ends of the DNA, thus forming stable protein-linked double strand DNA breaks (Meresse et al., 2004). VP-16 and mitoxantrone inhibit topo II DNA religation activity, leading to an increase in DNA scission (Liu, et al., 1983). The DNA breaks are believed to interfere with the progress of the moving replication fork (Qiu et al., 1996). Cells recognize drug-induced DNA damage and eliminate the damaged cells by apoptosis, but the pathway from DNA damage to cell death remains uncertain (Valkov and Sullivan, 2003). Etoposide mediated cell death is reported to occur when DNA-dependent protein kinase (DNA-PK) recognizes an accumulation of DNA double strand breaks and then phosphorylates p53 (Karpinich et al., 2002). Activated p53 then results in increased transcription of the pro-apoptotic protein Bax, which then translocates into the mitochondria and induces the release of cytochrome c.

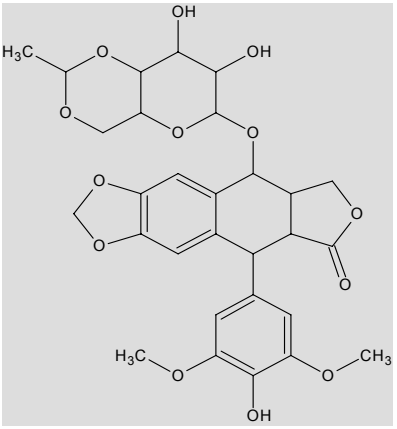


Figure 11. Etoposide (VP-16)

Mitoxantrone (MTX) is a synthetic anthracenediones that was developed as an analogue of doxorubicin (Fox, 2004). Mitoxantrone has been shown to have multiple mechanisms of action. In addition to being a topo II inhibitor (as previously described

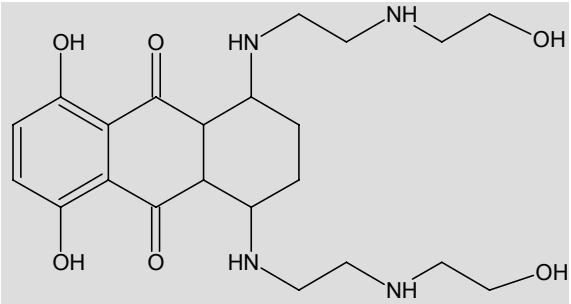


Figure 12. Mitoxantrone (MTX)

above), mitoxantrone also interferes with RNA synthesis, and intercalates with DNA.(Duff, 1984; Isabella et al., 1993) DNA intercalation results in aberrant DNA-protein crosslinks and DNA strand breaks (Hagemeister et al., 2005). Mitoxantrone has also been shown to inhibit antigen presentation and decrease the secretion of proinflammatory cytokines, which has important roles in modulating the immune response (Fox, 2004). MTX has also been shown to alter prostaglandin biosynthesis and calcium release (Ehninger et al., 1990). In summary, MTX has been shown to have multiple biological effects but, the drug's antitumor activity is mainly attributed to its interaction with DNA topoisomerase (Koeller and Eble, 1988). However, it is possible that these other cellular events could contribute to drug sensitivity to MTX. Mitoxantrone was approved by the Food and Drug Administration for the treatment of adult acute myeloid leukemia and symptomatic hormone-refractory prostate cancer. Mitoxantrone also has demonstrated beneficial activity in preclinical lymphoma models and appears clinically active against both aggressive and follicular lymphomas (Hagemeister e., 2005). Topo II targeting agents have been used effectively in the treatment of non-Hodgkin's lymphoma, leukemias, small-cell lung cancer and soft-tissue sarcomas. However, cancer cells have many mechanisms for evading topo II mediated drug cytotoxicity. Mechanisms of drug resistance to topo II targeting agents are reviewed in chapter six. .

4.5 Dual Inhibitors of DNA Topoisomerase.

Dual inhibitors of topo I and topo II are emerging as a new class of cytotoxic agents with promising anti-tumor activity (Denny and Baguley, 2003). The dual topo inhibitors have activity against both topo I and topo II enzymes and can be classified into three groups: DNA intercalators, non-DNA intercalators, and hybrid molecules (Denny and Baguley, 2003). The DNA intercalators physically intercalate between DNA bases, whereas the non-DNA intercalators recognize structural motifs present in topo I and II proteins. Two examples of dual topo I and II DNA intercalating agents include are N-[2-(dimethylamino) ethyl] acridine-4-carboxamide dihydrochloride (DACA) and the benzophenazine referred to as XR11576 (Mistry et al., 2002). I*n vitro* experiments using purified topo and DNA substrates show that DACA induces DNA breakage by both topo I and II (Finlay et al., 1996). However, the mechanism of DACA inhibition on topo I and II is still in question. The data suggests that DACA may act as a topo poison at a low drug concentration (0.5-5 μM) and as catalytic inhibitor at a higher drug concentration (≥ 10μM) (Spicer et al., 2000; Bridewell et al., 1999). Thus, topo-DNA cleavage complexes are not observed at the higher drug concentrations. This may be possible, if the intercalation of DACA into DNA at higher drug concentrations inhibits the binding of topo to DNA. DACA has completed phase I and II clinical trials (Dittrich et al., 2003; Caponigro et al., 2002; Twelves et al., 1999) and has been shown to overcome mechanisms of *in vitro* drug resistance in several multidrug resistant cell lines that express drug efflux pumps (i.e., P-glycoprotein, multidrug resistance protein, and multidrug resistance related protein) (Davey et al., 1997). DACA has also been shown to be active against solid tumors in mice (Bridewell et al., 1999).

Four examples of non-DNA intercalating agents have been described and include NK109, acetyl Boswellic acid, BN 80927, and F-11782. Acetyl-Boswellic acid (acetyl-BA) is isolated from the gum resin of *Boswellia serrata,* a tree that grows in Asia. Acetyl-BA inhibits topo I and IIα by binding directly to topo enzymes, rather than DNA-topo complexes, and is believed to compete with DNA for binding sites on topo (Syrovets et al., 2000). BN 80927 is a camptothecin analogue that has a seven-membered β-hydroxylactone ring, in contrast to other camptothecins that have a six membered α-hydroxylactone ring (Huchet et al., 2000, Demarquay et al., 2000). The α-hydroxylactone ring in BN 80927 is modified by the insertion of methylene (-CH2) group. When compared to camptothecin, topotecan, and irinotecan, BN 80927 has improved plasma stability because the additional methylene group undergoes slow and irreversible hydrolytic ring-opening (Demarquay et al., 2000). *In vitro* and *in vivo* experiments show that BN 80927 inhibits topo-I mediated supercoiled-DNA relaxation because it stabilizes topo I- cleavage complex (Lavergne et al., 1999). BN 80927 was also shown to inhibit topo II mediated supercoiled-DNA relaxation and DNA decatenation activity (Demarquay et al., 2000). In contrast to etoposide, BN 80927 does not stabilize topo II-DNA cleavage complexes (Demarquay et al., 2000). Thus, BN 80927 is a topo I poison and a topo II catalytic inhibitor. The fourth example of a non-intercalating dual topo I and II inhibitor mentioned above was F11782. Treatment of P388 murine leukemia cells with F11782 results in dose-dependent DNA fragmentation and activation of apoptotic pathways through a caspase 3/7 dependent mechanism. F11782 has also been shown to have improved *in vivo* anti-tumor activity, relative to three other cytotoxic agents (Kruczynski et al., 2004).

The third group of dual topo inhibitors is known as "hybrid molecules" because they consist of a topo I inhibitor biochemcially linked with a topo II inhibitor. The hybrid molecules are still in early stages of drug development and have not been formally reported in the literature (Denny and Baguley, 2003). The dual topo inhibitors are promising new drugs because they may evading toxicity by compensating one enzyme for another enzyme.

4.6 Conclusions.

Topo I and topo II are the molecular targets of several antitumor agents used for the treatment of hematological malignancies (Wang, 1996). Topo I is the major molecular target of the camptothecins (i.e., topotecan and irinotecan). The camptothecins are topo I poisons, and thus stabilize topo I-DNA cleavage complexes Topo II poisons, such as VP-16 and VM-26, stabilize topo II cleavable complexes, whereas the catalytic inhibitors of topo II do not stabilize cleavable complexes. The catalytic inhibitors prevent topo binding to DNA, or trap topo II in a closed formation. Dual inhibitors of topo I and topo II are promising new cytotoxic agents, but the focus of this investigation is on topo I and topo II poisons and topo II catalytic inhibitors. Drugs that act on other molecular targets besides topoisomerases are briefly described in following chapter.

Chapter Five

Non-topoisomerase Interacting Agents

5.1 Introduction.

Although some drugs interfere with DNA metabolism, they may do so without associating with a topoisomerase and should not be confused with the topoisomerase interacting agents described above. Thus, the primary mechanism by which these types of drugs exert their cytotoxic effect is independent of topo content or activity. For example, DNA alkylating agents such as Carmustine, a lipophilic nitosourea, undergoes hydrolysis *in vivo* to form reactive metabolites that produce lethal O^6-chloroethylguanine DNA interstrand crosslinks (Egyhazi et al., 1991). The non-topoisomerase interacting agents can be used in laboratory studies to help distinguish mechanisms of cellular drug resistance that are a result of alterations in the quality or content of topo from other non-topo related cellular events, such as alterations in drug transport. In some instances, exposure to one type of drug can result in cross-resistance to many other drugs that are structurally unrelated, a phenomenon known as multidrug resistance. We assessed human myeloma cell lines for cross-resistance to taxol, ara-c, cisplatinum, and carmustine. These cytotoxic agents were selected because of their differences in chemical structure and mechanism of action (figure 13; table 4). To improve drug sensitivity, the non-topoisomerase drugs are often given in combination with topo targeting cytotoxic

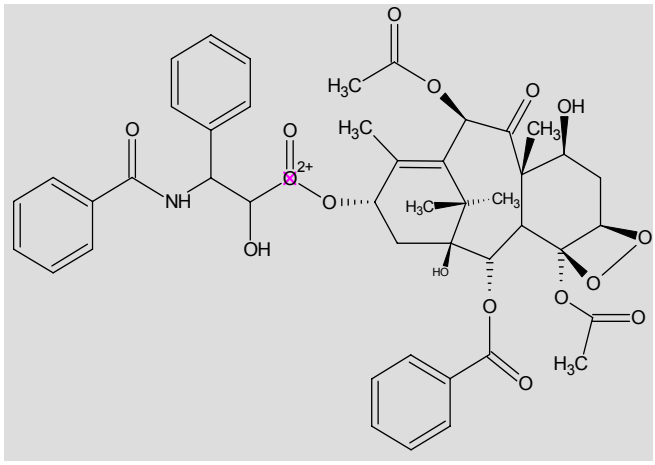
agents. For example, a combination of topotecan, ara-c, and cisplatin have reached phase II clinical studies for relapsed or primary refractory lymphomas (Coutinho et al., 2004). Unfortunately, multidrug resistance to combination chemotherapy is a limitation that needs to be overcome. Cisplatin, paclitaxel, ara-c, and carmustine are four examples of non-topoisomerase targeting agents described herein.

5.2 Cisplatinum.

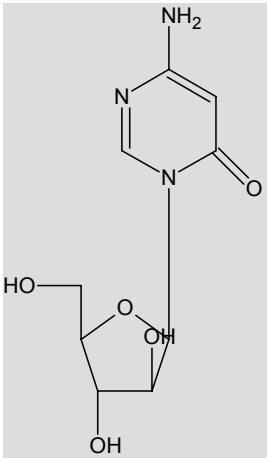
Cisplatin (*cis*-diamminedichloroplatinum; CDDP) and its synthetic analogues are DNA crosslinking agents and platinum containing compounds that exert their cytotoxic effects by inducing the formation of various types of DNA lesions (Crul et al., 2002; Iikima et al., 2004). Cisplatin is a DNA crosslinking agent with potent antitumor activity against various kinds of cancers including, non-small cell lung cancer (Wang et al., 2004). However, acquired drug resistance to CDDP is a common observation in these patients. The mechanisms of drug resistance to cisplatin are unknown, but enhanced DNA repair, altered drug transport, and drug inactivation by metallothionine have all been associated with decreased drug sensitivity to CDDP (Wang et al., 2004). Furthermore, sensitivity to DNA-crosslinking reagents are inversely proportional to topo levels and activity, and thus elevated levels of topo II correlate with resistance to cisplatin (Tan et al., 1987). This has been explained by the involvement of topo IIα in processing DNA damage induced by DNA crosslinking agents (Tan et al., 1987). A deficiency in topo IIα activity correlates with increased drug sensitivity as a result of an increase in drug-induced sister chromatid exchanges. These results suggest that the efficacy of CDDP in cancer chemotherapy can be improved through inhibition of topo IIα.

5.3 Paclitaxel.

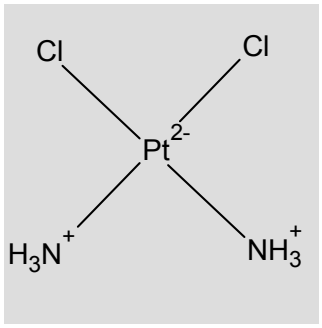
Microtubules are intracellular organelles, formed from the protein tubulin, involved in chromosome segregation, transport, motility, and cell structure (Jordan and



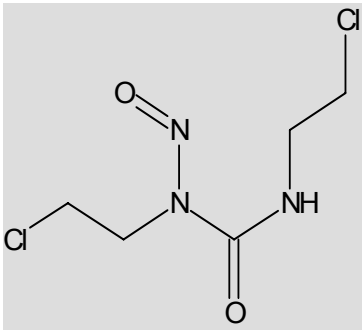
Paclitaxel (Taxol)



Cytarabine (Ara-c)



Cisplatinum (CDDP)



Carmustine (BCNU)

Figure 13. Paclitaxel (Taxol), Cytarabine (Ara-c), Cisplatinum (CDDP) and Carmustine (BCNU). Structures of the non-topoisomerase associated agents used in the soft agar cytotoxicity experiments (see "materials and methods") .

Table 4. Comparison of DNA Topoisomerase Inhibitors with Non-Topoisomerase Targeting Anticancer Agents

	Topoisomerase Associated			Non-topoisomerase Associated			
	Topotecan (TPT)	**Etoposide (VP-16)**	**Mitoxantrone**	**Paclitaxel (Taxol)**	**Cytarabine (Ara-C)**	**Carmustine (BCNU)**	**Cis-platinum (CDDP)**
Class	Alkaloid	Epipodophyllotoxins	Anthracenediones	Mitotic inhibitor	Anti-metabolite	Nitrosourea (Alkylating)	Alkylating
MW*	457.9	588.6	517	853.9	243.2	214.06	300
Mechanism of Action	Topo I inhibitor	Topo II inhibitor	Topo II inhibitor	Inhibits microtubule formation	DNA polymerase	DNA crosslinking	DNA crosslinking
Cell-cycle dependent?	yes, S-phase	yes, late S and G_2	yes	yes	yes, S-phase	some, G1 and G2/M	no
Examples of *In vitro* Mechanisms of Drug Resistance	Altered cellular content or activity of topo I; altered drug transport	Altered cellular content. location , or activity of topo IIα; altered drug transport	Altered cellular content , location, or activity of topo IIα; altered drug transport	Altered drug transport; gene mutations in β tubulin	Altered drug transport	Enhanced DNA repair	Drug inactivation; enhanced DNA repair; altered drug transport
Therapeutic Uses	Ovarian cancer, SCLC, gliomas, AML, CML, MM, MDS, neuroblastoma,	ALL, AML, Brain tumors, Ewing's sarcoma, Histiocytosis X	CML, AML, ALL, breast cancer, NHL, ovarian cancer	breast cancer, ovarian cancer, head and neck cancer, lung cancer	AML, ALL CML, NHL	brain tumors, Hodgkin's lymphoma, Non-Hodgkin's lymphoma	bladder cancer, adrenocortical cancer, lung cancer, ovarian cancer, testicular
References	Rasheed and Rubin, 2003	Meresse et al., 2004; Hande, 2003	Thomas and Archimbaud, 1997; Center, 1993	Jordan and Wilson, 2004	Wills et al., 2000; Abdel et al., 2000	Linfoot et al., 1988 Ueda-Kawamitsu, et al., 2002; Henderson et al., 1987	Brabec and Kasparkova, 2005; Arbuck, 1994

Wilson, 2004). Taxol is a tubulin-binding antimitotic agent with antitumor activity in a variety of cancers including breast cancer (Yvon et al., 1999; Fountzillas et al., 2004). Cells acquire drug resistance to taxol by decreasing intracellular concentration through protein transport pumps and by gene mutations in the beta tubulin gene that alter drug binding properties (Zhou et al., 2004; Hadfield, et al., 2003; Dumontet and Sikie, 1999).

5.4 Cytarabine (Ara-C).

Cytarabine (ara-C) is an antimetabolite and nucleoside analog of deoxycytidine (Frei et al., 1973; Kufe and Spriggs, 1985). Ara-c differs from deoxycytidine by the addition of a hydroxyl group on the 2' position of the sugar moiety. Once inside the cell, ara-C is converted into an active form, cytarabine-5'-triphosphate (ara-CTP) (Frei et al., 1973). The rate limiting step of ara-C activation is catalyzed by deoxycytidine kinase (Coleman et al., 1975). Ara-CTP competes with deoxycytidine triphosphate for incorporation into DNA, and thus is a competitive inhibitor of DNA polymerase (Kufe and Spriggs, 1985; Furth and Cohen, 1968). Incorporation of ara-CTP into DNA eventually results in inhibition of DNA synthesis and cell death (Mikita and Beardsley, 1988; Kufe et al., 1984). Incorporation of Ara-c into DNA is also associated with local conformational changes in DNA, inhibition of DNA polymerase, and a decreased ability of transcription factor binding to DNA binding elements (Zhang et al., 2004b; Mikita and Beardsley, 1988). Ara-C is often used in combination therapy for different types of leukemia (Szafraniec et al., 2004). Complete response rates in acute myelocytic leukemia (AML) reach 65-80%; however, patients usually relapse within 2-years and are resistant to subsequent chemotherapy (Cros et al., 2004). Resistance to ara-C has been attributed

to overexpression of the P-glycoprotein drug efflux pump, but some patients who are resistant to ara-C do not express P-glycoprotein (Galmarini et al., 2002a). Drug resistance in these patients has been partially attributed to deficiency in the human equilibrative nucleoside transporter 1 (hENT1), a transmembrane drug influx pump (Boleti et al., 1997). A lack of hENT1 presumably results in less intracellular concentrations of ara-C because of decreased intracellular drug import (Galamarini et al., 2002a; Lang et al., 2001).

5.5 Carmustine (BCNU).

Carmustine (BCNU) is a nitosourea compound with DNA alkylating activity. BCNU forms a chloroethyl adduct on the O^6 position of guanine, which spontaneously converts to N^1-O^6 ethanol-guanine (D'Incalci et al., 1988). The adjacent cytosine covalently binds to the N^1-O^6 ethanol-guanine to form an intrastrand cross-link that interferes with DNA replication and transcription (Erickson et al., 1980). Carmustine has antitumor activity against leukemias and gliomas, and is currently being evaluated in phase II clinical trials for recurrent glioblastoma (Brandes et al., 2004). A major limitation to BCNU effectiveness is that acquired drug resistance usually occurs after the first administration of the drug (Brandes et al., 2002). Acquired chemoresistance to BCNU has been attributed to the expression of the DNA repair enzyme, O (6)-alkylguanine-DNA- alkyltransferase (AGT), because this enzyme removes alkyl groups from DNA (Brandes et al., 2002).

5.6 Conclusions.

Topoisomerases are not the molecular targets of DNA crosslinking agents (i.e., cisplatin), anti-metabolites (i.e., ara-C), DNA alkylating agents (i.e., carmustine), or anti-microtubules (i.e., paclitaxel). Non-topoisomerase targeting agents are sometimes used in combination with topoisomerase targeting agents in cancer chemotherapy. Cancer cells have several molecular adaptations to evade drug induced cytotoxicity. Mechanisms of drug resistance to topoisomerase targeting agents are the focus of this investigation and are discussed in chapter five.

Chapter Six

In vitro Mechanisms of Topoisomerase Associated Drug Resistance

6.1 Introduction.

In the preceding sections, the family of DNA topoisomerases and commonly used chemotherapeutic drugs has been reviewed. The focus has been on the topoisomerase I and II as the molecular targets of topoisomerase targeting cytotoxic agents. In this chapter, the mechanisms of tumor cell resistance to topo targeting agents and especially topo II mediated cellular drug resistance are explained (reviewed in Engel et al., 2003).

Although the development of new drugs and new drug combinations has resulted in more effective treatment of cancer, a major factor limiting their usefulness is the presence of drug-resistant cancer cells that can exist prior to (intrinsic or *de novo* drug resistance) or arise during cancer therapy (acquired drug resistance) (Bellamy et al., 1990). Whether drug resistance is intrinsic or acquired, mechanisms conferring altered drug sensitivity may be categorized as involving a pre-target event, a drug-target interaction, or a post-target event (Larsen and Skladanowski, 1998). Mechanisms of drug resistance that result in a modification to the drug itself are called pre-target events because the cellular insult occurs before the drug reaches its intended molecular target. Drug metabolism is one example of a pre-target event because the drug becomes.

modified before reaching its intended molecular target. Modifications to the drug's intended target, however, can also occur and results in altered drug-target interactions. For example, sequestration of the drug's intended target into a cellular compartment different from the drug results in an inability of the drug to interact with its target. In other instances, neither the drug nor the drug's target is modified. Post-target events occur after the drug-target interaction occurs, and thus do not directly involve the drug or the drug's target. For example, altered cell-cycle progression can be a post target event, if the drug's target is not a cell-cycle regulatory protein. Pre-target events, drug-target interactions, and post-target events are discussed herein as they pertain to mechanisms of drug resistance to topoisomerase targeting agents. Figure 14 and figure 15 summarize the pre-target events, drug-target interactions, and post-target events that can lead to altered drug sensitivity to topo targeting agents. Each type of mechanism of drug resistance that is illustrated in these two figures is explained separately in the following sections. However, mechanisms of drug resistance are not mutually exclusive events and thus, there is a fair amount of overlap between the types of drug resistance mechanisms.

6.2 Pre-target Events: Altered Drug Transport.

Alterations in drug transport can either be a result of decreased drug import or increased drug efflux. Some hydrophilic drugs need to be transported across the plasma membrane and into cells by specific drug transporters (Cass et al., 1998). For example, cytarabine

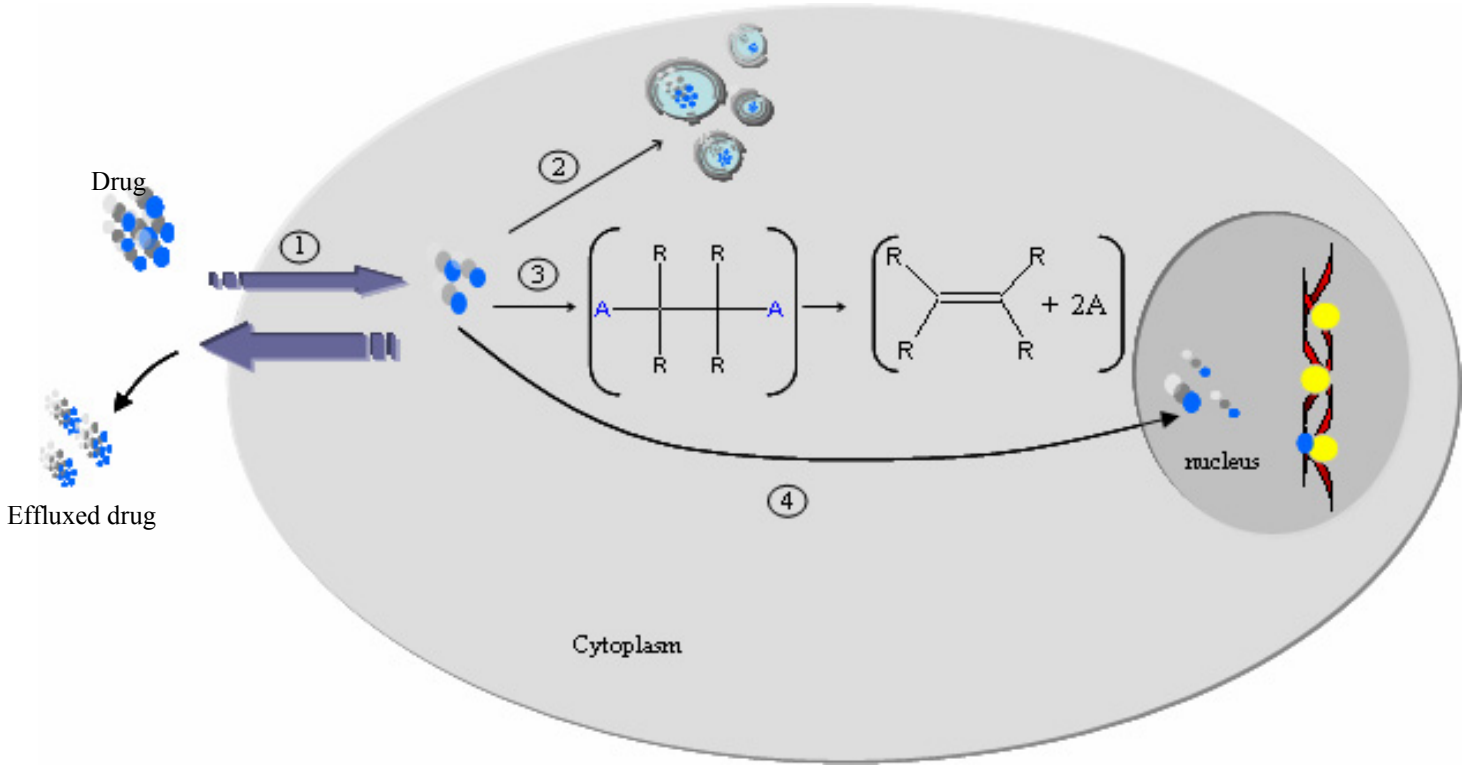


Figure 14. Alterations in Pre-target Events Associated with Drug Resistance. Mechanisms of cellular drug resistance to topoisomerase inhibitors can be categorized as pre-target events, drug-target interactions, and post-target events (see reference Larsen and Skladanowski, 1998). Multiple cellular factors can be involved in conferring altered drug sensitivity. Altered drug transport is shown in (1) as the expression of drug transport protein pumps located on the plasma membrane that can result in either decreased drug import or increased drug efflux from the cell. Once inside a cell, a drug can be sequestered into intracellular vesicles resulting in altered drug distribution as shown in (2). Some drugs undergo metabolic modifications, illustrated in (3), that alter their mechanism of action or result in an inactive form. Other drugs such as irinotecan are a prodrug that must be converted to an active metabolite. Cytotoxic agents that target topoisomerase must be move into the nucleus (4) where drug-target interactions occur. Mechanisms of drug resistance by alterted drug-target and post-target events are illustrated in figure 15.

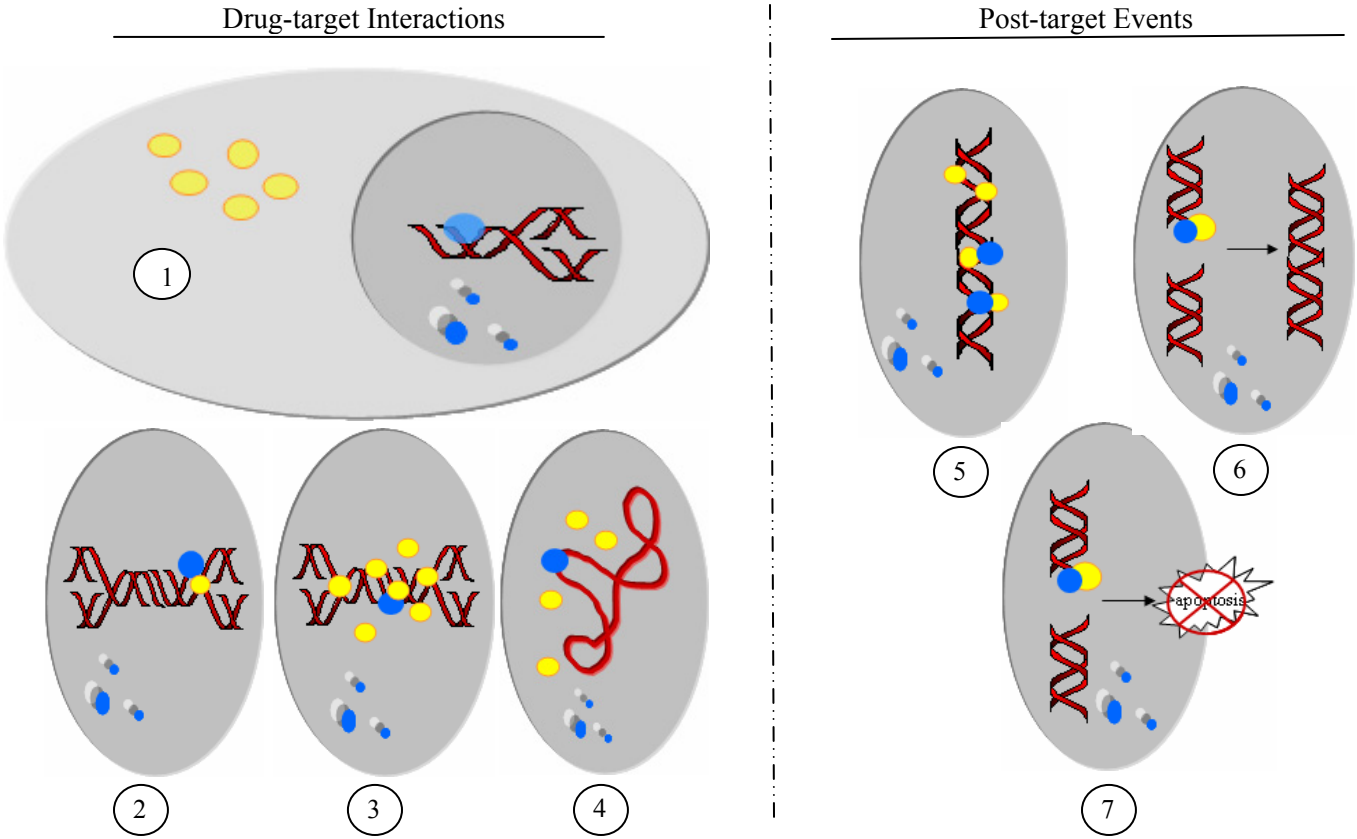


Figure 15. Alterted Drug-target Interactions and Post-target Events Associated with Drug Resistance. Altered drug-target interactions include (1) altered subcellular localization of nuclear enzyme that results in decreased amount of topo present in the nucleus (2) decreased amount of active enzyme in the nucleus is associated with resistance to topo poisons (3) increased amount of active topo enzyme is usually atttirbuted to decreased drug sensitivity to catalytic inhibitors of topo and (4) altered chromatin structure can result in altered topo-DNA binding activity. Post-target events associated with drug resistance to topo targeting agents include (5) decreased amount of replicating cells, (6) DNA-repair of cleavable complexes, and (7) decreased ability to initiate apoptosis. Topo is shown as yellow circles, the drug is shown as blue/grey circles, and DNA is shown as a red double helix except in (4) where DNA is shown in a relaxed conformation. In diagrams (5-7) the area of the cytoplasm is omitted because these events occur in the nucleus. For a review of this topic see reference (Larsen and Skladanowski, 1998).

(ara-C) is a hydrophilic nucleoside analogue that is transported into cells by the human equilibrative nucleoside transport protein 1 (hENT1), a drug influx pump that is expressed in the plasma membrane of some human cells (Boleti et al., 1997). *In vitro* cellular drug resistance to ara-C has been associated with lack of hENT1 mRNA expression in human T-lymphoblast CCRF-CEM cell lines selected for resistance to ara-C (Lang et al., 2001). Furthermore, shorter disease-free survival correlates with the absence hENT1 gene transcription in patients with acute myeloid leukemia (Galmarini et al., 2002b).

Topoisomerase targeting agents must also be taken up by cells and transported to the nucleus to be effective as cytotoxic agents. Therefore, reduced drug accumulation is a chief mechanism conferring drug resistance topoisomerase targeting agents in several drug-resistant cell lines (Matsumoto et al., 2005; Kamath et al., 1992; Hendricks et al., 1992). In general, topoisomerase targeting agents are presumed to enter cells by passive diffusion and are rarely found to be substrates of drug influx pumps. However, both active and passive transport of camptothecin has been found to occur in human intestinal cells (Gupta et al., 200). Influx of topotecan and SN-38 has also been suggested to occur by active transport in ovarian cells (Ma et al., 1998). Drug resistance in these cells was attributed to decreased drug accumulation independent of P-gp and MRP expression. Nevertheless, topo targeting agents are more commonly shown to be substrates for several drug efflux pumps. Therefore, tumor cells that express drug transport proteins involved in the extrusion of topo interacting agents are found to be drug resistant. Table 5 summarizes the chemotherapy related substrates for drug transport proteins.

Table 5. Drug Transporters and Chemotherapy Substrates

rug Name	Drug Transporters*	Member of ABC-Transporter Superfamily?	Drug Influx or Efflux?	References
Etoposide (VP-16)	P-gp/MDR1, MRP1, MRP2, MRP3,	Yes	Efflux	Lorico et al., 1995 Neuhaus et al., 1995
Teniposide	P-gp/MDR1, MRP3	Yes	Efflux	Kool et al., 1999
Mitoxantrone	MRP2, BCRP	Yes	Efflux	Neith and Lage, 2005
Topotecan (TPT)	BCRP	Yes	Efflux	Yang et al., 2000
***Cis*-platinum (CDDP)**	MRP2, MRP3	Yes	MRP2 and MRP3: drug efflux	Kool et al., 1997 Kawabe et al., 1999 Ishida et al., 2002
	CTR1	No, CTR1 is a plasma membrane carrier for nucleoside analogs.	CTR1: drug influx	
Sn-38 (active metabolite of CPT-11/ irinotecan)	BCRP	Yes	Efflux	Nakatomi et al., 2001
Paclitaxel (Taxol)	P-gp/MDR1, MDR2, BSEP	Yes	Efflux	Gerloff et al., 1998 Childs et al., 1998 Reinecke et al., 2000
Cytarabine (Ara-C)	hENT1	No, hENT1is an integral membrane proton belonging to the equilibrative nucleoside transport protein family	Influx	Boleti et al., 1997 Cass et al., 1998 Lang et al., 2001 Galmarini et al., 2002b
Carmustine (BCNU)	Not a substrate for known drug transporters	-	BCNU enters the cell by diffusion	Carter et al., 1972 Balcerczyk et al., 2003

Abbreviations used in this table: ABC-Transporter, ATP-binding cassette transporter; P-gp, P-glycoprotein; MDR, multidrug resistance; MRP, multidrug resistance-related protein; BCRP, breast cancer resistance protein; CTR1, copper transporter 1; BSEP, bile salt export pump; hENT1, human equilibrative nucleoside transporter1.
*Reviewed in reference, Ambudkar et al., 2003

Under normal conditions, ATP-binding cassette (ABC) transport proteins pump cytotoxic agents and xenobiotics out of the cells (van der Does and Tampe, 2004). Expression of ABC transporters in cancer cells results in decreased intracellular levels of the administered chemotherapeutic agent that is associated with the multi-drug resistance (MDR) phenotype (Ling, 1997). Multi-drug resistance describes the phenomenon that occurs when cells initially exposed to one class of cytotoxic agents become resistant to other unrelated drugs with various structural and chemical properties (Larsen and Skladanowski, 1998) The ABC transport proteins identified in humans include, multi-drug resistance protein-1/P-glycoprotein (MDR-1/P-gp) (Hoffmann and Kroemer, et al., 2004), multi-drug resistance-associated protein (MRP) (Hoffmann and Kroemer, et al., 2004), and breast cancer resistance protein (BCRP) (Doyle and Ross, 2003; Allen et al., 1999). The expression drug efflux pumps in cancer cells can reduce the intracellular accumulation of chemotherapeutic agents.

P-gp is a 170 kDa transmembrane glycoprotein that is able to expel a structurally heterogenous group of chemotherapeutic agents, including anthracyclines, vinca alkaloids and epipodophyllotoxins (Shustik et al., 1995). P-gp can extrude chemotherapeutic agents out of cells against a concentrations gradient (Lankelma et al., 1990). Overexpression of P-gp is estimated to occur in approximately 50% of all human tumors, and thus P-gp activity likely has a role in treatment related failure of some chemotherapy (Gottesman, 1993; Sikic, B.I. 1993). Overexpression of P-gp has been observed in many intrinsically drug resistant tumors derived from kidney, liver, adrenals, colon, and rectum (Goldstein et al., 1989). However, many drug resistant cells do not express P-gp,

suggesting a role for other drug efflux pumps in cellular drug resistance (Mirski et al., 1987, Zijlstra et al., 1987).

Some drug resistant cell lines express the multidrug resistance-associated protein (MRP) (Cole et al., 1992). MRP has seven family members conferring different substrate specificities. For example, methotrexate is a substrate for MRP1, but not MRP2 (Konno et al., 2003). Both P-gp and MRP are able to transport glutathione-S-conjugated chemotherapeutic agents (Müller et al., 1994). MRP expression is found in a wide range of tissues, but varies by family member. For instance, MRP1 expression is found in most tissues, whereas MRP6 expression is limited to the liver and kidney. The breast cancer resistance protein is another type of drug efflux pump that is found to be overexpressed in some drug resistant cell lines (Doyle and Ross, 2003). BCRP is expressed in breast tissue, placenta, intestine, and liver. Overexpression of BCRP results in cross-resistance to mitoxantrone and topotecan, but not to microtubular inhibitors, such as paclitaxel (Litman et al., 2000). The lung resistance related protein (LRP) is a major vault protein localized on lysosomes and in the nuclear pore complexes (Scheffer et al., 1995). LRP was initially described in drug resistant non-small cell lung cancer cell lines that were deficient in P-gp expression (Scheper et al., 1993). LRP modulates the transport of drugs between the nucleus and the cytoplasm. Expression of LRP is associated with the multidrug resistance and intrinsic drug resistance to doxorubicin, vincristine, and cisplatin (Mossink et al., 2003; Scheffer et al., 2000; Izquierdo et al., 1996). Drug uptake of [^{3}H-VP-16] is one method that is used in the laboratory to determine the cellular uptake of etoposide (or other cytotoxic agents) and drug transport protein activity.

6.3 Pre-target Events: Altered Drug Distribution.

After cellular drug uptake, cells may sequester drugs into intracellular vesicles and thus, withhold the drug from reaching its molecular target. Drug sequestration into cytoplasmic vesicles has been described *in vitro* in several drug resistant cell lines by following fluorescently labeled compounds, such as mitoxantrone (Schuurhuis et al., 1991; Gervasoni et al., 1991). Altered intracellular distribution of doxorubicin has also been described in leukemic cells obtained from patients with acute myeloid leukemia (Schuurhuis et al., 1995). Altered drug distribution is usually described as a shift in the amount of fluorescence from the nucleus to the cytoplasm. The pattern of fluorescence in the cytoplasm is consistent with vesicle formation. The apparent shift in fluorescently labeled compounds from the nucleus to the cytoplasm may be a result of LRP, the major vault protein found on the nuclear membrane that modulates nuclear-cytoplasmic drug transport. However, vesicle formation is not well understood (Slapak et al., 1992). One mechanism has been proposed for the sequestration of DNA topoisomerase targeting agents inside acidic vesicles (Larsen and Skladanowski, 1998). Accordingly, the cytoplasm of most cells is mildly alkaline, a requirement for the proper functioning of the *trans*-Golgi network and endocytotic secretory pathways (Tarakoff et al., 1983). Since some topo targeting agents such as anthracyclines, anthracenediones and amascarine act as weak bases, they can be protonated and retained inside acidic vesicles that are present in the cytoplasm (Larsen and Skladanowski, 1998). Drugs retained inside the lysosomes are then extruded from the cell via exocytosis (Tartakoff et al, 1983). Protonation and retention of topo targeting agents inside cytoplasmic lysosomes would theoretically inhibit drug transport into the nucleus. This hypothesis is supported by adriamycin-

resistant MCF-7 cells that sequester protonated compounds within vesicles, and secrete the drugs by endocytotic secretory pathways (Schindler et al., 1996). Hence, drug resistant tumor cells are able to maintain a pH gradient between the acidic environment of lysosomes and the alkaline environment of the cytoplasm, which ensures the entrapment of toxic substances inside acidic vesicles and subsequent extrusion of drugs out of the cell via the *trans*-Golgi network. Conversely, drug sensitive cells fail to establish a pH gradient, which disrupts proper formation of acidic vesicles and functioning of the *trans*-Golgi-network (Schindler et al., 1996). In summary, sequestration of chemotherapeutic agents inside vesicular compartments may have a role in altered drug sensitivity by reducing the amount of drug available to interact with its molecular target.

6.4 Pre-target Event: Drug Metabolism.

Once inside a cell, some topo I and II targeting agents can be modified by metabolic enzymes. Modifications to chemotherapeutic drugs may be necessary for activation of a prodrug or can result in a less effective cytotoxic agent. For example, CPT-11 is an inert drug that must be converted to the active compound SN-38 by carboxylesterases. Thus, tumor cells expressing low levels of carboxylesterases are resistant to CPT-11 (Danks et al., 1998). Furthermore, SN-38 can be detoxified when modified by one of several enzymes belonging to the family of UDP-glucuronosyltransferases (UGT) (Ciotti et al., 1999). The UGT enzymes can convert SN-38 into SN-38 glucuronide. Glucuronidation of SN-38 is associated with increased drug efflux from colon cancer cells and decreased drug sensitivity in colon, breast, and lung cancer cells (Cummings et al., 2002). Modification of DNA alkylating agents has also

been attributed to altered drug sensitivity. DNA alkylating agents can be detoxified when modified by the enzyme glutathione S-transferase (GST). GST belongs to a family of enzymes that catalyzes the conjugation of glutathione (GSH) to cancer drugs (Hayes and Pulford, 1995). Chemotherapeutic agents that are conjugated to glutathione are usually less active than their unconjugated counterparts. Glutathione conjugated drugs can also become substrates for the drug efflux pump, multidrug resistance protein 1 (MRP), and thereby pumped out of the cell (Paumi et al., 2001). Cellular drug resistance to anthracyclines is also associated with the intracellular amount of glutathione present because glutathione neutralizes free radicals. Some topo targeting agents, including the anthracyclines, exert their cytotoxic effect at least in part by producing free radicals. Glutathione is implicated in modulation of free radical formation by decreasing OH production. This is significant, because some human tumor cells have significantly higher levels of GSH. In summary, acquired drug resistance to DNA alkylating agents is attributed to deactivation by GST and extrusion from the cell by MRP, whereas drug resistance to anthracyclines is attributed to neutralization of free radicals by GSH. Additional mechanisms of cellular drug resistance to topo I inhibitors has also been associated with induction of hepatic cytochrome P-450, activation of nuclear factor kappa B (NF-κB), and activation of DNA-tyrosine phosphodiesterase (DTP1) (Frei et al., 2002; Yang et al., 1996; Harris and Hochhauser, 1992). DTP1 is an enzyme able to cleave the phosphodiester linkage between DNA and the active site tyrosine of topo I (Debethune et al., 2002). Yeast cells expressing DTP1 gene mutations are hypersensitive to CPT and thus, DTP1 may modulate CPT activity and affect cell sensitivity to topo I inhibitors (Cheng et al., 2002).

6.5 . Drug-target Events: Altered Quantity of Topoisomerase I or II Protein.

Generally, topo targeting agents turn topo into a lethal DNA damaging agent, so that the more target enzyme that is available to induce DNA damage, the more effective the agent. Thus, the amount of cleavable complex formation should increase as a function of increasing enzyme levels (Larsen and Skladanowski, 1998). This is supported by findings that the cytotoxicity of topo II poisons frequently correlates with topo II levels and the proliferative status of the cells *in vitro* (Sullivan et al., 1987a, 1987b; Chow and Ross, 1987). Since cancer cells often have a higher level of topo IIα protein and activity at all stages of the cell cycle, topo II is a frequent target of antineoplastic agents. Tumor cells that are undergoing high rates of DNA replication are more sensitive to topo inhibitors because topo IIα is preferentially transcribed in S-phase cells and cells that are in S and G_2/M have higher levels of topo IIα enzyme than non-proliferating cells. This has been demonstrated in breast cancer cell lines that are drug resistant to etoposide because of lower amounts of topo IIα protein relative to the parental cell line (Potmesil et al., 1988). Colorectal tumors show a 5-35 fold increase in topo I protein levels compared to normal colorectal cells. Topo I levels are also increased 2-10 fold in prostate tumors compared to benign prostate tissues. Alterations in the expression of topo protein may be due to loss or gain of gene copies or due to altered gene transcription. For example, methylation of the topo I gene may account for downregulation of topo I protein content in resistant cells (Fujimori et al., 1996).

6.6 Drug Target Interactions: Altered Quality of Topoisomerase I or II Protein.

The phosphorylation status may contribute to alterations in cellular drug sensitivity. Hyper and hypophosphorylation of topo II has been associated with reduced cleavable complex formation and decreased drug sensitivity to topo II targeting agents (Ritke et al., 1994). Changes in topo II phosphorylation are also associated with both increased and decreased enzyme activity and a decrease in the cellular content of topo II protein (Kroll, 1997). For example, hyperphosphorylation of topo IIα by casein kinase II is associated with decreased protein levels and decreased drug sensitivity to etoposide in breast cancer cells (Matsumoto et al., 1997). However, topo II phosphorylation does not always result in attenuation of topo II content. HL60 cells selected for resistance to doxorubicin were found to be cross-resistant to etoposide because of a reduction in the formation of VP-16 stabilized cleavable complexes, when compared to the parent cell line that were sensitive to both doxorubicin and etoposide (Ganapathi et al., 1996). However, the attenuation of cleavable complex formation was not a result of either a reduction in the amount or activity of topo II protein in these cells. The reduction in VP-16 stabilized cleavable complex formation was attributed to a 3-fold decrease in the phosphorylation status of topo IIα. Further investigations determined that, unlike topo IIα, there is a concomitant hyperphosphorylation of topo IIβ in the HL60 cells selected for doxorubicin resistance (Grabowski et al., 1999). Furthermore the changes in the phosphorylation status of both topo IIα and IIβ correlated with an increase in drug sensitivity to *m-AMSA*, a DNA intercalating agent that inhibits topo II. The increased drug sensitivity to m-AMSA was attributed to a 2-fold increase in topo IIβ stabilized cleavable complex formation as compared to VP-16 -topo IIβ cleavable complex

formation. These results suggest that the phosphorylation status of the different topo II isoforms may have a role in drug sensitivity that depends on the specific mechanisms of drug action.

Although topo I has been shown to be differentially phosphorylated during mitosis (D'Arpa and Liu, 1995), whether the changes in topo I phosphorylation have a role in conferring differences in drug sensitivity to topo I targeting agents has not been established. Human purified topo I enzyme activity is inactivated by phosphatases and can be reactivated by casein kinase II or protein kinase C, suggesting that phosphorylation regulates topo I enzyme activity (Kaiserman et al., 1988; Pommier et al., 1990). However, differently phosphorylated forms of topo I have been shown to cleave chromosomal DNA and relax supercoiled DNA with about equal topo I activity (D'Arpa and Liu, 1995). Alternatively, sumoylation followed by redistribution of topo I from the nucleoli to the nucleoplasm in response to topotecan or camptothecin exposure has been reported (Mo et al., 2002; Rallabhandi et al., 2002). It is not clear whether the translocation of topo I from the nucleoli to the nucleoplasm results in altered drug sensitivity to topotecan or camptothecin. Another cellular mechanism that could confer cellular resistance to topo I poisons appears to be the degradation of DNA-linked topo I, which is mediated by ubiquitination and small ubiquitin related modifiers (SUMO) (Desai et al., 1997). Proteins modified with ubiquitin molecules are frequently destroyed by the proteasome, whereas sumoylation has been shown to modulate gene transcription, protein-protein interactions, subcellular localization, and protein stability. When Chinese hamster ovary cells are treated with camptothecin, topo I-DNA cleavable complexes are multi ubiquitinated and destroyed by the 26S proteasome mediated pathway. However,

only 5-10% of the total amount of cellular topo I that was trapped in the cleavable complex after CPT treatment becomes conjugated to ubiquitin. Thus, it appears that a fraction of topo I cleavable complexes are marked for ubiquitin mediated proteolysis. Additional investigations are needed to address whether ubiquitin modified topo I cleavable complexes can result in altered drug sensitivity to camptothecin and whether proteasome mediated degradation of topo I cleavable complexes is specific to CPT and topo I. Camptothecin also induces sumoylation of topo I on Lys-117, located in the amino terminal region of topo I (Rallabhandi et al., 2002). Sumoylation of Lys-117 is associated with the clearing of topo I from the nucleoli to the nucleoplasm in response to CPT treatment.

There are numerous examples found in the National Library of Medicine that describe posttranslational modifications of topo I and II occurring in cell lines that have been selected for resistance to topo targeting drugs (Yu et al., 2004; Matsumoto et al., 2001, 1999; Yanase et al., 2000; Yu et al., 1997; Kasahara et al., 1992). However, it is beyond the scope of this investigation to review the results of all of these *in vitro* studies. A reasonable conclusion that can be inferred from the collective results of all of these investigations is that post-translational modification of topo I or II likely has a role in conferring alterations in drug sensitivity by altering the amount or activity or the enzyme, cleavable complex formation, or the stability of cleavable complexes. *In vivo* drug resistance is likely to be manifested by any one or more of these methods that is dependent on the drug class, enzyme targeted, and cell type.

6.7 Drug Target Interactions: Topoisomerase Gene Mutations.

In some cell lines, cellular drug resistance to topo I and II targeting agents has been attributed to both point mutations and deletions. Mutations in the topo I gene usually confers *in vitro* cellular drug resistance to one class of topo I targeting agents (Chang et al., 2002; Yanase et al., 2000). This is in contrast to topo II gene mutations that usually result in cells being cross-resistant to all classes of topo II targeting agents (Larsen and Skladanowski, 1998). However, some point mutations in the topo I gene, including tyrosine 723 to phenylalanine (T723F) and tyrosine 727 to phenylalanine (T727F), result in cells that are resistant to CPT and the indolocarbazole derivates of the antibiotic rebeccamycin (Woo et al., 2002). Topo I gene mutations conferring resistance to the camptothecins have been found in drug resistant human cell lines, yeast cells, and in tumor tissue from patients treated with irinotecan (Tsurutani et al., 2002). Mutations in the topo I gene appear to cluster either around the active site tyrosine residue (Tyr723) or around amino acids 361 through 364 (Larsen and Skladanowski, 1998). Gene mutations that involve amino acids 361, 362, 363, and 364, confer drug resistance to CPT and are believed to alter the DNA-enzyme binding properties of CPT (Rasheed and Rubin, 2003; Larsen and Skladanowski, 1998). Point mutations that occur in the core domain of topo I have been attributed to cellular drug resistance to camptothecin because of a reduction in topo I-DNA interactions (Kingma et al., 1999). Camptothecin resistant P388 murine leukemia cells contain both gene rearrangements and hypermethylation in one allele of the topo I gene, resulting in a reduction of topo I enzyme activity and mRNA transcripts (Tan et al., 1989).

Mutations in the topo IIα gene that confer altered cellular drug sensitivity to topo targeting agents have been studies in both yeast and mammalian cell lines (Larsen and Skladanowski, 1998). Point mutations in yeast cells have been shown to confer drug resistance to etoposide (Kingma et al., 1999). A point mutation in yeast topo II that results in histidine 1012 changed to a tyrosine (H1012Y) residue corresponds with a 3-4 fold increase in drug resistance to etoposide as compared to yeast cells expressing non-mutated topo II enzyme (Kingma et al., 1999). The observed drug resistance in these cells was explained by a decreased affinity of etoposide-enzyme binding as compared to non-mutated enzyme (Kingma et al., 1999). In human cell lines, selection for drug resistance to etoposide or teniposide has been shown to result in point mutations in the topo IIα gene (Patel et al., 1993; Bugg et al., 1991). Mutations found in human topo IIα in drug resistant cell lines tend to cluster either around ATP binding domain or the active site tyrosine residue (Nitiss and Beck, 1996). Furthermore, since topo II forms dimers, it is possible that heterodimers exist that consist of one drug-sensitive and one drug resistant subunit. Many cell lines that have been selected for resistance to topo II poisons *in vitro* have been shown to carry mutations in the gene for topo IIα, but mutations are rarely reported in patient samples (Wessel et al., 2002). Alterations in topo gene copy number can also occur in human cancers. For example, the topo IIα gene is coamplifed with the HER-2/neu oncogene in breast, ovarian, and bladder cancer cells because the two genes are in close proximity to each other (Simon et al., 2003; Hengstler et al., 1999). This is significant because an increase in topo IIα gene copy number is associated with cancers that have increased sensitivity to topo II poisons such as doxorubicin, while a deletion of one copy of the topo IIα gene is associated with resistance to doxorubicin.

The only mutation in topo IIβ that has been reported to date is derived from drug-resistant cell lines which contain topo IIβ gene mutations that completely abate the activity of the enzyme (Dereuddre et al., 1995). No study has been reported that assesses mutations in topo IIβ from clinical specimens.

6.8 Drug Target Interactions: Altered Chromatin Structure.

The topological structure of DNA modulates the level of topoisomerase binding (Zechiedrich and Osheroff, 1990; Howard et al., 1991). In general, eukaryotic topoisomerase I and II type enzymes prefer positive and negatively supercoiled DNA over relaxed DNA substrates (Osheroff, 1986). Modifications such as glycosylation and methylation have been shown to influence DNA conformation; however, the molecular mechanism leading from DNA modification to cellular drug resistance is not well documented (Stopper and Boos, 2001; Lopez-Baena et al., 1998). In mammalian cells, DNA methylation of cytosine in CpG islands is an important regulatory mechanism of gene expression. DNA methylation has also been shown to influence local conformational changes in the structure of DNA (Zacharias et al., 1988; Diekmann, 1987) DNA methylation is associated with remodeling of euchromatin to heterochromatin, suggesting that methylated DNA somehow alters the accessibility of DNA to transcription factors and regulatory proteins (Hori, 1983). Conformational changes that occur in methylated DNA have been also been suggested to alter the binding and DNA cleavage properties of topo I and II (Boos and Stopper, 2001; Leteurtre et al., 1994). For example, topo I and II cleavage patterns are modified *in vitro* by CpG methylation in c-myc gene DNA fragments (Leteurtre et al., 1994). Topo I and II

cleavage activity is shown to be dependent on the location of the methylated cytosine on the DNA in relation to the scissile bond. Methylated cytosine has both suppressive and stimulatory activity on topo I, whereas topo II cleavage activity is mostly suppressed by methylated cytosine. DNA methylation of CpG islands been proposed to have a role in tissue selectivity and efficiency of DNA topo targeting agents (Leteurtre et al., 1994). DNA methylation has also been shown to alter topo II decatenation activity in vitro, which may result in genomic instability, but whether this contributes to cellular drug resistance is not known (Zwelling et al., 1989).

6.9 Post-target Events: DNA Repair.

Both topo I and topo II have roles in DNA damage recognition and repair processes, but how cells repair drug-stabilized DNA cleavable complexes is poorly understood. Sensitivity to DNA-crosslinking reagents is inversely proportional to topo levels and thus, elevated levels of topo II correlate with resistance to DNA cross-linking agents such as melphalan and Cisplatin (Dvorakova et al., 2002; Hirota et al., 2002). This has been explained by the involvement of topo IIα in processing DNA damage induced by melphalan (Hirota et al., 2002). A deficiency in topo IIα activity correlates with increased drug sensitivity because of an increase in melphalan-induced sister chromatid exchanges (Karpinich et al., 2002). These results suggest that the efficacy of melphalan in cancer chemotherapy can be improved through inhibition of topo IIα. Similar observations have been reported in H69 small-cell lung cancer cells resistant to radiation and cisplatin, in part because of an increase in topo IIα expression (Hennes et al., 2002). The results suggest that increased topo IIα expression may confer radiation

and drug resistance because of increased DNA repair, and that radiation followed by a topo IIα targeting agent may be a potential strategy for overcoming resistance in small cell lung cancer. DNA repair pathways have also been implicated in conferring sensitivity to etoposide and camptothecin in human colorectal cancer cells (Jacob et al., 2001; Aebi et al., 1997). DNA mismatch repair (MMR) is normally involved in correcting base/base mismatches during DNA replication but it is also implicated in double strand DNA break repair and for modulating the induction of apoptosis as a result of various types of drug induced DNA damage. Cell lines defective in DNA mismatch repair pathways show increased sensitivity to both CPT and etoposide as compared to MMR proficient colorectal cell lines and sensitivity to these drugs does not correlate with endogenous levels of topo I or topo II (Jacob et al., 2001). In summary, differences in the processing and repair of DNA lesions are likely to have a role in drug sensitivity to topo targeting agents. It is plausible that the way cells remove and repair drug-stabilized cleavable complexes could influence cellular sensitivity to DNA damaging agents.

6.10 Post-target Events: Alterations in Cell Cycle Progression.

The cellular content of topo IIα protein is highest during G_2/M and lowest during early G1 of the cell cycle (Meyer et al., 1997; Nakajima et al., 1996). Topo IIα protein is also markedly decreased in growth-arrested cells as compared to proliferating cells (Holden et al, 1990, Turley, et al, 1997). In contrast, topo I and topo IIβ have a relatively uniformed expression throughout the cell cycle as compared to topo IIα (Drake et al., 1989). The cell-cycle and proliferation dependent fluctuations of topo IIα gene expression may also explain why some slow growing cancers may be intrinsically

resistant to topo inhibitors. Therefore, the application of drugs specifically directed against topo IIβ could be useful for tumors that express a mutant form of topo IIα or have a large fraction of cells in Go/G_1. In these cases, topo IIβ could be a preferred molecular target because unlike topo IIα, topo IIβ expression is not dependent on the cell cycle or proliferative stage of the cell. XK469 is a new anticancer agent that is reported to specifically target topo IIβ, although it may also have an anti-proliferative role involving cell-cycle related proteins (Snapka et al., 2001; Gao et al., 1999). XK469 exerts its cytotoxic effect by arresting cells in prophase as a result of an irreversible accumulation of cyclin B1 in the G_2/M phase of the cell cycle (Ding et al., 2001). XK469 has been shown to have activity against a broad spectrum of murine solid tumors, including tumors that express the multi-drug resistance gene (LoRusso et al., 1998-99).

6.11 Post-target Events: Altered Cell Death Pathways.

Certain cellular conditions such as glucose starvation, hypoxia, and DNA damage, are associated with drug resistance to topo II inhibitors, such as etoposide and adriamycin, because of a decrease in topo IIα gene expression. A decrease in transcription of topo IIα gene correlates with increased levels of Sp-3, YB1, and p53 status. Several studies have investigated the role of p53 in response to topo mediated DNA damage (reviewed in Valkov and Sullivan, 2003). The p53 protein has been shown to interact with topo II *in vitro* and stimulates the decatenation activity of topo II (Cowell et al., 2000). The p53 protein can arrest cell cycle progression and can commit cells to programmed cell death (Bladosklonny, 2002). Since p53 is mutated in a large percentage of cancers, this could suggest that a failure to induce apoptosis results in resistance to

DNA-damaging agents (Bunz et al., 1999). Since topo-targeting agents kill cells by inducing DNA damage, p53 deficient cells could be resistance to topo poisons. Other alterations in the apoptotic pathway may also confer cellular drug resistance to topo inhibitors. For example, an elevated level of the anti-apoptotic protein bcl-2 is associated with decreased drug sensitivity in cancer cells (Real et al., 2004).

6.12 Conclusions.

In summary, cancer cells have multiple different ways to evade the cytotoxic effects of topo targeting drugs. Tumor cells with *de novo* resistance fail to respond to initial drug treatment.. In contrast, tumor cells with acquired drug resistance are initially sensitive to drug exposure, but then become resistant with future drug exposures. The most commonly observed mechanisms of *in vitro* drug resistance to topo targeting agents usually describes alterations in drug transport or alterations in the amount or activity of topo IIα protein that may or may not be accompanied by topo IIα gene mutations.

Chapter Seven

In vivo Mechanisms of Topoisomerase Associated Drug Resistance

7.1 Introduction.

The mechanisms of drug resistance described for topo I and topo II inhibitors in model human cell lines have been well established, but the actual *in vivo* situation in patients with solid tumors and hematologic malignancies is still uncertain. Mechanisms of *in vivo* drug resistance to topoisomerase targeting agents are usually related with alterations in drug transport and the quantity of topo protein or mRNA in clinical preparations. However, *in vivo* drug resistance to topo inhibitors is likely to be a phenomenon influenced by a network of physiological factors, such as drug dosage, presence of the molecular target, the tumor microenvironment, and presence or absence of specific genetic aberrations (reviewed in Lehnert, 1996). The clinical activity of chemotherapy is also dependent on hepatic drug metabolism, extracellular drug concentration, intracellular drug transport, and ability of the drug to access the cancer cells. Furthermore, antitumor agents differ in their ability to diffuse through different tissues. For example, scar tissue from surgery or radiotherapy reduces response rates with chemotherapy, especially in head and neck cancers (Rooney et al., 1985). Furthermore, some tumors can be poorly vascularized and some drugs are unable to cross

the blood brain barrier. Therefore, various *in vivo* factors can influence how much active drug reaches the molecular target withincancer cells. Cellular drug resistance in humans is complex and appears to be both multifactorial and variable amongst individual patients.

The work presented herein definitively describes an *in vitro* mechanism of drug resistance observed to topo II targeting agents in a human leukemia and myeloma cell lines. Although clinical applications are speculated from the *in vi*tro data presented in this work (refer to pages 201-203 herein), a complete description of *in vivo* mechanisms of cellular drug resistance in the clinical environment was outside the limits of this investigation (reviewed in Engel et al., 2003). The focus here is to briefly describe our current understanding of clinical drug resistance to topoisomerase targeting chemotherapeutic agents. This chapter focuses on gene mutations, cellular content of topo I and II, and the tumor microenvironment as mechanisms of clinical drug resistance to topo targeting chemotherapeutic agents.

7.2. Gene Mutations.

There has been a constant search for point mutations, deletions, truncations, and atypical isoforms of topo in a wide variety of clinical samples and diverse tumor types. Several genetic aberrations, such as allelic inactivation, deletions, and point mutations, have been reported to occur in the gene for topo I and II in human cell lines. However, topo I and II gene mutations are rarely detected in patient samples. Thus far, the bronchial aspirate of one patient with small cell lung cancer treated with either etoposide-containing chemotherapy is the only reported case to have identified two point mutations

in the topo IIα gene (Kubo et al., 1996). DNA sequence analysis of this tumor show two transversions at codons 486 (G to A) and 494 (A to G), resulting in two missense mutations (arginine to lysine and glutamate to glycine, respectively). More frequently, chemotherapy with topo II inhibitors leads to gross genomic rearrangements, including gene amplifications and deletions of the topo IIα gene (Järvinen and Liu, 2003). For example, a recent clinical trial has detected topo IIα gene amplification by fluorescence in situ hybridization (FISH) in clinical samples from primary breast cancer tumor samples, which correlates with response to topo II inhibitors (Järvinen et al., 1999). Breast cancer seems to be one disease where amplification or deletion of the topo IIα gene frequently occurs (Järvinen and Liu, 2003). Furthermore, topo IIα has been shown to be a marker of tumor aggressiveness and invasive potential in breast cancer, but the complexity of the response of breast tumors to chemotherapy is usually determined by evaluation of several markers (Cardoso et al., 2001; Nakopoulou et al., 2000).

There have been a limited number of clinical studies reporting topo I gene mutations (Ohashi et al., 1996). One patient with large cell lung carcinoma who did not respond to cisplatin or irinotecan was found to have a missense mutation at codon 737 in the topo I gene (Tsurutani et al., 2002). However, it was not determined if the missense mutation altered CPT induced topo I cytotoxicity. Colorectal carcinomas have a high expression of topo I protein that is due to an increased topo I gene copy number, reaching up to eight copies (Boonsong et al., 2002, 2000). For this reason, irinotecan, a topo I inhibitor, is being evaluated in clinical trials for the effective treatment of colorectal carcinoma. Additional clinical studies are needed to determine if gene mutations found in drug resistant cell lines are clinically relevant.

7.3 Topoisomerase I and II Gene and Protein Expression.

The amount of topo protein has been investigated as a predictive factor for clinical response to topo I and topo II targeting chemotherapeutic agents. All studies point to a substantial interpatient variability in the amount of topo content and often fail to correlate the amount of topo enzymes with either the prognosis or with the clinical response to topo I and topo II inhibitors used in different combinations (McKenna et al., 1994). For example, in acute lymphoblastic leukemia (ALL), Western blot analysis, polymerase chain reaction (PCR), Northern blot analysis and immunohistochemistry demonstrate that topo I and topo II gene and protein expression are variable (Stammler et al., 1994; McKenna et al., 1994). In these studies, a total of 60% of the blasts are shown to be positive for topo II while the levels of topo I are in the range of 25-85% of the control cell line. However, no correlation is found between the level of topo and clinical response.

7.4 Tumor Microenvironment.

Additional physiological conditions exist *in vivo* that contribute to decreased drug sensitivity. For example, under physiological conditions in the bone marrow, tumor cells aggregate to form tumor cell masses that confer drug resistance. This is supported by *in vitro* studies demonstrating that when cancer cells are cultured in monolayers they are more susceptible to cytotoxic agents than when cultured as 3D aggregates, a phenomenon referred to as multicellular drug resistance (Jianmin et al., 2002; Desoize and Jardillier, 2000)). Cell adhesion mediated drug resistance (CAM-DR) is another phenomenon whereby tumor cells bind stromal cells or extracellular matrix proteins (Dalton, 2003).

CAM-DR represents an intrinsic mechanism of drug resistance used by malignant cells (Dalton, 2003). Collectively, these findings suggest that the resistance to anticancer drugs that are often observed in the clinic is likely to be a result of many physiological conditions that act simultaneously (Mattern and Volm, 1993). This hypothesis is supported by in vitro findings that cells selected for drug resistance have multiple mechanisms conferring decreased drug sensitivity (Hazlehurst et al., 1999; Harker et al., 1995). An example of an *in vitro* cell model that supports this theory comes from the mitoxantrone resistant 8226/MR4 8226/M20 myeloma cells (Hazlehurst et al., 1999). When compared to the drug-sensitive parental cell line, the 8226/MR4 cells were 10-fold resistant to mitoxantrone and 4-fold resistant to etoposide, whereas the 8226/M20 cells were 37-fold resistant to mitoxantrone and 15-fold resistant to etoposide. Drug resistance in both cell lines was associated with decreased drug accumulation in a P-gp and MRP independent mechanism. Decreased drug accumulation in these cells was attributed to an ATP dependent ouabain-insensitive drug transport mechanism. Drug resistance could be reversed by the chemosensitizer, fumitrenogin C. However, drug resistance in the 8226/M20 cells was also attributed to a 70-88% reduction in topo IIα and topo IIβ protein content and reduced topo II enzyme activity, which was not observed in the 8226/MR4 cells (Hazlehurst et al., 1999). Thus, cancer cells have numerous mechanisms for overcoming the drug cytotoxicity.

7.5 Conclusions.

Resistance to chemotherapy is a common problem in patiens with cancer and a major obstacle that must be overcome for cancer therapy to be effective. *In vivo* drug resistance is likely to be a multifactorial problem. Tumor cells can manipulate their tumor microenvironment to gain growth and proliferative advantages and some tumor cells may have gene mutatins that contribute to altered drug sensitivity. Therefore, clinicians must consider numerous biological variables that may differ between patients to optimize the effectiveness of cancer chemotherapy.

Chapter Eight

Nuclear-cytoplasmic Trafficking of Proteins

8.1 Introduction.

The separation of the nucleus from the cytoplasm plays a central role in regulating the subcellular localization of proteins, but is also a variable in drug delivery and cytotoxicity. Generally, topo poisons convert the enzyme into a lethal DNA damaging agent, such that the more active enzyme that is present in the nucleus, the more effective the poison (reviewed in Engel et al., 2003). Without the co-existence of the ternary drug-DNA-topo complex, topo inhibitors are less effective as cytotoxic agents. Thus, investigating the selective movement of macromolecules across the nuclear envelope represents a recent area of drug development (Dean et al., 2003). The potential of the DNA topoisomerases to shuttle between the nucleus and cytoplasm has been one area of recent investigations (Engel et al.2004; Valkov et al., 2000).

8.2 The Nuclear Pore Complex.

Recent investigations have elucidated several molecular pathways for the nuclear import and export of proteins across transport passageways or nuclear pore complexes (NPCs) (Weiss, 2003). The NPCs are located in the nuclear envelope where the inner

and outer membranes are fused, providing a link between the cytoplasm and the interior of the nucleus (Davis, 1995; Doye and Hurt, 1997; Görlich and Mattaj, 1996). The NPC is a large (125 MDa) (Reichelt et al., 1990) multimeric protein structure that perforates the nuclear envelope and channels proteins greater than 40 kDa into or out of the nucleus (Pante and Kann, 2002; Hinshaw et al., 1992). The NPC consists of a central ring-spoke structure flanked by filaments emanating from its nuclear and cytoplasmic surfaces (Hinshaw and Milligan, 2003; Akey and Radermacher, 1993). The pores are 25 nm in diameter and form 9 nm long channels (Peters, 1986). The number of NPCs per nucleus varies with the organism, cell type, and growth conditions (Maui, 1977). It is common for cells to have between 10^2 and 5 X 10^7 NPCs present per nucleus, depending on the metabolic or differentiation state of the cell. Mammalian cells typically have 3,000-5,000 NPCs, whereas yeast cells have approximately 120 NPC per nucleus (Winey et al., 1997).

8.3 Nucleoporins.

The NPCs are composed of approximately eight copies of 30 different proteins, the nucleoporins (Nups) (Rabut et al., 2004). A number of membrane and nonmembrane anchored Nups have been described in yeast and mammalian cells by mass spectroscopy (Cronshaw et al., 2002). Nup210 and glycoprotein 121 (gp121) are believed to play critical roles in anchoring the NPC to the nuclear envelope (Wozniak et al., 1989). The Nups are phosphorylated in a cell-cycle dependent manner and are hyperphosphorylated during M-phase (Favreau et al., 1996). During mitosis, the nuclear membrane breaksdown and the NPC is disassembled. Phosphorylation of nucleoporins is associated with the disassembly of the NPC as well as reformation of the NPC when cell division is

complete. Nucleoporins contain characteristic domains featuring multiple repeats of short peptide sequences ending in the amino acids phenylalanine and glycine (FG repeats) (Bayliss et al., 2000). The FG repeats provide docking sites for protein- receptor cargo complexes (Radu et al., 1995). Binding of transport receptors to the FG domains on the nucleoporins triggers the pore to dilate (Shahin et al., 2005; Shulga and Goldfarb, 2003). One model suggests that multiple FG rich repeats in the NPC channel form a meshwork through weak hydrophobic interactions, which can function as a sieve (Ribbeck and Görlich, 2001). Hydrophobic receptor-cargo complexes can transiently interact with the hydrophobic repeats, and thus dissolve the meshwork and translocate thorough the NPC. In contrast, hydrophilic proteins remain excluded, explaining the high selectivity of the NPC. Transport through the NPC machinery appears to be regulated by mitogenic signals, phosphorylation, and Ca^{2+} concentrations within the endoplasmic reticulum and nuclear lumen (Harreman et al., 2004; Greber and Gerace, 1995). For example, Ca^{++} stores in the nuclear envelope of *Xenopus laevis* oocytes, is suggested to regulate NPC gating (Perez-Terzie et al., 1996). In these cells, conformational changes in the NPC require calcium, such that when the Ca^{++} stores are depleted, a central plug occludes passage of nuclear transport processes. Similar results have been observed in HeLa cells grown in the presence of the calcium pump inhibitor, thapsigargin, which blocks signal-mediated protein import (Greber and Gerace., 1995). These results suggest that calcium stores present in the lumen of the endoplasmic reticulum, which is contiguous with the nuclear envelope, may have a role in regulating nuclear–cytoplasmic trafficking.

8.4 Nuclear and Cytoplasmic Targeting Sequences.

Since 1999, when Günter Blobel received the Noble Prize in Physiology and Medicine for discovering that proteins have intrinsic signals that govern transport and localization in cells, there have been significant advancements in understanding the mechanisms of nuclear-cytoplasmic transport of proteins (Raju, 2000; Shields, 2001). Investigative reports now describe many different types of nuclear-cytoplasmic targeting sequences, including nuclear localization sequences (NLS), nuclear export sequences (NES), nuclear retention sequences (NRS), cytoplasmic retention sequences (CRS), and bi-directional shuttling sequences (Jans et al., 1998). Proteins targeted for receptor-mediated transport across the NPC must either contain an NLS or NES. However, some proteins that do not contain a nuclear-targeting sequence still traverse the NPC by piggy-backing with another protein containing a NLS or NES.

8.5 Nuclear Localization Signals.

The first NLS, P**KKKRK** was identified in the Simian Virus 40 (SV 40) large T antigen (Kalderon et al., 1984). The NLS found in the SV40 large T antigen was later referred to as a monopartite sequence to distinguish it from the bipartite sequence, **KR**PAATKKAGQA**KKKK**LD, found in *Xenopus* nucleoplasmin (Robbins et al., 1991). The monopartite sequence is often referred to as the "classical" NLS and consists of a short cluster of basic amino acids, often preceded by an acidic amino acid or proline residue. Human DNA topo I has a classical monopartite NLS between amino acids 150-156 (Stewart and Champoux, 1996). The amino acid sequence of the monopartite NLS in topo I is KKIKTED. Recently, a second non-classical monopartite signal was identified

in topo I between amino acids 117-146 (Mo et al., 2000). In contrast to the basic amino acid residues found in a classical NLS, this NLS is unique because it is characterized by two clusters of acidic amino acids at amino acids 117-146 and 151-156. The first cluster of acidic amino acids is KDEPEDDG followed by a spacer region of 16 amino acids, and then the second cluster of acid amino acids is DEDDAD. Thus, topo I has two independent NLS with opposite charged amino acids. The precise amino acid sequence that targets topo IIβ to the nucleus has not been determined, but the last 116 amino acids found in the C-terminal domain in necessary and sufficient for nuclear localization of topo IIβ in mammalian cells (Cowell et al., 1998). The C-terminal domain is presumed to have a bipartite NLS, which has been implied by the presence of several bipartite NLS type motifs found in the C-terminal domain of topo IIβ.

Bipartite NLS, a second type of NLS, consists of two clusters of basic amino acids separated by a spacer region of approximately ten amino acids, often flanked by a neutral or acidic amino acid (Robbins, et al., 1991). Topo IIα contains a bipartite NLS located in the carboxy-terminus between amino acids 1454-1497 that consists of a spacer region of 21 amino acids (Mirski et al., 1997). The amino acid sequence for the NLS in topo IIα is KPDPA**K**T**K**N**RRKRK**PSTSDDSDSNFEKIVSKAVTS**KK**S**K**GESDD (Mirski et al., 1997). The two clusters of basic amino acids are shown in bold text. Computer software programs are available that can scan amino acid sequences for potential bipartite NLS (Shelagh et al., 1999). Additionally, previously described NLS are annotated in SWISS-Prot and PIR and can be retrieved at the NLS database located at the Predict NLS server (Nair et al., 2003).

8.6 Nuclear Export Signals.

Classic protein NES are hydrophobic rich sequences that have a characteristic spacing of leucine, isoleucine, valine, and/or phenylalanine amino acid residues (Elfgang et al., 1999). The Center for Biological Sequence Analysis at the Technical University of Denmark has prepared an NES predictor, called the NetNes 1.1 server, accessible to the public and found on the worldwide web (la Cour et al., 2004). The NetNes 1.1 server uses a prediction algorithm based on previously described NES, but also uses a "hidden Mark model" to identify non-hydrophobic residue that are not included in the consensus sequences for classical NES. To date, approximately 80 experimentally validated protein-NES have been identified and compiled on the NESbase version 1.0 database, which is also accessible on the worldwide web (la Cour et al., 2003). NES have been identified based on the fact that mutation or deletion of the NES renders it inactive in mediating nuclear export of the protein carrying it, or that the NES is active in effecting export from the nucleus of a carrier protein such as beta-galactosidase (Jans et al., 1998).

8.7 Retention Signals.

Some proteins contain nuclear retention or cytoplasmic retention signals, which do not specifically target a protein to the nuclear or cytoplasmic compartments, but assures their retention once there (Jans et al., 1998). The nuclear retention signals usually consist of an arginine rich motif and may include an arginine rich α-helical structure. One example of a NRS has been described in an SR protein. The SR proteins are RNA splicing factors that contain RNA recognition motifs and a C-terminal domain rich in alternating serine (S) and arginine (R) amino acids, known as the RS domain. The RS

domain can become phosphorylated and direct the subcellular localization and nucleocytoplasmic shuttling of individual SR proteins (Cazalla et al., 2002). The human SC35 SR protein, however, contains a dominant nuclear retention signal located in the RS domain, and thus is not shuttled between the nucleus and cytoplasm. The nuclear retention signal for the SC35 SR protein is, PPPVSKRESK**SRSRS**KSPPKSPEEEGAVSS (Cazalla et al., 2002). The adenovirus E4orf6 protein also contains an amphipathic arginine rich α-helical region with the amino acid sequence ARRTRRLMLRAVRIIAE, which has been associated with E4orf6 nuclear retention. The cytoplasmic retention signals are less well characterized (Satoshi et al., 2003). For example, cyclin B1 contains a cytoplasmic retention signal that consists of a stretch of 45 amino acids located in the amino terminus (Pines and Hunter, 1994). The CRS is situated within a CRM1 dependent NES of cyclin B1 (Yang et al., 1998). During interphase, cyclin B1 is exported to the cytoplasm via the NES and is retained in the cytoplasm through its cytoplasmic retention signal. Cyclin B1 is then imported back into the nucleus by NLS phosphorylation (Hagting et al., 1999; Li et al., 1997). Cytoplasmic retention signals have been identified in human and *Xenopus* cyclin B1 and *Xenopus* cyclin B2 (Yoshitome et al., 2003; Pines and Hunter, 1994). The amino acid sequence alignments for CRS and NES for human and Xenopus cyclin B are shown in Figure 16.

Human cyclin B1	142	PVKEEKLSPEPI LVDTASPSPMETSGCAPAEED**LCQAFSDVIL**AVN	154
Xenopus cyclin B1	91	EP SSPSPMETSGCLPDE **LCQAFSDVLIHVKDV**DADD	126
Xenopus cyclin B2	89	PSPVPMDVSLKEEE **LCQAFSDALTSVEDI**DADD	121

Figure 16. Cytoplasmic Retention Signals and Nuclear Export Sequences for Cyclin B. The CRS in human cyclin B1 consists of a stretch of 45 amino acids (residues 142-154) and a NES between amino acids 142-151. The CRS for Xenopus cyclin B1 and B2 contains amino acid residues 91-126 and 89-121, respectively. All of the NES (shown in **bold** text) that have been found in either cyclin B1 or B2 are nested within the CRS. (Yoshitome et al., 2003; Pines and Hunter, 1994).

8.8 Bi-directional Shuttling Signals.

Another type of shuttling signal is characterized by the M9 sequence of heterogeneous nuclear ribonucleoprotein A1, which consists of a 38-amino acid stretch that is enriched in aromatic residues and glycine (Pollard et al., 1996). The M9 sequence functions in both nuclear import and nuclear export and is now referred to as a "shuttling sequence" to distinguish it from the unidirectional targeting sequences (NLS and NES).

8.9 Signal-mediated Nuclear Transport.

Active transport between the nucleus and cytoplasm involves primarily three classes of macromolecules: substrates, adaptors, and receptors. In general, protein import occurs when the transport receptors, importin-α and importin-β, form a complex with the protein-NLS and escort the protein cargo across the NPC into the nucleus (Figure 17) (Nigg, 1997; Görlich and Mattaj, 1996; Moroianu et al., 1996). The protein cargo is released into the nucleus when the guanine nucleotide exchange factor, RCC1/RanGEF, replaces Ran bound GDP with GTP. In the presence of GTP. importin-α binds the export receptor, cellular apoptosis susceptibility protein (CAS), and is transported back into the cytoplasm. Importin-β remains bound to GTP and is recycled back to the cytoplasm.

Once in the cytoplasm, RanBP1 and RanGAP1 hydrolyze the bound GTP to GDP (Gasiorowski and Dean, 2003).

Protein export occurs when Ran-GTP and the nuclear export receptor, chromosome region maintenance-1 (Crm-1 or exportin-1), bind to a protein bearing an NES and transports the protein cargo into the cytosol (Figure 18) (Fukuda et al., 1997). The protein cargo is released into the cytoplasm when Ran-GTP is hydrolyzed by RanBP1 and RanGAP1, small GTPase activating proteins (Yamada et al., 2004; Görlich et al., 2003). In this manner, continued nuclear-cytoplasmic shuttling occurs by maintaining a gradient of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm (Görlich et al., 1996).

Several notable exceptions to this general mechanism have been reported (Miyamoto, 2002). For example, HIV-1 Rev proteins are recognized by importin-β rather than the importin-α/importin-β heterodimer (Tiganis et al., 1996). Moreover, the accessory protein Tat of HIV-1 Rev is not recognized by either importin α or β (Efthymiadis et al., 1998), but rather, by other receptors related to importin β. At least 21 different importin β family members have been identified in humans. Furthermore, Crm-1 belongs to the growing Kapβ family of proteins (table 6) (Moroianu, 1998). Crm-1 was identified as a general receptor for proteins bearing hydrophobic rich NES, but table 6 establishes that receptor mediated nuclear export may be more specific (Jans et al., 2000). Many more import and export receptors are believed to exist, so it is possible that nuclear import and export pathways show sufficient specificity to be considered targets for therapeutic intervention. The precise nuclear transport pathways for topo enzymes has not been elucidated (Engel and Turner et al., 2004; Mirski et al., 2003).

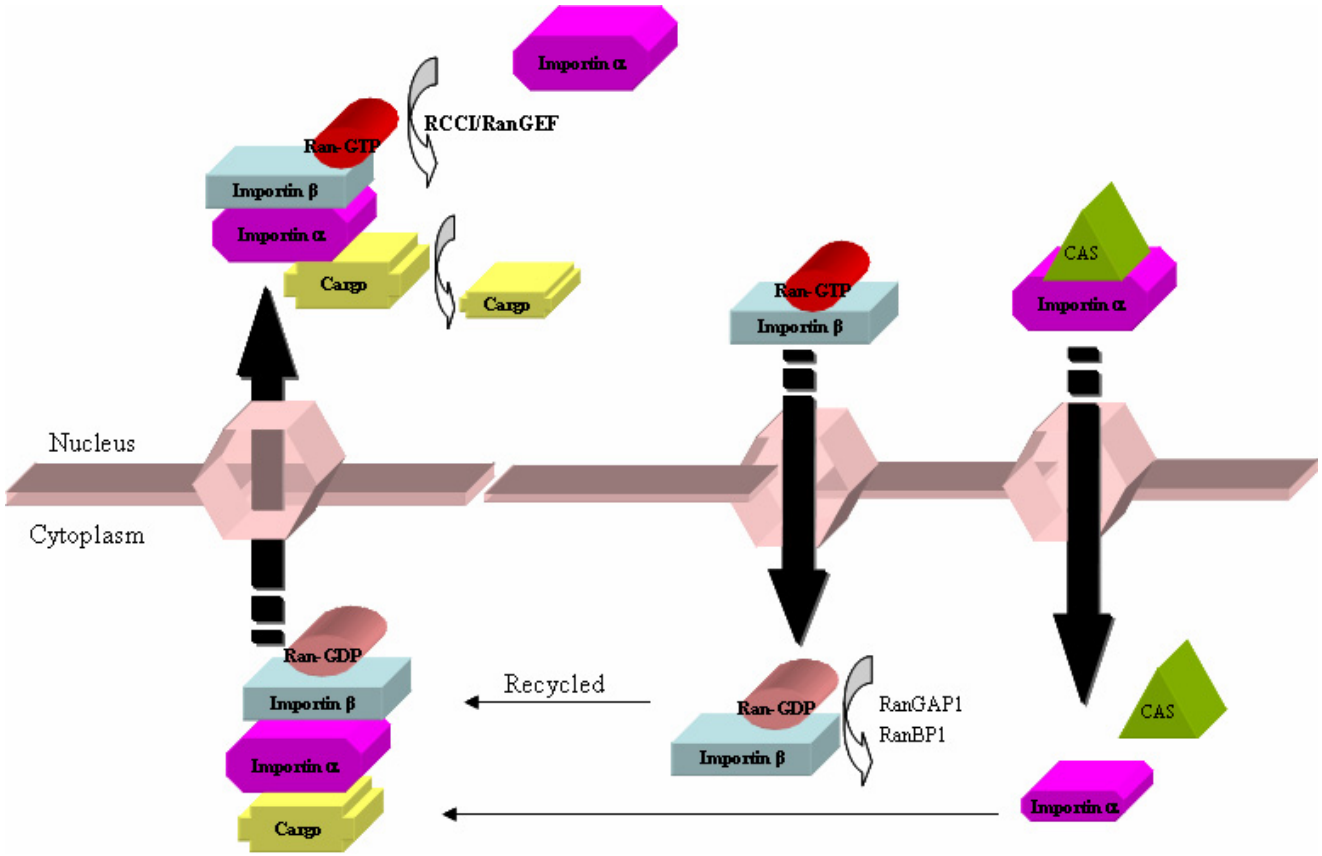


Figure 17. Nuclear Import Pathway. Protein cargo containing a nuclear localization signal binds to the nuclear import receptor, importin α. Importin β-bound to Ran-GDP forms a complex with the importin-α protein cargo and escorts the entire complex across the nuclear pore complex. Once inside the nucleus, the nucleotide exchange factor, RCC1, replaces Ran-GDP with Ran-GTP. Importin-β-Ran-GTP is transported back to the cytoplasm.where GTPases recycle Ran-GTP back to Ran-GDP. Importin-α is exported from the nucleus by the export receptor, cellular apoptosis susceptibility protein (CAS).

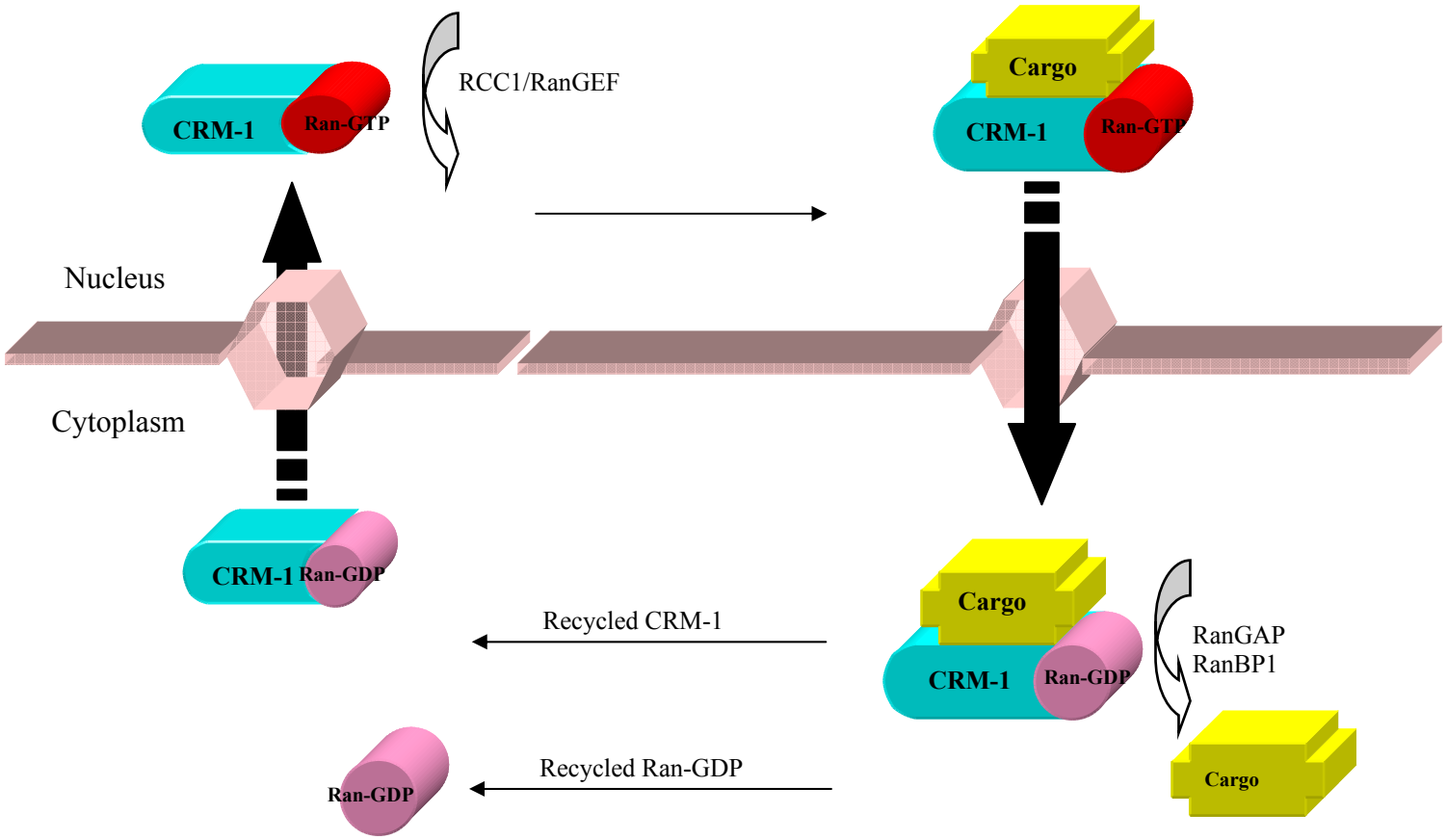


Figure 18. Nuclear Export Pathway. The presence of guanine nucleotide exchange factors in the nucleus maintains a high concentration of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm. In the nucleu, Ran-GTP binds the export receptor, Crm-1 and a NES-containing protein cargo. Crm-1 escorts the complex across the nuclear por complex. In the cytoplasm, Ran-GTPases hydrolyze GTP to GDP. Crm-1 and Ran-GDP are recycled.

Table 6 . Nuclear Export Receptors.

Mammalian Kapβ Family Export Receptors	
Protein Receptor	Export Cargo
CRM-1 (exportin-1)	general export receptor for leucine rich export sequences
CAS	importin-α
Importin-13	eukaryotic translation factor-5A
Exportin-4	hypusine-modified eukaryote translation factor-5A*
Exportin-5	dsRNA binding proteins
Exportin-6	actin export
Exportin-7	p50RhoGAP
Non-Kapβ Family Nuclear Export Receptors	
Mex67p (yeast)	mRNA
TAP/NXF (metazoans)	mRNA

*hypusine, [N(epsilon)-(4 amino-2-hydroxybutyl)lysine], is a rare amino acid. Hypusine is formed when a lysine residue is post-translationally modified by the transfer of a butylamine from spermidine followed by hydroxylation reaction (Mehta et al., 1994; Shiba et al., 1971).

8.10 Regulating Nuclear-cytoplasmic Transport.

Signal mediated transport has been shown to be regulated by several cellular processes that are summarized in Figure 19. Phosphorylation (Engel et al., 1998), ubiquitination (Lohrum et al., 2001), and sumoylation (Salinas et al., 2004) are the most widely reported mechanisms of transport regulation. Like ubiquitination, sumoylation is a three-step process involving an EI-activating enzyme heterodimer Aos/Uba2, the E2-conjugating enzyme Ubc9 and substrate-specific E3 ligases (Gill, 2004). The nucleoporin Nup358 (or RanBP2) is an example of an E3 ligase involved in modifying nucleo-cytoplasmic shuttling proteins with small ubiquitin-like modifiers (SUMO)

(Azumo and Daso, 2002). This finding illustrates the intimate association of SUMO modification in regulating nuclear-cytoplasmic protein transport.

Several drugs and molecular agents have been used to decipher transport processes in the laboratory (Table 15 appearing on page 213). LMB is an unsaturated branched-chain fatty acid isolated from *Streptomyces pombe* (Hamamoto et al, 1983a). LMB is an antifungal antibacterial agent with anti-tumor activity (Hamamoto et al., 1983b) and was also shown to be an inhibitor of the nuclear export receptor, chromosome region maintenance protein-1 (Crm-1) (Kudo et al., 1998). LMB attacks the sulfydryl groups on cysteine-529 of Crm-1 in a Michael-type reaction (Kudo et al., 1999). In this way, LMB blocks all Crm-1-mediated nuclear export. The ratjadones represent a new class of natural compounds which inhibit proliferation in eukaryotes by blocking nuclear export (Meissner et al., 2004). Like LMB, the ratjadones covalently bind to Crm-1. Additional compounds are being screened for their ability to block nuclear-export of proteins at different points in the export pathway.

8.11 Conclusions.

The nuclear-cytoplasmic trafficking proteins have potential applications in drug delivery and drug-target interactions. The nuclear pore complexes are the gateway between the nuclear anc cytoplasmic compartments. Protein transport across the NPC requires the presence of nuclear localization or nuclear export signals. Crm-1 has been identified as the major export receptor that transports NES containing proteins out of the nucleus. Leptomycin B specifically inhibits Crm-1 mediated nuclear export of proteins and is being investigated for its therapeutic potential in the treatment of cancer.

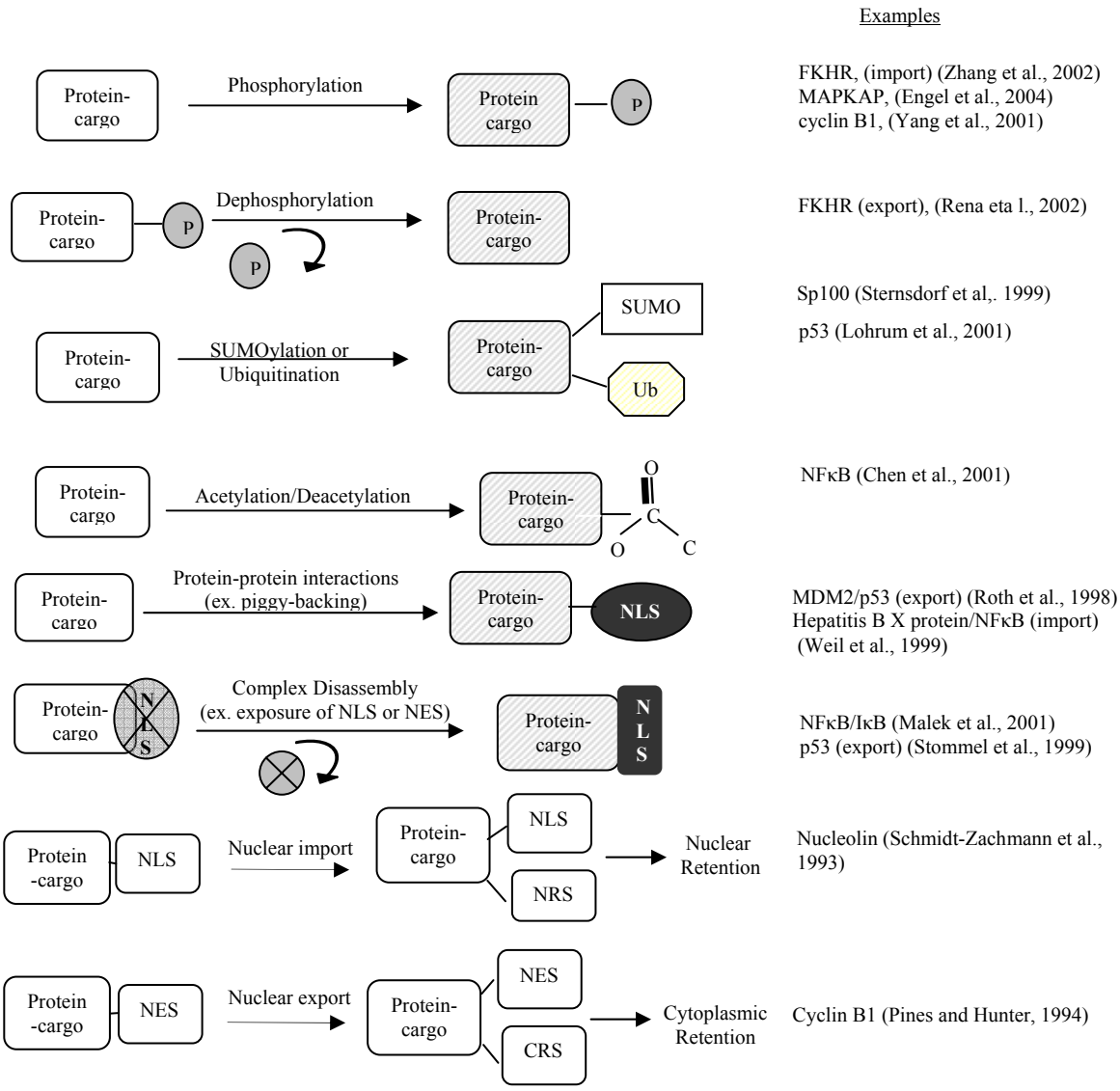


Figure 19**.** Mechanisms of Nuclear-cytoplasmic Transport Regulation. NLS, nuclear localization signal; NES, nuclear export signal; NRS, nuclear retention signal; CRS, cytoplasmic retention signal; FKHR, fork head transcription factor; Ub, ubiquitin; and SUMO, small ubiquitin-like modifier (reviewed in Jans et al., 2000).

Chapter Nine

Experimental Objectives and Rationale

In vitro experiments demonstrate that cancer cells may exhibit multiple different mechanisms to evade drug cytotoxicity. We were interested in identifying mechanisms of cellular drug resistance to DNA topoisomerase targeting agents. Altered drug transport and alterations in the quantity or quality of topo protein, which may or may not be a result of topo gene mutations, have already been described in cell lines selected for drug resistance. In culture, most cell lines also display a differential sensitivity to topo II inhibitors, which depends on cell density (Sullivan et al., 1986, 1987; Chow and Ross, 1987; Markovits, 1987). It is well established that when cell lines become confluent and maintain extensive cell-cell interactions they become intrinsically drug resistant (Croix and Kerbel, 1997). How cell adhesion controls cell growth, apoptotic signal, cell cycle progression and drug resistance remains to be elucidated. One mechanism of *in vitro* drug resistance to topo II inhibitors has been described to occur when cells transition from log to plateau cell density. For example, in non-transformed cell lines, the transition from log to plateau density may lead to an attenuation of cellular topo IIα amount and activity, with concomitant drug resistance (Sullivan et al., 1987). However, tumor cell lines do not necessarily downregulate topo protein content at plateau cell density, yet the cells are resistant (Sullivan et al., 1986, 1987). Therefore, tumor cell lines must have other ways besides the degradation of topo II protein for evading drug

cytotoxicity. Thus, we investigated the differences in drug sensitivity of several leukemia and myeloma cell lines at three levels of increased cell densities, called log, plateau, and accelerated-plateau, and then explained any observed drug resistance experimentally. Therefore, we investigated possible alterations in the cell cycle, drug transport, topo II enzyme activity, cleavable complex formation, and subcellular distribution of topo I, topo IIα, and topo IIβ.

Much of our current understanding of drug resistance is derived from cell lines that have been selected for drug resistance by applying a continuous drug pressure, and then correlating the total cellular content of topo or expression of drug efflux pumps with drug resistance (Davis et al., 1998). Our data establish a novel mechanism of drug resistance in human myeloma cell lines that is detectable prior to any drug exposure and independent of protein expression or P-gp mediated drug resistance. We demonstrate that topo IIα translocates to the cytoplasm in a cell-density dependent manner and that the nuclear export of topo IIα has a role in decreased drug sensitivity to VP-16 and other topo targeting agents, but less so with non-topoisomerase inhibitors. The cytoplasmic localization of topoisomerase IIα has thus far been attributed only to C-terminally truncated proteins that have lost a critical nuclear localization signal (Wessel et al., 1997). Our data are the first to describe a nuclear export signal of topo IIα by cells expressing full-length topo IIα-GFP. We also demonstrate that a cytoplasmic transport of endogenous topo IIα in human myeloma cells can be abrogated in the presence of the nuclear export inhibitor, leptomycin B. The clinical significance of these findings is the treatment of multiple myeloma is suggested.

Chapter Ten
Materials and Methods

10.1 Materials.

Etoposide (VP-16), phenylmethylsulphonyl fluoride (PMSF), *cis*-platinum, paclitaxel (taxol), carmustine (BCNU), ara-C (cytosine 1-β-D-arabinofuranosyl hydrochloride), dimethyl sulphoxide (DMSO), 2-mercaptoethanol, and leptomycin B, NonidetP-40 (NP-40), phenylmethylsulphonyl (PMSF), adenosine triphosphate (ATP), dithiothreitol (DTT), ethylenediaminetetroactetic acid (EDTA), MTT, and vincristine, were all obtained from Sigma Chemical (St. Louis, MO). Mitoxantrone was generously provided by Immunex Corp (Seattle, WA). Topotecan was generously provided by SmithKline Glaxo (Philadelphia, PA).

10.2 Cell Culture.

All cells were obtained from the American Type Culture Collection (Manassas, VA). The RPMI-8226 cell line is from the peripheral blood of a 61 year old male with multiple myeloma and the NCI-H929 cell line is from the malignant effusion of a 62 year old Caucasian female with myeloma. The HL-60 cell line was obtained from a 36-year old Caucasian female with acute promyelocytic leukemia. This cell line is positive for the myc oncogene. The KG-1a human acute myelogenous leukemia cells are a subline variant of the KG-1 cell line. The parental KG-1 cells were obtained from a 59-year old Caucasian male with erythroleukemia that evolved into acute myelogenous leukemia.

After 10 passages, the KG-1 cells were split and cultured by two different laboratories in the same department and under identical conditions. After 35 passages, one of the cultures exhibited different morphological characteristics (reviewed in ATCC) and were called KG-1a cells. The CCRF cell line was obtained from the peripheral blood buffy coat of a 4-year old Caucasian female with lymphoblastic leukemia. The L1210 cells were derived from a female mouse with lymphocytic leukemia. HeLa cells are epithelial adenocarcinoma cells that were obtained from the cervix of a 31 year old black female.

HeLa cells were grown in Alpha Minimal Essential Media (Gibco) containing 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) (Hyclone). The HL60, KG1a, RPMI-8226, and CCRF cells were grown in RPMI medium containing100 U/ml penicillin and 100 μg/ml streptomycin from Gibco BRL (Gaithersburg, MD) and 10% FBS (Hyclone). The NCI-H929 cells were grown in RPMI medium containing 100 U/ml penicillin, 50 μM β-mercaptoethanol (Sigma) and 100 μg/ml streptomycin from Gibco BRL (Gaithersburg, MD). The L1210 cells were grown in RPMI medium containing 100 U/ml penicillin, 100 μg/ml streptomycin from Gibco BRL (Gaithersburg, MD) and 20% FBS. The Chinese Hamster Ovary (CHO) cells were grown in Alpha Minimal Essential Media (Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum (FCS). Flow fibroblast 2000 cells were grown in Alpha Minimal Essential Media (Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum.

10.3 Log, Plateau, and Accelerated-plateau Cell Model.

Cell density and viability were determined by staining cells in a 1:1 dilution with trypan blue and counting them with a hemacytometer. Growth curves were performed on all cell-lines described above (HL60, KG1a, RPMI, 8226, CCRF, L1210, CHO) to determine log and plateau phase cell density. Log-phase was defined as 3.0 X 10^5 to 4.0 X 10^5 cells/ml with ≥ 85% viability. The log cell densities for the CHO and Flow fibroblasts were 2.0 X 10^4 cells/ml for 10 ml of media in a 25-cm^2 flask, whereas plateau phase was reached at 8.0 X10^5 cells/ml for CHO cells and 2.0 X 10^5 cells/ml for the Flow fibroblast cells.

When the log growing cells reached confluence, they were referred to as "natural-plateau". Natural-plateau is defined as cell viability ≥ 85% (by trypan blue dye exclusion) and no net gain in cell number. The plateau density was different for each cell line: 9.0 X 10^5 cells/ml for RPMI 8226, 1.0 X 10^6 cells/ml for H929, 1.6 X 10^6 cells/ml for HL-60, 2.0 X 10^6 cells/ml for CEM-CCRF and L1210, and 8.0 X10^6 cells/ml for KG-1a cells.

For convenience, the "accelerated-plateau" model was established by concentrating log-phase H929, 8226, HeLa, CCRF, or HL-60 cells to a super-confluent cell density for 16 h or 24 h before experimentation (Valkov et al., 2000; Engel et al., 2004). Log-phase cells were centrifuged at 100 x g for 5 min at room temperature and resuspended in fresh media to obtain a superconfluent cell density and grown with 5% CO_2 at 37°C. The super-confluent cell density was between 2.0 x 10^6 and 2.5 x 10^6 cells/ml for RPMI-8226, H929, and HeLa cells. The super-confluent cell density for HL-60 and CEM-CCRF was set between 3.0 X 10^6 cells/ml and 4.0 X 10^6 cells/ml. KG-1a,

Chinese hamster ovary, and Flow Fibroblast cells were not used in the accelerated-plateau cell model.

10.4 Bone Marrow Samples.

Bone marrow samples were obtained after informed consent from patients with multiple myeloma on a high-dose chemotherapy study, approved by the Institutional Review Board. Approximately 5 ml of bone marrow aspirate was collected in EDTA tubes and diluted to 30 ml with PBS. This was then layered over 15 ml of Ficoll-Paque Plus (Pharmacia Biotech) and centrifuged at 400 x g in a swinging bucket rotor at 4°C for 30-min. The mononuclear cell interface was collected and washed twice with cold PBS. The cell number was determined and 10^5 cells were cytospun onto double cytoslides. The cells were fixed with 4% formaldehyde and stored at -85°C. Aliquots of cells were preserved in freezing media consisting of 40% RPMI-1640, 50% FCS, and 10% DMSO in liquid nitrogen. Where noted, samples were also obtained in a similar manner but from two untreated myeloma patients. Mononuclear fractions of these samples were obtained by centrifuging the bone marrow aspirates at 700 x g over a Ficoll-Hypaque density gradient (Valkov et al., 2000).

10.5 Clonogenic Cytotoxicity Assays.

Cytotoxicity assays were performed by treating 1.0 X 10^5 cells from either log, plateau, or accelerated plateau cell suspensions (Valkov et al., 2000; Engel, 2004). Drug treatment was for 1 hour with various concentrations of etoposide, mitoxantrone, topotecan, and cis-platinum (diluents were DMSO, water, MeOH, and DMSO,

respectively). Cells were treated with paclitaxel for 24 hours and the drug was diluted in DMSO. Cells were treated with carmustine for 4 hours and the drug was diluted in ethanol. Cells were treated with ara-C (cytosine 1-β-D-arabinofuranosyl hydrochloride) for 20 hours and the drug was diluted in water. Cells were exposed to γ-irradiation (Cs-137 gamma irradiator) (0-800 rads). Controls had the same concentration of dilutent. A total of 2500 drug-treated cells were plated in triplicate in 0.3% select agar (Gibco BRL) in RPMI-1640 containing 15% FBS for 14-17 days at 37°C in the presence of 5% CO_2. Colonies > 50 cells were counted manually.

10.6 Flow-cytometric Cell Cycle Analysis.

The cell cycle was analyzed by measuring BrdU incorporation and total amount of DNA by propidium iodide staining (Engel et al., 2004). To measure BrdU incorporation, one million log and accelerated-plateau cells each were treated with 30 μg/ml BrdU (Becton Dickinson, San Jose, CA) in serum free media for 30 min. Cells were washed twice in PBS and resuspended in 4 ml of ice cold PBS. While vortexing, 6 ml of ice cold 100% ethyl alcohol was slowly added and the cells fixed overnight at -20ºC. At least 1 X 10^6 cells were centrifuged at 100 x g for 5 min and resuspended in pepsin solution (0.04% pepsin in 0.1% HCl) for 1 h at 37º C with gentle shaking. Whole cells were pelleted at 100 x g for 5 min, resuspended in 3 ml of 2 N HCl, and then incubated for 30 min at 37ºC. To this solution, 6 ml of 0.1M sodium borate was added, vortexed for 20 seconds and then recentrifuged. Cells were washed once with a wash buffer containing 0.5% BSA and 0.5% Tween-20 in PBS and brought to a final volume of 200 μl FITC-conjugated anti-BrdU antibody (Becton Dickinson, San Jose, CA) was

added in a 1:20 dilution and allowed to incubate in the dark for 60 min at room temperature. These same cells were washed once with wash buffer and resuspended in 10 µg/ml PI in wash buffer to achieve 1 X 10^6 cells/ml. Cells were treated with 250 µg of RNase (Sigma) from a 10 mg/ml solution in PBS for 30 min at 37ºC. The sample was passed through an 18 G needle and transferred to a Falcon 2054 vial and read by FACS-Scan (Becton Dickinson, San Jose, CA). The experiment was repeated three times and the mean values were plotted using Prism 2.01 (GraphPad Software, San Diego, CA).

In addition to the above, DNA content was also determined by measuring propidium iodide staining alone in three separate flow cytometry experiments. To measure DNA content by propidium iodide staining, one million log and accelerated-plateau cells each were centrifuged at 1000 r.p.m. for 5 min at 4ºC and resuspended in 100 µl of ice cold PBS. The cells were fixed with 70% ice cold ethanol by slowly trickling the ethanol into the cell suspension with constant vortexing. The cells were fixed overnight at -20ºC, centrifuged at 1,400 r.p.m. for 5 min at room temperature, resuspended in 200 µl of PBS, and transferred to a 10 X 75 mm tube (Fisher Scientific, Pittsburgh, PA). An RNase (Sigma) stock, equivalent to 100 K units/mg solid, was diluted to 10 mg/ml in PBS and added to the cell suspension in a 1:4 dilution. A 1 mg/ml propidium iodide stock solution was prepared in ethanol and added to the cells in a 1:10 dilution immediately before reading and DNA histograms were determined by FACS-Scan (Becton Dickinson, San Jose, CA). The experiment was repeated 3 times.

10.7 Gel Electrophoresis and Immunodetection.

Log-phase H929 and 8226 cells were seeded at 2.0 X 10^6 cells/ml, returned to the incubator and then lysed either 4h, 16h, or 24 h later (Engel et al., 2004). Whole cell lysates were prepared from log and accelerated-plateau cells by centrifuging 2.0 X 10^6 cells at 100 x g. for 5 min at 4°C. The pellet was washed once with ice cold PBS and then resuspended in 250 µl of H_2O (pH 9.0; adjusted with 0.1 M borate buffer). Cells were sonicated on ice with 15 pulses from a Branson sonifier (output 4.0, duty cycle 30%), and then boiled for 3 min in 1X sample buffer (2% SDS, 0.1M DTT, 10% glycerol, and 0.025% bromphenol blue). Protein concentration was determined by using the BCA protein assay kit (Pierce, Rockford, IL). A total of 50-100 µg of protein were loaded onto a 7.5% SDS-page gel, electrophoresed under 7 watts and transferred at 65 volts overnight onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in 0.05% dry milk in TBST buffer containing 0.05% Tween-20 , 0.5M NaCl, 20 mM Tris, pH 7.5. Primary antibodies used were polyclonal antibody 454 against topo IIα diluted 1:5000 (Hochhauser et al., 1999), polyclonal antibody JB1 against topo IIβ diluted 1:5000 (Valkov et al., 2000; Austin et al., 1995), or C-21 murine monoclonal IgM antibody directed against the C-terminus of topo I (a generous gift from Dr. Y.-C. Cheng, Yale University Medical School, New Haven, CT) diluted 1:2500, or polyclonal anti-LDH (Research Diagnostics, Flanders, NJ) diluted 1:500 in blocking buffer. Membranes were blocked for 1 h, and treated with either the polyclonal antibody 454 against topo IIα or JB1 against topo IIβ for 1 h or blocked overnight at 4°C and treated with the monoclonal antibody C21 for 2 h. Membranes were washed in TBST every 15 min for 1 h and then

treated with secondary antibody for 1 h at room temperature. Secondary antibodies used were either donkey anti-rabbit (topo IIα and topo IIβ), sheep anti-mouse (topo I) (Amersham, Arlington Heights, IL) or mouse anti-goat (LDH) (Santa Cruz Biotechnology, Santa Cruz, CA) conjugated to horseradish peroxidase. The topo IIα and IIβ secondary antibodies were diluted 1:3000 and the LDH secondary antibody was diluted 1:500 in blocking buffer. The signal was detected with ECL (Amersham) and exposed to film. The bands were quantified with Adobe PhotoShop 5.0 imaging software. The experiment was done one time for topo IIβ and repeated 3 times for topo IIα and topo I in NCI-H929 and RPMI-8226 cell lines (Engel et al., 2004).

10.8 Nuclear-cytoplasmic Separation.

Subcellular fractions was prepared from 1.0 X 10^7 cells as previously described (Oloumi et al., 2000) with the following modifications (Engel et al., 2004; Valkov et al., 2000). Cells were washed in PBS and resuspended into cold Buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM sucrose, 1 mM EDTA (pH 8.0), 1 mM DTT, 0.2% NP-40, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml protease inhibitors mixture (Sigma), and 0.1 mM Na_3VO_4). The cells were incubated in Buffer A on ice for 15 min., sheared with a pipette 20 times, and then centrifuged for 10 min at 830 x g at 4°C. The supernatant was reserved and the nuclear pellet was prepared for protein determination and gel electrophoresis as described above (refer to "Gel Electrophoresis and Immunodetection") except that the DNA was sheared with 10 pulses from a Branson sonifier after being resuspended in Buffer B (250 mM Tris-HCl (pH 7.9), 5 mM $MgSO_4$,

250 mM sucrose, 2 mM NaTT, 1% thiodiglycol, 1% NP-40, 1mM PMSF, 10μg/ml PI, and 0.1 mM Na_3VO_4).

10.9 Immunofluorescence Microscopy.

Slide preparations from log and accelerated-plateau cells were immunostained as previously described (Engel et al., 2004; Valkov et al., 2000). Log and accelerated plateau cell suspensions were diluted to 5.0 X 10^4 cells/ml with PBS and cytocentrifuged for 3 min at 500 r.p.m. onto double cytoslides (Shandon, Pittsburgh, PA). The cells were fixed in 4% paraformaldehyde for 4 min at room temperature and permeablized with 0.5% Triton X-100 and 1% glycine in PBS overnight at 4°C. After permeablization, the slides were washed in fresh PBS every 15 min for 1 h. The slides were treated with the following combinations of primary antibody: either rabbit polyclonal antibody 454 against topo IIα (Hochhauser et al., 1999) was used simultaneously with a mouse monoclonal antibody against histone (Roche Molecular Biochemicals) or a C-2 murine monoclonal IgM antibody against topo I (a generous gift from Dr. Y.-C. Cheng, Yale University Medical School, New Haven, CT) was used simultaneously with the polyclonal antibody JB1 against topo IIβ (Austin et al., 1995). The primary antibodies were diluted 1:100 with staining solution containing 0.1% NP-40 and 1% BSA in PBS and the incubation period was for 1 h at room temperature. Slides were washed in PBS every 10 min for 1 h and then incubated with goat anti-rabbit IgG-TRITC labeled antibody (Sigma) diluted 1:80 and goat anti-mouse IgG FITC-labeled antibody (Sigma) diluted 1:64 in staining solution as above for 35 min in the dark at room temperature. The slides were washed in PBS every 15 min for 2 h, air dried and covered with

antifade/DAPI (Vector, Burlingame, CA). Immunofluorescence was observed with a Leitz Orthoplan 2 microscope and images were captured using a CCD camera with Smart Capture program (Vysis, Downers Grove, IL).

10.10 Quantitative Measurement of the Immunofluorescence of Topo IIα.

Slide preparations from log and accelerated-plateau cells were immunostained as previously described (Valkov et al., 2000; Engel et al., 2004). Topo IIα immunofluorescence was analyzed as previously described (Valkov et al., 2000; Engel et al., 2004) using Adobe PhotoShop 5.0 (Adobe Systems, San Jose, CA) and Prism 2.01 (GraphPad Software, San Diego, CA). The nuclear/cytoplasmic ratios were calculated for each cell by dividing [the number of pixels X (mean intensity - background)] of the nuclear compartment by [the number of pixels X (mean intensity – background) of the cytoplasmic compartment. At least 50 cells per experiment were analyzed and the experiment was repeated 3 times.

10.11 Equilibrium concentrations of [^{3}H]-VP-16.

[^{3}H]-VP-16 was obtained from Moravek Biochemical (Brea, CA). Equilibrium concentrations of [^{3}H]-VP-16 were measured as previously described (Sullivan et al., 1986, 1987). Briefly, equal numbers of log and accelerated-plateau cells were incubated in the presence of 5 to 50 μM of [^{3}H]-VP-16 diluted with cold etoposide for 1 h at 37ºC. Whole cells were centrifuged at 100 x g for 10 min and washed with cold PBS. Drug uptake for log and accelerated-plateau cells was determined as cpm/mg of cells.

10.12 Band Depletion Assay.

Cleavable complex formation was measured in log and accelerated-plateau H929 cells as previously described (Engel et al., 2004) with few modifications (Kaufmann et al., 1997). Briefly, 2.0 X 10^7 cells were centrifuged at 1,000 r.p.m. for 5 min in a swinging bucket rotor. The pellet was resuspended in 10 ml of buffer A (10 mM HEPES, pH 7.4 in serum free media). 2.0 X 10^6 cells were transferred to a 1.5 ml microcentrifuge tube and treated with VP-16 in DMSO for 45 min in a 37ºC water bath and mixed by inverting the tubes every 15 min. After the incubation period, the cells were centrifuged at 3200 X g for 1 min. Proceeding one tube at a time, the supernatant was aspirated and 1 ml of lysis buffer (6M guanidine HCl, 0.25 M Tris, pH 8.5 with HCl, 10 mM EDTA, 1% 2-BME, and 1X protease inhibitors containing (20 mM PMSF, and 20 µg/ml each of antipain, aprotinin, chymostatin, leupeptin, soybean trypsin inhibitor, benzamidine, and pepstatin A) was added with brisk vortexing. The cells were incubated in lysis buffer overnight at 20ºC. Genomic DNA was sheared with 20 pulses from a Branson sonifier (output 4.0, duty cycle 35%). To each sample, 100 µl of 150 mM iodoacetamide in lysis buffer without 2-BME was added and allowed to incubate in the dark for 1 h at room temperature. After incubation, 10 µl of 2-BME was added to each sample. The samples were dialyzed at 4ºC in the dark as follows: 3 X 90 min in 1 L of 4M Urea, 2 X 90 min in 0.1% SDS, and 1 X overnight in 0.1% SDS. Samples were lyophilized and stored at -20ºC. Immediately before gel electrophoresis, protein was solublized by heating the samples in sample buffer (4M Urea, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.03% bromphenol blue and 1% 2-BME) for 20 min at 65-70ºC. Proteins were separated

by gel electrophoresis on a 10% bis-acrylamide gel for western analysis. The experiment was repeated two times.

10.13 Comet Assay.

DNA damage was analyzed by the comet assay as previously described with some modifications (Engel et al., 2004; Kent et al., 1995). Briefly, 2.5 X 10^5 cells/ml were treated with VP-16 for 1 h at 37ºC. After treatment, 5,000 cells were removed and washed in cold PBS. The supernatant was aspirated and 1.5 ml of 1% agar (Nusieve GTG) was added to 500 µl of PBS, mixed with the pellet, and spread onto a frosted glass microscope slide. The slides were solidified at room temperature for 2 min and then 4 min at 4ºC. Cells were lysed for 1 h at 4ºC in batches (6-8 slides) in 200-250 ml lysis buffer containing 0.5% SDS (w/v), 30 mM EDTA, pH 8.0, and Proteinase K was added (375 units/100 ml) immediately before use. After 1 h, the slides were incubated in the same lysis buffer overnight at 37ºC for 12-16 h. The lysis buffer was washed off with 200 ml of 1X TBE (pH 8) for 2 h, changing buffer every 15 min. The cells were electrophoresed in 1X TBE under 25 volts for 20 min. The DNA was stained with a 1:10,000 dilution of Syber Green (Molecular Probes) in 1X TBE for 25 min in the dark at room temperature. Slides were washed two times with 1X TBE for 5 min each, stored in a dark humidified box at 4ºC, and viewed within 24 h with a Leitz Orthoplan 2 microscope. Images were captured using a CCD camera with Smart Capture program (Vysis, Downers Grove, IL). Fifty single cells with no overlapping tails were captured per drug dosage per condition and the experiment was repeated three times. The comet

moments were captured and analyzed with Optimas and calculated by using the formula: $\sum_{0\rightarrow n}$ (Intensity X Distance)/Total Intensity.

10.14 Putative NES Peptides.

The complete amino acid sequence for human topo IIα (accession number NP 001058) was downloaded from the National Center for Biotechnology Information database and searched for matches to the NES consensus sequence from table 12 (Engel and Turner et al., 2004). Six amino acid sequences in topo IIα matched the NES consensus sequence (refer to Table 11, page 163), and were synthesized as native (nt) or mutated (Δ) peptides. The mutated peptides contained alanine in place of those hydrophobic residues suspected of being critical for nuclear export (leucine, isoleucine, or valine). To facilitate conjugation with preactivated sulfosuccinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate (SMCC-BSA) (Pierce) (Figure 20), the NES-peptides were designed with a cysteine residue at the amino terminus. The peptides obtained from the Biopeptide Company (San Diego, California) were as follows: ($ntNES_{80\text{-}91}$), $C^{80}GLYKIFDEILVN^{91}$; ($\Delta NES_{80\text{-}91}$), $C^{\Delta 80}GAYKAFDEAAAN^{91}$; ($ntNES_{230\text{-}241}$), $C^{230}SLDKDIVALMVR^{241}$; ($\Delta NES_{230\text{-}241}$), $C^{\Delta 230}SADKDAAAAMAR^{241}$; ($ntNES_{467\text{-}476}$), $C^{467}TLAVSGLGVVG^{477}$; ($\Delta NES_{467\text{-}477}$), $C^{\Delta 467}TAAASGAGAAG^{477}$, ($ntNES_{1017\text{-}1028}$), $C^{1017}DILRDFFELRLK^{1028}$; ($\Delta NES_{1017\text{-}1028}$),

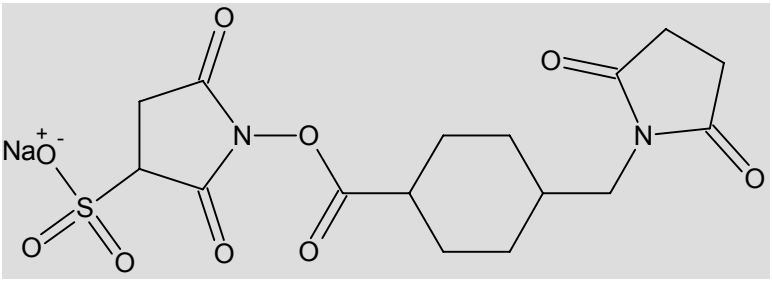


Figure 20. Sulfosuccinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate (Sulfo-SMCC). Sulfo-SMCC is a water soluble non-cleavable crosslinking agent.

$C^{\Delta 1017}$CDIARDAFEARAK^{1028}. Peptides (ntNES$_{569\text{-}580}$), C^{569}FLEEFITPIVKV^{580}; (ΔNES$_{569\text{-}580}$), $C^{\Delta 569}$AAEEAATPAAKA^{580}; (ntNES$_{1054\text{-}1066}$), C^{1054}FILEKIDGKIIIE^{1066}; and (ΔNES$_{1054\text{-}1066}$) $C^{\Delta 1054}$FIAEKADGKAIAE^{1066} were obtained from the University of Florida Protein Chemistry Core Facility (Gainesville, FL). All peptides were HPLC purified to ≥ 95% and analyzed by mass spectroscopy. In addition, peptide sequences and purity were confirmed by Rick Feldhoff, Ph.D at the University of Louisville, School of Medicine (Louisville, KY).

10.15 Generation of BSA-Peptide-FITC Conjugates.

Peptides were crosslinked to BSA and FITC. Figure 21 illustrates the plausible binding of peptides and FITC to BSA. First, a total of 2 mg Imject® Maleimide activated sulfosuccinmidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate bovine serum albumin (Sulfo-SMCC BSA) (Pierce, Rockford, IL) was reconstituted in 200 µl distilled water for a final concentration of (0.1 M sodium phosphate buffer, 0.15 M NaCl, 0.1 M EDTA, pH 7.2). Then, a molar excess (1-2 mg) of native or mutated topo IIα NES peptide in 400 µl of conjugation buffer (83 mM sodium phosphate buffer, 0.1 M EDTA, 0.9 M NaCl, 0.002% sodium azide, pH 7.2) was mixed with the Sulfo-SMCC BSA and reacted for 30 min at room temperature.

The reaction was quenched by adding 40 mM cysteine solution in deionized water to the peptide-SMCC-BSA solution to obtain a molar excess of cysteine to peptide sample (approximately 7 nmoles cysteine/nmole of SMCC-BSA-peptide). The conjugates were purified by size exclusion chromatography at room temperature using the Pharmacia P-

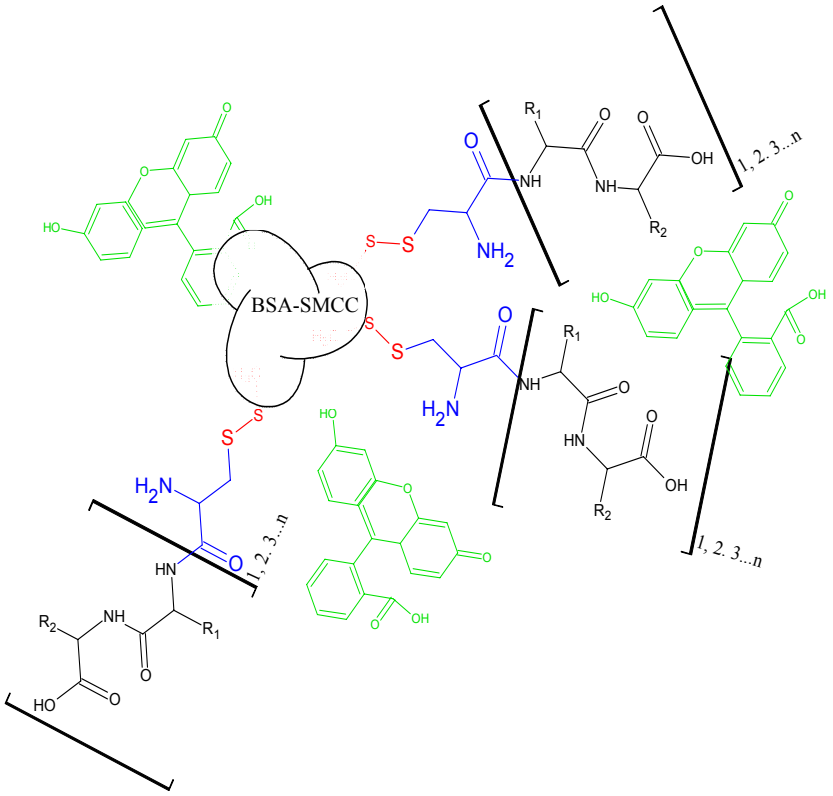


Figure 21. An Illustration of BSA-FITC-Peptide Complexes. Multiple peptides covalently bind BSA-SMCC ( )via disulfide bonds (red) formed by the placement of a cysteine residue (blue) at the carboxyl terminus of the peptide. Here the peptide is illustrated as the backbone of any amino acid residue (shown in black brackets [ ] FITC is shown in green. FITC covalently binds to any primary amine, whether located on the BSA or peptide molecules. Theoretically, multiple FITC and peptides will be crosslinked per mole of BSA .

500 FPLC system with LKB control Unit UV-1. The high resolution column (10 mm inner diameter and 30 cm length) (Amersham Pharmacia, Piscataway, NJ) was packed at 2.0 ml/min with Superdex 200 prep grade (Amersham Pharmacia) in filtered and degassed PBS, pH 7.4. The peptide conjugates were loaded onto the column using a 500 µl Superloop and were run at 0.5 ml/min in degassed dH_2O. The 500 µl fractions were collected with a Fraction-100 collector (Pharmacia Biotech) and stored at 4°C overnight. Total protein was estimated in peak samples by measuring absorbance at 562 nm using the Fisherbrand Protein Assay. Approximately 25 µg of protein from eluted fractions were loaded onto a 10% SDS-page gel and electrophoresed with 7 watts for 2-3 h. Peptide conjugation was confirmed by silver stain analysis. Similar fractions of crosslinked BSA-peptide were pooled and concentrated on a microsep 30k filter (Pall Corporation) by centrifuging at 5,000 x g in an SS-34 rotor until dry. The samples were eluted with 400 µl of PBS, pH 7.4 to obtain approximately 2 mg/ml peptide-conjugate solution. FITC was solubilized in DMSO to 1 mg/ml and added to the peptide sample in four 5 µl aliquots until a total of 20 µl of FITC (Sigma) was added. FITC was reacted with the peptides for 6 h at 4°C, and then 23 µl of 1 M NH_4Cl in PBS, pH 7.4 was added to the sample and incubated for 2 h at 4°C. FITC-BSA-peptide conjugates were separated from unincorporated label by FPLC. The ratio of fluorescein to protein was determined by measuring the absorbance at 495 nm and 280 nm. The nuclear control, tetramethylrhodamine-bovine serum albumin (TRITC-BSA), was obtained from Sigma.

10.16 Microinjection.

To promote cell adherence, Fisherbrand glass coverslips were pretreated with 1N HCl for a minimum of 4 hours at 50°C and then rinsed extensively with deionized water. Coverslips were washed in 100% ethanol and dried between pieces of Whatman paper. Subconfluent HeLa cells growing in Alpha Minimal Essential Medium (Gibco) containing 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS (Hyclone) were plated onto the center of glass coverslips in NUNC brand petri dishes and incubated at 37°C for 24-48 h preceding microinjection. Prior to microinjection, cells were gently rinsed with sterile PBS warmed to 37°C and replaced with Leibovitz's L-15 Medium containing no phenol red.

FITC labeled BSA-NES peptide conjugates were centrifuged at 13,000 x g for 30 min at 4°C, and the supernatant then loaded into Eppendorf Femptotips (diameter of 0.5 µm ± 0.2 µm). All cells were injected using the semi-automated Eppendorf Injectman NI2 and Femtojet microinjector on a Nikon TE 2000 inverted microscope (Figure 22) under exactly the same conditions (injection pressure (P_i), 100 hPa; compensation pressure (P_c), 30 hPa; injection time (I_t) 0.2 sec, atomospheric conditions). Following injection, the cells were incubated at 37°C for up to 90 min, washed with Leibovitz's L-15 Medium, and then fixed with 4%

Figure 22. Eppendorf FemptoJet and Micromanipulator on a Nikon TE 2000 Inverted Microscope.

paraformaldehyde for 3 minutes at room temperature, rinsed in PBS and mounted onto Shandon microscope slides with mounting medium containing DAPI. Fluorescence was observed with a Leitz Orthoplan 2 microscope and images were captured using a CCD camera with Smart Capture program (Vysis, Downers Grove, IL).

10.17 Topoisomerase IIα Cloning and Site Directed Mutagenesis.

Topo IIα primers were designed which contained the eight amino acid FLAG peptide preceded by a start codon and a Kozak motif (5'-ATG GAC TAC AAA GAC GAT GAC GAC AAG GAA GTG TCA CCA TTG CAG CCT GTA AAT GAA AAT ATG-3' forward primer, 5'-ATG CGG CCG CTT AAA ACA GAT CAT CTT CAT CTG ACT CTT C-3' reverse primer). Amplification was performed with an enzyme mixture of *Taq* and *Pyrococcus* species GB-D thermostable DNA polymerases (Elongase, Invitrogen), in 60 mM Tris-SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 200 μM dNTP mixture and 200 nM each primer. Forty cycles were performed (94°C for 30s, 60°C for 30s, and 5.5 min at 68°C), and the PCR products were agarose gel-purified and ligated to a pcDNA3.1 vector using a TOPO-TA cloning system (Clontech). The 5' end of the new FLAG-topo IIα fusion protein vector was sequenced to ensure that the DNA was in-frame. Site-directed mutagenesis was performed using a Quickchange XL site-directed mutagenesis kit (Stratagene). Briefly, 100 ng of template dsDNA were mixed with 125 ng of each oligonucleotide primer, 2.5 units of *PfuTurbo* DNA polymerase, in a reaction mixture containing 2 mM dNTPs. Eighteen cycles were performed (95°C for 50 s, 60°C for 50s, and 68°C for 20 min), after which the parental plasmid was digested with methylation specific enzyme *Dpn-I*. Ultra-competent cells

were transformed with mutated plasmid and clones were sequenced to determine the presence of desired mutations. DNA sequencing was performed at the H. Lee Moffitt Cancer Center Molecular Biology Core Facility.

10.18 Transfection Protocol.

Human myeloma H929 and human leukemia HL-60 cells (ATCC) were plated at log phase density ($2x10^5$ cells/ml) two days prior to transfection. Transfection was performed as previously described (Van den Hoff et al., 1992). Briefly, 40 µg of wild-type or mutated topo IIα plasmid in 300 µl tris-EDTA (TE) buffer was precipitated by the addition of 30 µl of 5 M NaCl and two volumes of 95% ethanol on ice for ten minutes. Plasmid was pelleted by centrifugation for fifteen minutes at 20,000 x g at 4°C, washed with 75% ethanol and re-centrifuged. All remaining ethanol was removed by pipette and the DNA immediately resuspended in 50 µl of cytomix buffer containing 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP, and 5 mM glutathione, pH adjusted to 7.6 by the addition of KOH. ATP and glutathione were made fresh and added prior to each transfection (26). Two days prior to transfection, human myeloma H929 cells and leukemia HL-60 cells were placed in fresh growth media (RPMI/10% FBS/pen-strep) at a concentration of $2 x10^5$ cells/milliliter. Cells were collected and $1.6x10^7$ cells pelleted by centrifugation at 1500 x g for 5 minutes. Cell pellets were washed twice in 10 ml of sterile PBS, resuspended in 350 µl of cytomix buffer (4°C), mixed with prepared DNA, and placed in a 4 mm electroporation cuvettes. Electroporation was at 250V/750 capacitance, after which cells

were split into even groups and plated at log and plateau growth conditions for twenty hours in 5 % CO_2 incubator at 37°C with RPMI medium containing a 5% FBS.

10.19 Immunofluorescence of FLAG-Topo IIα.

Twenty hours post-transfection, live cells were isolated by centrifugation for 20 minutes at 20°C on a ficoll gradient, and then washed with PBS. Transfected cells were plated on a glass microscope slide using cytospin funnels and fixed with 4% paraformaldehyde at 20°C for ten minutes. The fixation was stopped by washing in PBS and cells were permeabilized for twenty-four hours in a solution containing 1% glycine and 0.25% Triton X-100 in PBS. Slides were stained with anti-FLAG M2 monoclonal antibody-FITC conjugate (Sigma) diluted 1:100 with 0.1% NP-40 and 1% BSA in PBS, and incubated one hour at room temperature. Slides were washed in PBS, dried briefly and counterstained with Vectashield mounting media antifade/DAPI (1:1) (Vector Laboratories Inc., Burlingame, CA). Immunofluorescence was observed with a Leitz Orthoplan 2 fluorescent microscope and images were captured by a CCD-camera with Smart Capture program (Vysis, Downers Grove, IL). Quantitation of FITC fluorescence was performed using the Adobe Photoshop 7.0 program.

10.20 Western Blot of FLAG-Topo IIα.

Hela cells grown in RPMI media containing 5% FBS were transfected directly on 100 cm^2 tissue culture plates. Plasmid DNA (10 µg) was mixed with 60 µl of Superfect transfection reagent (Qiagen) in 300 µl of serum-free media for 10 minutes, followed by 600 µl of serum containing media, and the entire mixture was then added directly to cell

culture plates. Transfection was allowed to proceed for three hours at 37°C in a 5 % CO_2 incubator, and terminated by the removal of transfection solution and the addition of 15 ml of 5% FBS containing RPMI media. After incubation for twenty-four hours, the cells were harvested by the addition of 0.53 mM EDTA, washed with cold PBS, and lysed in SDS buffer (2% SDS, 10% glycerol, 0.06 M Tris, pH 6.8). Protein from $2x10^5$ cells per lane was separated on an 8% SDS-PAGE gels and electrobloted (Biorad) onto nitrocellulose membranes (Amersham). The blots were blocked for one hour at ambient temperature in a blocking buffer containing 0.1M Tris-HCl buffered saline, 0.5% Tween-20, and 5% non-fat milk. Blots were stained by the direct addition of anti-FLAG M2 (Sigma) antibody and incubated overnight at 4ºC. Membranes were washed three times for ten min with 0.1M Tris-HCl buffered saline and incubated with anti-mouse IgG antibody (Sigma) in blocking buffer containing 0.1M Tris-HCl buffered saline, 0.5% Tween-20, and 5% non-fat milk for sixty minutes at room temperature. Antibody binding was visualized by ECL (Amersham) on autoradiography film (Kodak).

Chapter Eleven

Experimental Results

Part I: Cell density-dependent VP-16 Sensitivity of Leukemic Cells is Accompanied by the Translocation of Topo IIα from the Nucleus to the Cytoplasm.

11.1 Preliminary Results.

The results of the data described immediately below were obtained prior to my entering the laboratory, but are important because they established a foundation for my doctoral work on mechanism of drug resistance to topoisomerase targeting agents. A complete description of the data is given in Valkov et al., 2000. I became a contributing author of Valkov et al., 2000 for my immunostaining and microscopy work on log and plateau 8226 and CCRF cell lines, which included the development of an *in vitro* cell model for investigating the nuclear-cytoplasmic distribution of topo IIα.

11.2 Drug Resistance Phenotype of Plateau-phase Tumor Cell Lines.

Most cell lines display a differential sensitivity to topo II targeting agents such that when cells reach confluence they become drug resistant to topo II poisons (Sullivan et al., 1987, 1986; Chow and Ross, 1987; Markovits, 1987). Drug resistance to topo II poisons in non-transformed plateau cell lines have been attributed to attenuations in the amount and activity of topo IIα protein. However, the transition from log to plateau cell density does not necessarily lead to a decreased cellular content of topo IIα protein in

transformed cell lines (Sullivan et al., 1987). Thus, we were interested in explaining the observed drug resistance in transformed cell lines at plateau density, which is independent of the cellular content of topo IIα.

First, the differences in drug sensitivity between log and plateau cell densities of several transformed and non-transformed cell lines were investigated. To do this, the cytotoxic effects of two topoisomerase II poisons (i.e., VP-16 and MTX), one topo I poison (TPT), and two non-topoisomerase targeting agents (i.e., cisplatin and vincristine) in several log and plateau cell lines was determined. A comparison of the drug sensitivity of four leukemic cell lines (CCRF, HL-60, KG-1a, L1210), one myeloma cell line (RPMI-8226) and non-transformed human Flow 2000 fibroblasts and Chinese hamster ovary (CHO) cells shows that, at plateau cell density, all cell lines become resistant to VP-16 (Table 9). The magnitude of cross-resistance to another topo II inhibitor, mitoxantrone, was uniformly less in all cell lines, but followed a similar pattern of resistance as that observed with VP-16. The mouse leukemia L1210 and Chinese hamster ovary (CHO) cell lines showed significant plateau phase drug resistance to the topo I inhibitor topotecan. Resistance to drugs that act by mechanisms other than the inhibition of topo was low, except for the resistance of human leukemia KG-1a cells to vincristine. These data suggest that the log to plateau phase transition involves specific changes in topo in these leukemic and non-transformed cell lines. Altered drug sensitivity to VP-16 has been shown to result from a cell-cycle dependent degradation of topo IIα protein. It is well established that the cellular content of topo IIα is maximal in G_2/M of the cell cycle, and thus alterations in the cell cycle can result in altered drug sensitivity to topo II poisons. The specific contributions of topo IIβ in conferring drug

resistance to topo II poisons are less well understood. Attenuation in the cellular content of topo I protein, however, has also been attributed to decreased drug sensitivity to topo I targeting agents. Furthermore, topotecan is reported to be S-phase specific cytotoxic agent. Thus, we examined the cell cycle distribution by flow cytometric analysis and the cellular content of topo I, topo IIα, and topo IIβ by Western blot analysis in log and plateau cells.

Table 7. Drug Resistance of Natural Plateau Phase Non-transformed and Tumor Cell Lines

	VP-16 IC_{50} * (µmol/l)	R_f **	MTX IC_{50} (µmol/l)	R_f	TPT IC_{50} (µmol/l)	R_f	CDDP IC_{50} (µmol/l)	R_f	Vincristine IC_{50} (µmol/l)	R_f
Fibroblasts	11.5±6.6[a].	5.2±0.2[a]	17, 17.1[b]	2.6	2.4±0.6	3.3±0.1	ND	ND	ND	ND
CHO	4.0[**]	15.0[d]	25.0[††]	8.0	0.18±0.15	274±168	3.0[c]	1.0	ND	ND
CRRF	0.63±0.61	39.0±11.5[e]	1.37±0.66	7.7±3.9[g]	0.37±0.28	16.8±12.6	0.71±0.29	1.9±0.05	1.25±0.91	1.3±0.4
HL-60	0.55±0.04	7.6±0.3	18.0[c]	2.2	0.06±0.02	1.0±0	ND	ND	ND	ND
8226	0.57±0.26	10.4±2.1	8.5±0.5	2.6±0.2	0.95±0.55	2.0±1.0	1.15±0.35	1.1±0.1	1.8	1.3
L1210	1.33±0.29	17.7±6.6[f]	7.5±0.5	4.9±0.5	0.30±0.29	282±156	0.55±0.55	1.3±0	3.55±0.05	1.3±0.1
KG-1a	5.67±1.7	3.5±1.4	22.3±12.2	1.1±0.1	1.25±0.75	2.5±0	3.1±0.1	1.1±0.1	1.0, 1.1[b]	27.5, 25.3[b]

*IC_{50}, drug concentration that inhibits growth by 50% as determined by the colony forming assay; values reported are for log phase cells
**R_f, the resistance factor or ratio of IC_{50} values of plateau and log phase cells
[a]Mean ± SEM
[b]n = 2, the experiment was done two times and both values are shown
[c]n = 1, the experiment was done one time and the individual value is shown
[d]From Sullivan et al., 1986
[e]Similar results found with MTT assay (n = 3), where R_f = 26.0 ± 4.9.
[f]Similar results found with MTT assay (n = 3), where R_f = 10.5 ± 0.5
[g]Similar results found with MTT assay (n = 3), where R_f = 6.0 ± 4.3

11.3 Cell Cycle Distribution and [^{3}H]-thymidine Incorporation.

The drug resistance to topotecan observed in the L1210 and CHO cell lines may be explained in part by the decrease in the number of S-phase cells at plateau density (Table 10), as topotecan is thought to be an S-phase specific agent. However, the magnitude of the decrease in S-phase cells at confluence did not parallel the degree of resistance. The fibroblast cells were only minimally resistant to topotecan, and yet had the second greatest decrease in number of S-phase cells at confluence. Although the HL-60 cells showed no decrease in the number of S-phase cells at confluence, they had 6.5 fold less [^{3}H]-thymidine incorporation, suggesting a significant decrease in the number of cycling cells. The sensitivity of HL-60 cells to topotecan, however, paralleled the flow cytometry results (no apparent change in S-phase). Factors other than a simple change in percentage of S-phase are probably involved in the sensitivity to topotecan in this log and plateau cell model.

Table 8. Analysis of S-phase and [^{3}H]-thymidine Incorporation in Log and Natural Plateau Phase Cell Lines*

	BrdU Incorporation Log S (%)	**BrdU Incorporation Plateau S (%)**	**Log [3H] thymidine (cpm/10^6 cells)**	**Plateau [3H] thymidine (cpm/10^6 cells)**
Flow fibroblasts	29.6 ± 6.9[a]	10.1 ± 10.3	ND[b]	ND
CHO	37.4 ± 5.1	14.9 ± 9.2	ND	ND
CCRF-CEM	34.7 ± 8.9	25.9 ± 8.2	20760 ± 371 (P < 0.0001)[c]	2089 ± 256
HL-60	30.0 ± 8.9	30.5 ± 4.9	2289 ± 401 (P = 0.003)	352 ± 33
RPMI-8226	25.4 ± 7.8	15.5 ± 2.3	6819 ± 351 (P = 0.41)	6504 ± 39
L1210	37.3 ± 9.5	27.9 ± 8.1	10570 ± 750 (P = 0.0004)	2306 ± 188
KG-1a	21.6 ± 7.1	11.7 ± 7.9	2860 ± 120 (P <0.0001)	695 ± 50

*Cell cycle distribution was determined by flow cytometry of log and plateau phase cells in three to five independent experiments, while incorporation of [^{3}H] thymidine was measured in appropriate cells in three or four separate experiments.

[a]Mean ± SEM

[b]Not determined

[c]P-values relative to uptake in plateau phase

11.4 Cellular Content of Topo in Log and Plateau-phase Cell Lines.

The overall quantity of topo was determined by Western blot analysis of whole cell lysates of log and plateau phase leukemic, myeloma and non-transformed cell lines. A notable difference between malignant and non-transformed cell lines was the near disappearance of topo IIα from CHO and fibroblasts cells at a time when they reach confluency and lose their proliferative potential (Figure 23). In contrast, all tumor cell lines preserved their total cellular topo IIα content, independent of cell density. Topo IIβ did not show any substantial changes in the cell lines studied and was not downregulated in either normal or neoplastic cells. The content of topo I was not found to be significantly changed by the log to plateau phase transition in fibroblasts, 8226, HL-60, KG-1a, CCRF, or L1210 cells. Furthermore, a reduction in the total cellular amount of topo IIα could not account for the decreased sensitivity to VP-16 and mitoxantrone in transformed cell lines. The relatively high level of resistance of plateau phase L1210 cells to topotecan also does not appear to result from a downregulation of cellular topo I content.

Thus far, we have been able to attribute the drug resistance to topo VP-16 and MTX observed in non-transformed cell lines to attenuation of topo IIα protein. However, we have not been able to fully describe the mechanism(s) of drug resistance to VP-16 or mitoxantrone in the transformed cell lines at plateau cell density. Although alterations in the cell cycle may have a role in the decrease drug sensitivity of L1210 cells to topotecan, the data obtained from analysis of the cell cycle was not always consistent with the drug sensitivity. Furthermore, there were discrepancies between the percent of S-phase cells as determined by flow cytometric analysis and the number of cycling cells determined by

[^{3}H] thymidine incorporation. For example, plateau HL-60 cells have minimal resistance to topotecan, which is consistent with the analysis of S-phase by flow cytometry. However, HL-60 cells demonstrated relatively large decrease in the amount of [^{3}H] thymidine incorporation. Thus, we speculated that other cellular events must be occurring that have a role in drug sensitivity to topoisomerase targeting agents. We questioned whether transformed cell lines may alter their subcellular distribution of topo IIα protein to evade drug-target interactions with VP-16 or mitoxantrone. To address this possibility, we compared the subcellular distribution of topo IIα in 8226 and CCRF cells at log and plateau density by immunofluorescence microscopy.

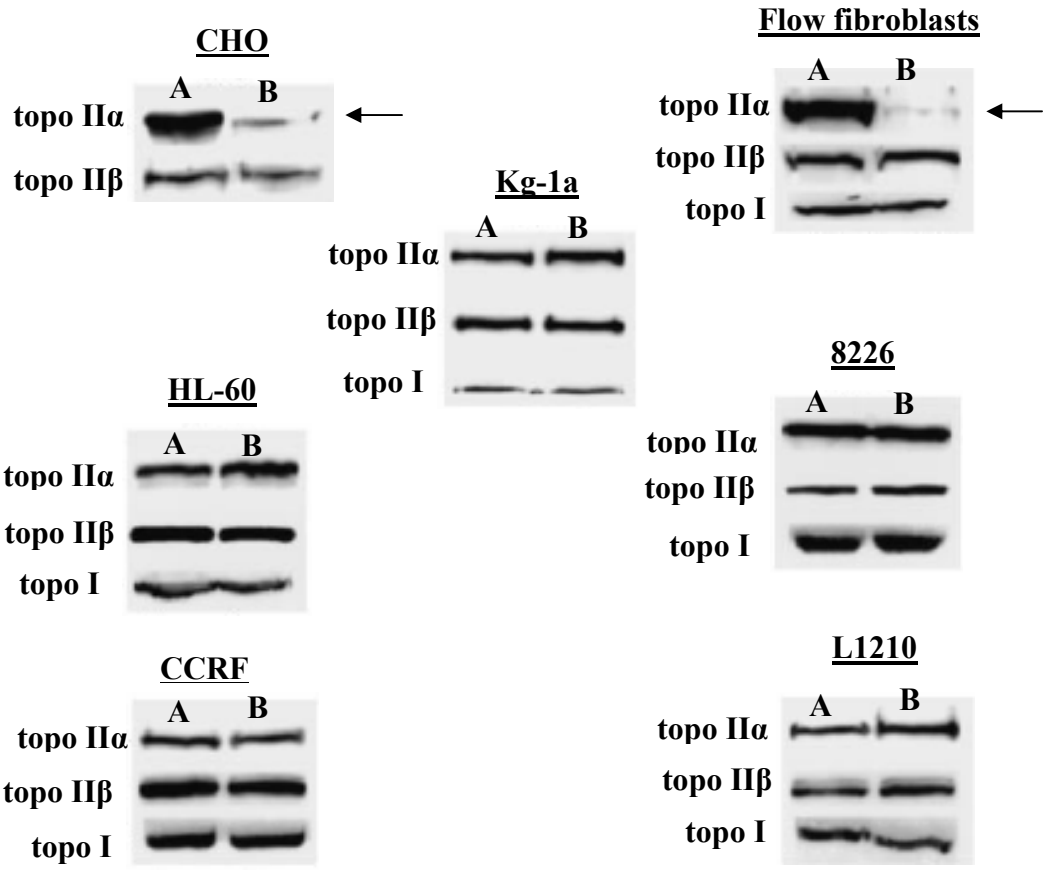


Figure 23**.** Western Blot Analysis of Topo I, Topo IIα, and Topo IIβ from Whole-cell Lysates of Cell Lines at Log and Plateau Densities. One million cells were loaded in all lanes, and the ECL signal was quantified by densitometry. These results are representative of experiments performed a minimum of three times for each cell line. Lane A, log-phase cells; lane B, plateau-phase cells.

11.5 Subcellular Distribution of Topo in Log and Plateau-phase Cell Lines.

At log density, topo IIα was strictly nuclear, while at plateau cell density, the tumor cell lines demonstrated an increase in topo IIα present in the cytoplasm (Figure 24). In order to evaluate the amount of topo IIα at the single cell level statistically, the pixel intensity of nuclear and cytoplasmic topo IIα in 200 individual cells (50 cells/experiment four times) at log, midlog and plateau densities was determined (complete data not shown here, refer to Valkov et al., 2000). CHO cells had a proportional decrease in the nuclear/cytoplasmic ratio, such that at plateau phase the ratio was less than one (<1). This suggested that for the remaining undegraded topo IIα there was relatively more in the cytoplasm than in the nucleus. From early log phase to plateau phase, there was a significant decrease in the topo IIα nuclear/cytoplasmic ratio for all cell lines except KG-1a. Thus, at confluent densities, all cell lines (except KG-1a) had significantly more topo IIα in the cytoplasmic compartment. The immunolabelling of three cells lines (CCRF, L1210, and HL-60) for topo I and topo IIβ demonstrated no significant trafficking of these topoisomerases to the cytoplasm during the transition from log to plateau phase (data not shown).

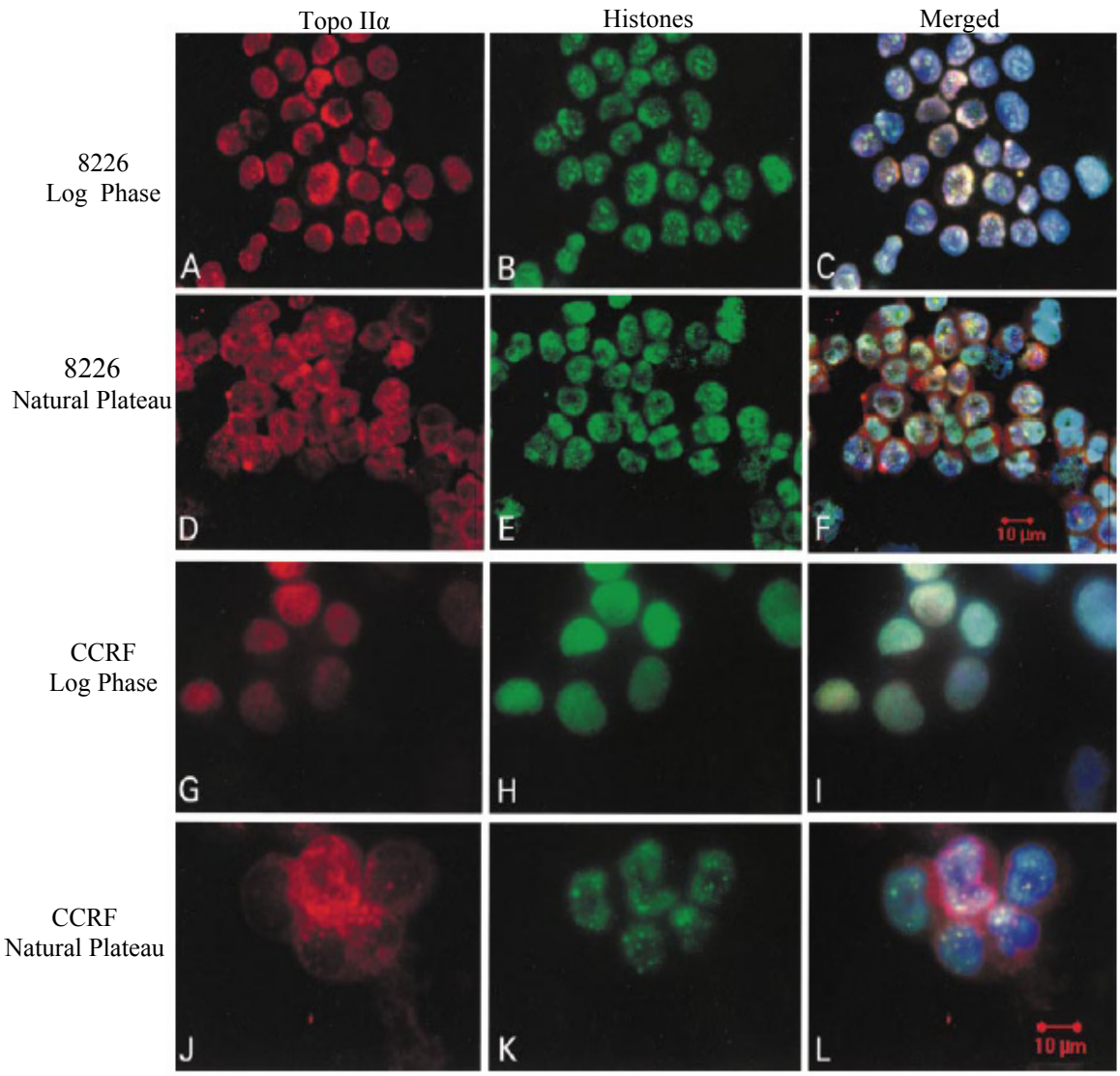


Figure 24. Immunofluorescent Staining and Confocal Microscopy. Immunofluorescent staining for topo IIα (A, D, G, J, histones (B,E, H, and K), and merged images (C, F, I,and L) in log and plateau phase CCRF and 8226 cells. Log phase 8226 cells (A-C) were at a density of 2.0 X 10^5 cells/ml; plateau-phase 8226 cells (D-F) at 9.0 X 10^5 cells/ml; log phase CCRF cells (G-I) at 2.0 X 10^5 cells/ml; and plateau phase CCRF cells (J-L) at 1.6 X 10^6 cells/ml. Cell viability in all experiments was ≥ 95% and the immunofluorescence was observed with a Leitz Orthoplan 2 microscope and captured digitally using the Smart Capture program.

11.6 Conclusions.

The resistance of several leukemia and myeloma cell lines (CCRF, L1210, HL-60, KG1a, and RPMI-8226) to VP-16 was found to increase with cell density and to be maximal (3.5 to 39-fold) in plateau phase cell cultures, as measured by clonogenic and MTT assays. Non-transformed confluent Flow 2000 human fibroblasts and Chinese hamster ovary (CHO) cells were also five and 15-fold resistant to VP-16, respectively. The transition from log to plateau phase was accompanied by a drastic decrease in topoisomerase IIα content in CHO cells and human fibroblasts, while the leukemic cells maintained constant cellular levels of topo IIα and topo IIβ. The nuclear-cytoplasmic ratio of topo IIα may be critical in determining the sensitivity of leukemia cells to topo II inhibitors.

Chapte Twelve

Experimental Results

Part II: The Cytoplasmic Trafficking of DNA Topoisomerase IIα Correlates with Etoposide Resistance in Human Myeloma Cells

12.1 Introduction.

The data described in Part I, suggests that the nuclear-cytoplasmic ratio of topo IIα may be critical in determining the sensitivity of myeloma and leukemia cells to topo II inhibitors. However, many questions remain. Does a nuclear-cytoplasmic trafficking of topo IIα contribute to drug resistance *in vitro*? Are plateau leukemia and myeloma cells cross-resistant to other classes of antitumor agents besides topo targeting agents? How does a cytoplasmic topo IIα contribute to altered drug sensitivity? Is the cytoplasmic distribution of topo IIα a result of deceased nuclear import or increased nuclear export? Can we manipulate the nuclear-cytoplasmic trafficking of topo IIα by either inducing its cytoplasmic location or by blocking the cytoplasmic relocation of topo IIα in plateau cells? These question, and others established the purpose of my doctoral work.

12.2 Accelerated-plateau Human Myeloma Cell Line Model.

To more precisely define the role that the nuclear-cytoplasmic shuttling of topo IIα has in modulating drug sensitivity to topo inhibitors and other classes of anti-cancer

agents, we developed a human multiple myeloma cell line model. The data presented inthe log and plateau cell models suggest that the nuclear-cytoplasmic trafficking of topo IIα could be cell-density dependent, and thus influenced by cell-cell contact. To exploit cell-cell contact, log-phase (defined as 3.0 x 10^5 cells/ml) human myeloma cell lines (NCI-H929 and RPMI-8226) and the human leukemia HL-60 cell line were concentrated to a superconfluent cell density (2.0 x 10^6 cells/ml) and grown at this density for up to 24 h before experimentation. After growing at the superconfluent density for as long as 24 h, the accelerated-plateau cells were still > 85% viable by trypan blue exclusion.

The superconfluent cell density was initially determined by obtaining growth curves for NCI-H929 (Figure 25) and RPMI-8226 (data not shown) cells to establish the cell-density

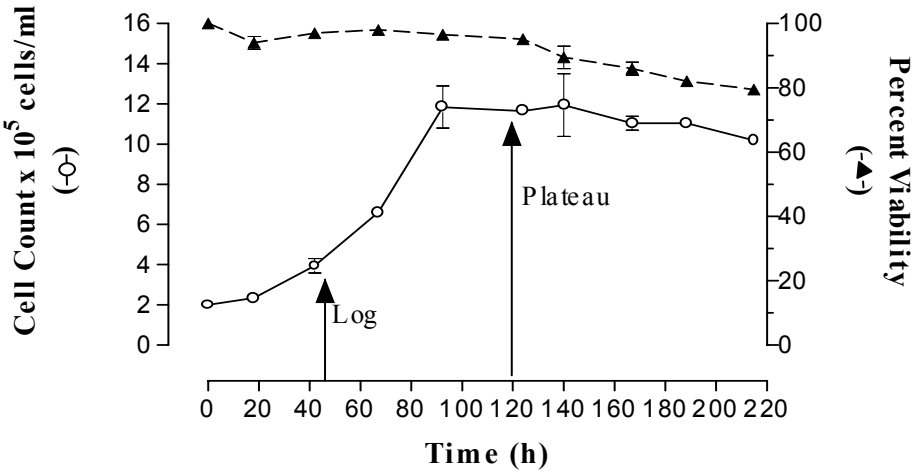


Figure 25. Growth Curves**.** Growth curves for human myeloma H929 and 8226 (data not shown) cells were plotted to determine log and plateau cell densities. H929 cells enter logarithmic growth approximately 2 days after being seeded to an initial cell density of 2.0 x 10^5 cells/ml in fresh media. After growing continuously for 2 days, H929 cells reach "natural-plateau" near 1.2 x 10^6 cells/ml The graph illustrates the relatively long plateau phase (~4-5 days) characteristic of these cell lines. Cell density as cell count x 10^5 cells/ml (o); Percent viability, (▲).

of "naturally-plateaued" H929 and 8226 cells. For example, after being seeded at 2.0 X 10^5 cells/ml, H929 cells require approximately 48 hours to reach log-phase, and then The remain in log-phase for an additional 36-48 hours before reaching plateau phase. H929 cells remained in plateau phase for approximately 2 days. The H929 cells reach confluence when their cell density nears 1.2 X 10^6 cells/ml (natural plateau). Thus, the superconfluent or accelerated-plateau was set to approximately double the natural-plateau cell density (2.0 x 10^6 -2.5 x 10^6 cells/ml).growth curve also illustrates the relatively long natural plateau-phase (approximately 4-5 days) characteristic of H929 human myeloma cells. Other cell densities were also analyzed (1.0 X 10^6 cells/ml, 5.0 x 10^6 cells/ml, and 1.0 x 10^7 cells/ml) however, 2.0 x 10^6 cells/ml was determined to be an optimal cell-density that preserved cellular viability and demonstrated topo IIα export in a measurable period of time.

12.3 Drug Sensitivity of Human Myeloma Cell Lines in Accelerated-plateau.

To determine if topo IIα protein trafficking correlates with cellular drug resistance to other topo targeting agents as well as other classes of antineoplastic agents, the toxic effects of several classes of antineoplastic agents were examined in log and accelerated-plateau H929 and 8226 cells. We expanded the drug sensitivity data that was described for log and plateau cells (in Valkov et al., 2000) to include an antimetabolite (ara-C), a nitosourea DNA crosslinking agent (BCNU), a platinum containing DNA crosslinking agent (cisplatin), an antimicrotubule (Paclitaxel), and γ-irradiation. A comparison of drug sensitivity of log-phase and accelerated-plateau phase H929 and 8226 (data not shown) cells shows that the log-phase cells are more sensitive to topo inhibitors, such as

VP-16 and mitoxantrone, compared to cells seeded at the plateau-phase density for 16 h or 24 h, as determined by colony forming assays (similar results were obtained with 8226 cells) (Table 11). The accelerated-plateau cells were most resistant to VP-16, 10-fold at16 h and 24-fold at 24 h). The accelerated-plateau H929 cells demonstrated less resistance to the non-topo inhibitors such as ara-C, taxol, BCNU, and γ-irradiation, but were found to be 3-fold resistant to topotecan. Thus, the observed drug resistance appears to be more specific for topo inhibitors than other classes of chemotherapeutic agents. These data suggest that the principal mechanism of drug resistance involves quantitative or qualitative changes in topoisomerase. However, other cellular events, such as alterations in either drug transport or cell-cycle can also contribute to a decrease in drug sensitivity to topo poisons. To explain the mechanism(s) of observed drug resistance to topo poisons in accelerated-plateau cells, changes in total amount of topo protein, drug uptake, cell cycle distribution, and subcellular localization were examined.

Table 9. Drug Sensitivity of Log and Accelerated-plateau H929 Cells to Cytotoxic

Antitumor Drug[b]	**Log-phase IC_{50}**[c]	**Resistance Factor**[d] **(R_F)**	
		16 h	**24 h**
VP-16 (DMSO, 1 h)	0.360 ± 0.08[e]	9.6 ± 3.3	23.5 ± 5.1
Mitoxantrone (water, 1 h)	0.0009 ± 0.0001[f]	5.3 ± 0.2	5.1 ± 0.2
Topotecan (methanol, 1 h)	3.32 ± 1.0[g]	3.1 ± 0.4	3.5 ± 0.3
Taxol (DMSO, 24 h)	1.40 ± 0.92	1.2 ± 0.3	2.8 ± 0.2
γ-Irradiation	75 cGy ± 8.6	0.33 ± 0.2	1.3 ± 0.1
***Cis*-platinum** (DMSO, 1 h)	7.00[h]	0.43	0.5
Ara-C (water, 20 h)	1.20[h]	1.3	5.0
BCNU (ethanol, 4 h)	1.0[h]	1.5	1.0

[a]Unless otherwise noted, three to five independent experiments were performed for each drug in triplicate. The mean values ± SEM are shown.
[b]Solvent for drug dissolution and hours of drug exposure are shown in parentheses.
[c]IC_{50} is the concentration of drug that inhibits growth by 50% and the values reported are for log- phase cells in μM concentrations.
[d]Fold resistance (R_F) is the ratio of 50% inhibition concentration values (IC_{50}'s) obtained from colony-forming assays.
[e]Similar results were found in 8226 cells at 24 h, IC_{50} = 0.460 ± 0.06 (n =3), where R_F = 10.3 ± 1.6.
[f] Similar results were found in 8226 cells at 24 h, IC_{50} = 0.003 ± 0.001 (n =3), where R_F = 5.0 ± 0.5.
[g] Similar results were found in 8226 cells at 24 h, IC_{50} = 1.1 ± 0.5 (n =3), where R_F = 2.0 ± 1.1.
[h]The colony-forming assay was performed one time in triplicate.

12.4 Cell-cycle Analysis.

The cellular amount and activity of topo IIα is regulated in a cell-cycle and proliferation dependent manner, and alterations in the amount and activity of topo IIα correlate with cellular drug resistance to topo inhibitors. Therefore, it was necessary to separate the contributions of the cell cycle to the observed drug resistance from other cellular events. The cell cycle was analyzed over a 24 h period for changes in the S-phase cell population from the time that the log-phase cells were concentrated to 2.0 X 10^6 cells/ml (time zero) (Figure 26). Analysis of BrdU incorporation in H929 cells indicates that 19.4% ± 5.0% of log-phase cells are in S-phase compared to 14.4% ± 1.3% of

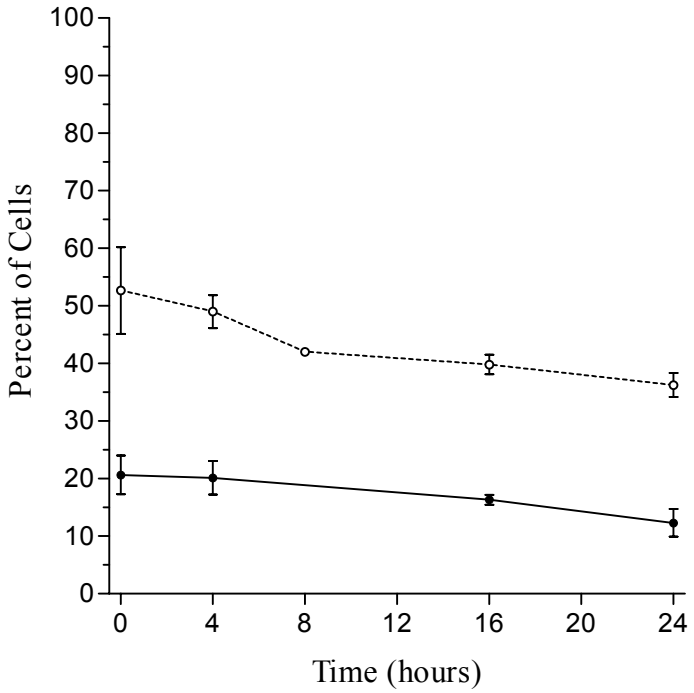


Figure 26. Percent of Accelerated-plateau Cells in S-phase. BrdU incorporation was measured to determine the percent of NCI-H929 and RPMI 8226 cells in S-phase. The experiment was repeated three times and the mean values with standard deviation are shown (o), 8226 cells; (•), H929 cells.

accelerated-plateau cells at 16 h. These data suggest that the changes in the cell cycle distribution that occur within 16 h of being plated at 2.0 x 10^6 cells/ml are not likely to be the main mechanism contributing to the drug resistance observed to topo inhibitors. A greater reduction in the S-phase population of accelerated-plateau cells was observed at 24 h and this difference could account for the increase in drug resistance observed with ara-c (1.3 fold to 5.0 fold) and taxol (1.2 fold to 2.8 fold). To more specifically determine the contribution that topo IIα trafficking plays in drug sensitivity, subsequent experiments were performed within 16 h to minimize contributions of cell cycle changes to drug sensitivity in the accelerated-plateau cell model.

12.5 Cellular Amount of Topoisomerase by Immunoblotting.

An attenuation of topo enzymes can contribute to drug resistance by decreasing the amount of drug target. To determine if there are any changes in the total cellular amount of topo protein in accelerated-plateau cells that could contribute to the observed drug resistance, the amount of topo protein in whole cells was determined by Western blot analysis. No significant changes in the amount of topo II protein were observed in whole cells when compared to log-phase cells 4-24 h after seeding at the super-confluent cell density (Figure 27). Thus a decrease in the total amount of topo II protein is unlikely to account for the observed drug resistance in accelerated-plateau H929 and 8226 cells to VP-16 and mitoxantrone, while a decrease in topo I (20-30%) may contribute to the resistance to topotecan.

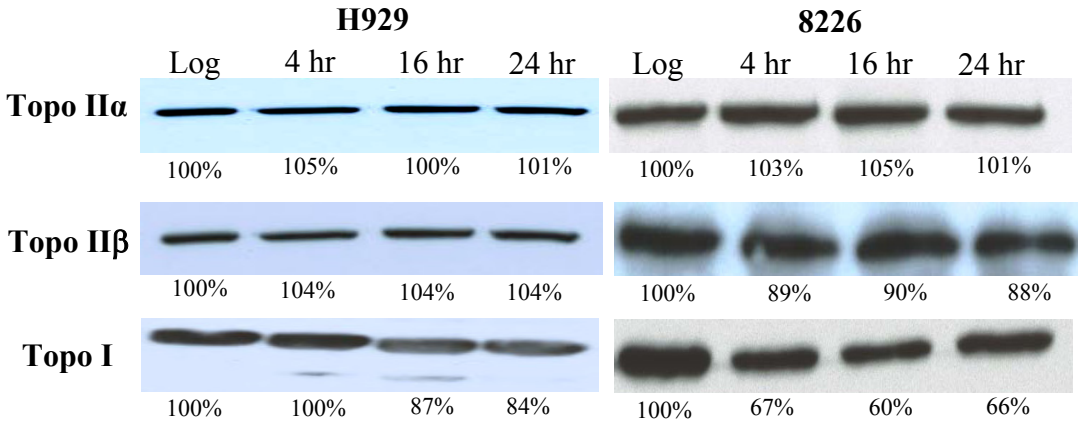


Figure 27. Western blot Analysis of DNA Topoisomerase I and II. The total cellular amount of topo I, topo IIα, and topo IIβ protein in whole cell lysates from 1.0 x 10^6 log and accelerated-plateau H929 and 8226 cells was determined by Western blot analysis. There are no significant differences in the amount of topo IIα or IIβ protein up to 24 h by densitometry. A small decrease in topo I is seen in H929 cells ≥ 16h, while a 30% decrease in topo I is seen in 8226 cells at times ≥ 4 h. Hours refer to the time after concentrating log-phase cells to super-confluent densities. The expermiment was performed one time in duplicate for topo IIβ and repeated three times each for topo I

The observed decrease in topo I may also contribute to the decrease in drug sensitivity to ara-C because recent data shows that topo I cleavable complexes can be trapped by ara-C incorporation into DNA (Pourquier et al., 2000). Inhibition of topo I by ara-C is suggested to occur by structural modifications that occur in the DNA helix when ara-CTP is incorporated. Ara-c incorporation into DNA results in structural modifications in the DNA helix and inhibition of DNA synthesis (Pourquier et al., 2000) Ara-c incorporation into DNA is believed to result in misalignment of the DNA 5'-hydroxyl with the enzyme-DNA tyrosyl phosphodiester bond, which is necessary for nucleophilic attack and DNA religation (Pourquier et al., 1997, 1998, 2000). Thus, it

appears that ara-C cytotoxicity is a result of both inhibition of DNA polymerization and topo I poisoning. Inhibition of topo I by ara-C could explain the 5-fold resistance of plateau cells to ara-C at 24 hours because there is a concomitant decrease in the total cellular amount of topo I protein in plateau cells. Thus, it is possible that cell death induced by ara-C in the log growing cells was due in part to the formation of topo I cleavable complexes in addition to cessation of DNA synthesis.

12.6 Drug Transport in Tumor Cell Lines.

Reduced drug accumulation is a major mechanism conferring drug resistance in several drug-resistant cell lines, and many important mammalian drug transport proteins are ATP-dependent proteins that belong to the ABC superfamily (reviewed in Leonard et al., 2002). Under normal conditions, these pumps protect cells from accumulating toxic substances by actively pumping out cytotoxic substances. Thus, topo I and II inhibitors may be effectively eliminated from cells by a variety of these drug transporters. To determine if the differences in log and accelerated-plateau cells in drug sensitivity to topo inhibitors was a function of altered drug transport, cellular uptake of [^{3}H]-VP-16 was measured as described in "Materials and Methods" (Sullivan et al., 1987). Briefly, H929 cells were incubated in the presence of [^{3}H]-VP-16 for 1 h to achieve steady state, washed, and pelleted by centrifugation. The pelleted cells were transferred to drying paper and dried overnight, and then weighed. After weighing, the pellets were dissolved in a buffer, and then ^{3}H content was determined by scintillation counting. Drug uptake for log and accelerated-plateau cells was determined as cpm per milligram of cells. No

considerable difference was observed in the uptake of [^{3}H]-VP-16 between log (1.7 x 10^4 counts/mg of dry weight) and accelerated-plateau cells (1.8 x 10^4 counts/mg of dry weight). We have previously shown that there are no differences between several log and natural-plateau human myeloma and leukemia cell lines (Valkov et al., 2000; Sullivan et al., 1986, 1987). Thus, an alteration in drug transport of VP-16 is not likely to be the main mechanism conferring drug resistance to topo inhibitors in the accelerated-plateau model.

12.7 Subcellular Distribution of Topoisomerase IIα.

Although the total amount of topo protein does not appear to change in the whole cells, a change in the subcellular distribution of topo could alter the amount of topo protein located in the nucleus. Therefore, we examined the subcellular distribution of topo IIα (28) and topo I and topo IIβ (29) was examined by immunofluorescence microscopy and confirmed by scanning confocal microscopy.

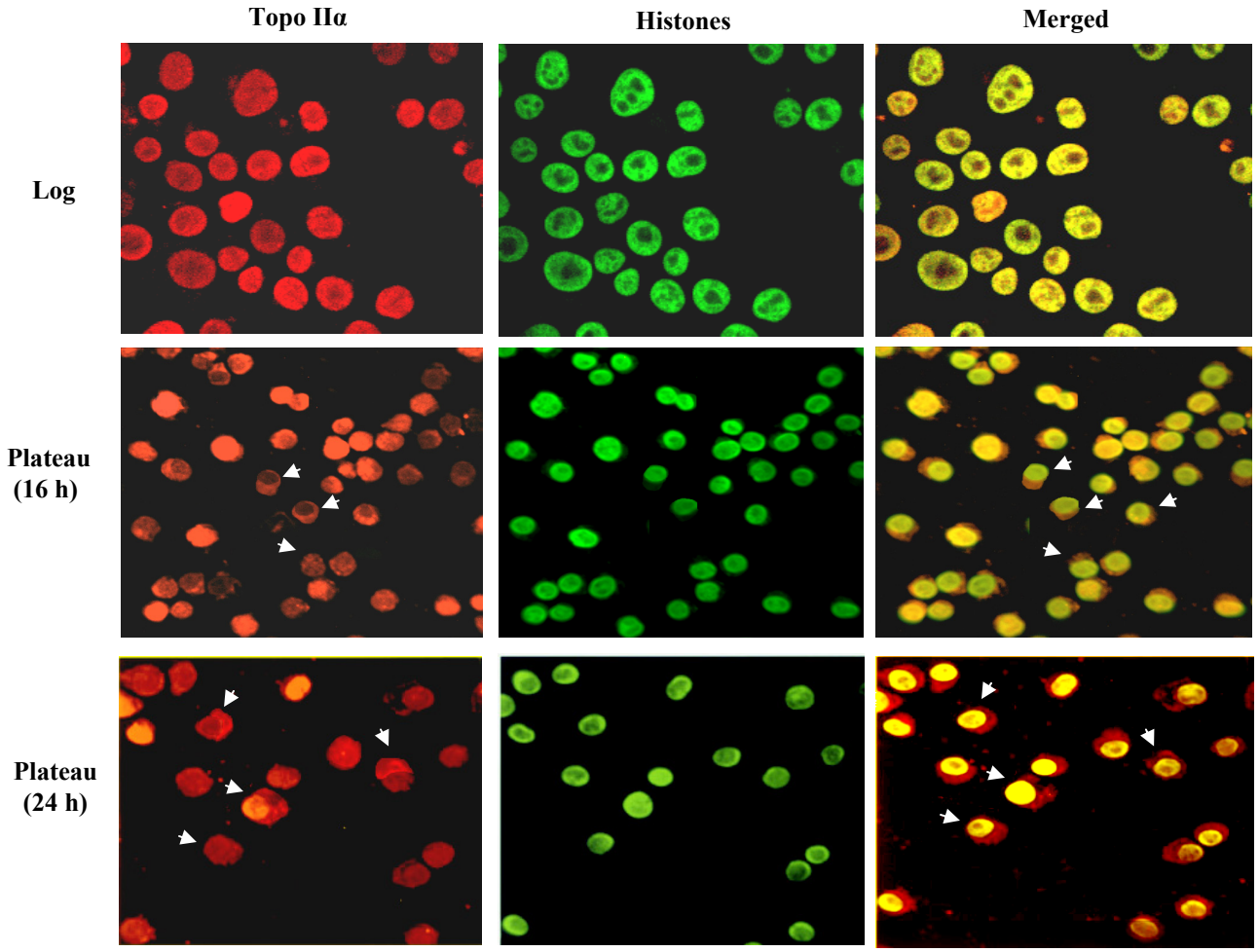


Figure 28. The Subcellular Distribution of Topo IIα in Log and Accelerated-plateau H929 Cells at 16 h and 24 h. Histones are non-shuttled proteins that define the area of the nucleus. Topo IIα is nuclear in log-phase cells and has a markedly increased cytoplasmic distribution in accelerated-plateau cells at 16 h and 24 h. Arrows point to cells with cytoplasmic topo IIα.

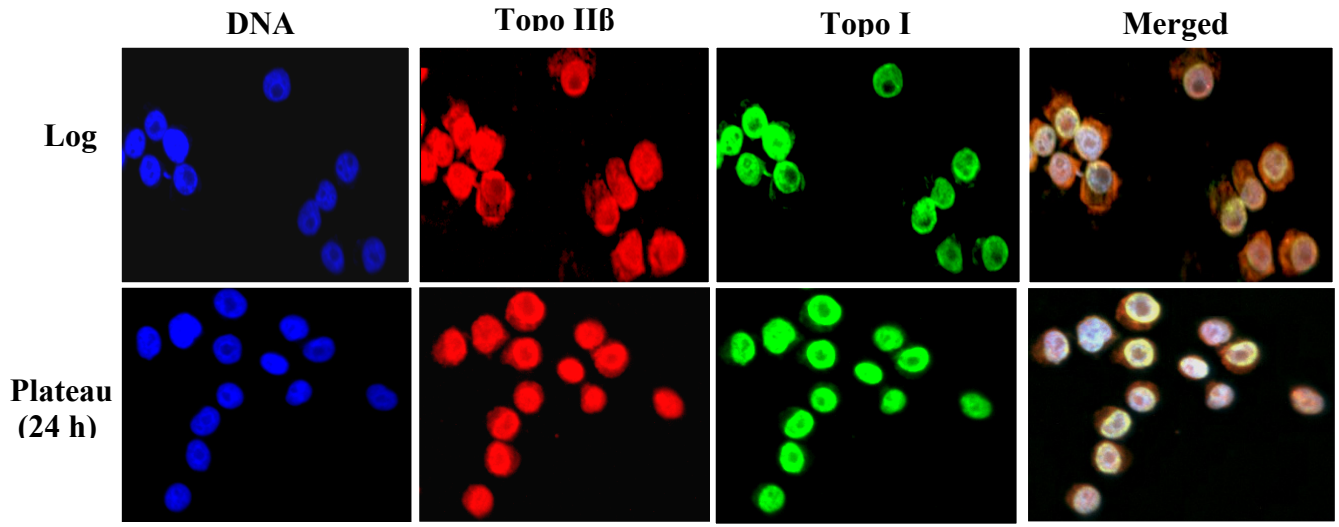


Figure 29. The Subcellular Distribution of DNA, Topo I, and Topo IIβ in Log and Accelerated-plateau H929 Cells at 24 h. DNA was stained with DAPI which defines the area of the nucleus. Topo I appears nuclear in log and accelerated-plateau cells. Topo IIβ is found in the nucleus and cytoplasm of log and plateau cells.

A least 50 cells per experimental condition (i.e., log or plateau) were randomly picked by 4'-6-diamidino-2-phenylindole (DAPI) staining of the nucleus. Cells were numbered as 1-50 and then, analyzed for the pixel intensity of either tetramethyl rhodamine isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) labeled antibody as described in Figure 30. Topo IIα is predominately nuclear in log-phase cells, but there is a time-dependent translocation of topo IIα in the accelerated-plateau cells after 16 h. Similar results were observed in the natural plateau cells (data not shown).

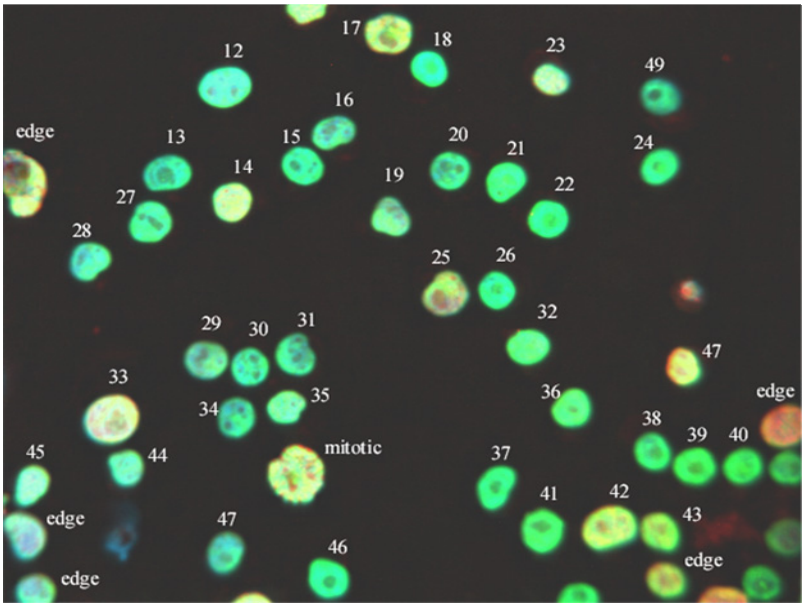


Figure 30. Method for Selecting Cells by Confocal Microscopy. Cells were selected randomly by positive DAPI staining and images captured using a CCD camera as described in"Materials and Methods". The cells were numbered 1-50. Mitotic cells and cells touching the edge of the field of vision were not included in the data. Furthermore, any cell staining positive for DAPI but histone negative were presumed to be cell debris and also were not included in the data.

The subcellular distribution of topo protein was quantified in log and accelerated-plateau H929 and 8226 cells by calculating the nuclear/cytoplasmic ratio (N/C) from the pixel intensity of the individual compartments from 50 cells per time point. The staining for nuclear topo I and II was internally controlled in each cell by DAPI staining of DNA and histone staining. Figure 31 illustrates the method, as described in "materials and

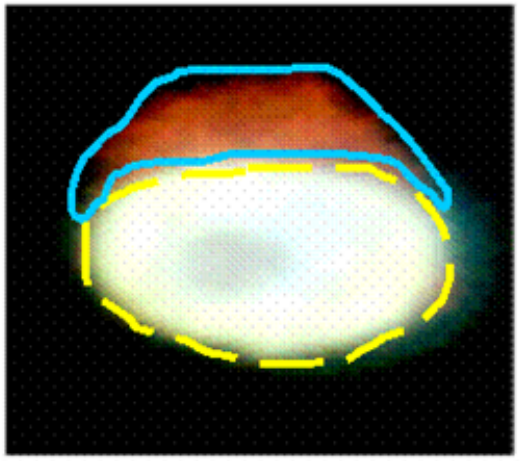


Figure 31. Determination of Nuclear-cytoplasmic Ratios (N/C). Using AdobePhotoshop, we were able to zoom-in on each cell. To obtain the value of the pixel intensity between the nuclear and cytoplasmic compartments for either TRITC or FITC labeled antibody, the nuclear and cytoplasmic compartments were outlined using the "lasso" feature. In the cell shown here, the cytoplasm is outlined in blue, whereas the nucleus is outlined in yellow. The N/C ratio was determined from the following equation: number of pixels in nucleus x (mean-background)/number of pixels in cytoplasm x (mean - background).

methods", for analyzing the subcellular localization of topo by immunofluorescence. A least 50 cells per experimental condition (i.e., log or plateau) were analyzed. These experiments were performed in duplicate and repeated three times in both H929 and 8226 cells. The median N/C ratio of topo IIα is 29.9 ± 9.1 for log-phase H929 cells and decreases to 1.3 ± 1.3 for the accelerated-plateau cells after 16 h and to 0.90 ± 1.1 after 24 h. The median N/C ratio of topo I and IIβ did not change. The median N/C ratio of

topo I was 27.0 ± 3.2 in log-phase H929 cells and 29.2 ± 4.1 at 16 h accelerated plateau. The median N/C ratio of topo IIβ was 10.1 ± 5.5 in log-phase and 8.9 ± 5.4 at 16 h accelerated plateau. The subcellular distribution of histones was monitored as a nuclear control and also does not change in the accelerated-plateau cells. Similar results were obtained in 8226 cells (data not shown). For example, the median N/C ratio in 8226 cells of topo IIα in log-phase was 9.5 ± 6.3 versus 2.1 ± 1.6 at 16 h accelerated plateau.

The subcellular distribution of topo IIα in H929 cells was confirmed by biochemical separation of the nuclear and cytoplasmic compartments with subsequent western blot analysis (Figure 32). The subcellular locations of topo I and LDH were used as nuclear and cytoplasmic controls, respectively. The amount of topo IIα found to be distributed to the cytoplasmic compartment in plateau-phase H929 cells varied between 25% and 50% of the total cellular amount. This experiment was repeated seven times and the majority of the experiments demonstrated 40-50% cytoplasmic topo IIα in plateau-phase cells. These results agree with the microscopy findings above, which demonstrated a N/C ratio of 1.3 ± 1.3 for 16 h plateau-phase H929 cells. The partial degradation of topo IIα that occurred during the nuclear-cytoplasmic separation was not observed when cells were immediately immunoblotted, and was not prevented by the addition of several protease inhibitors (see "Materials and Methods"). Similar immunoblot results were obtained in HL-60 cells (data not shown) and 8226 cells. Thus, it appears that topo IIα, but not topo I or IIβ, is shuttled to the cytosol when H929 cells are grown at a super-confluent cell density.

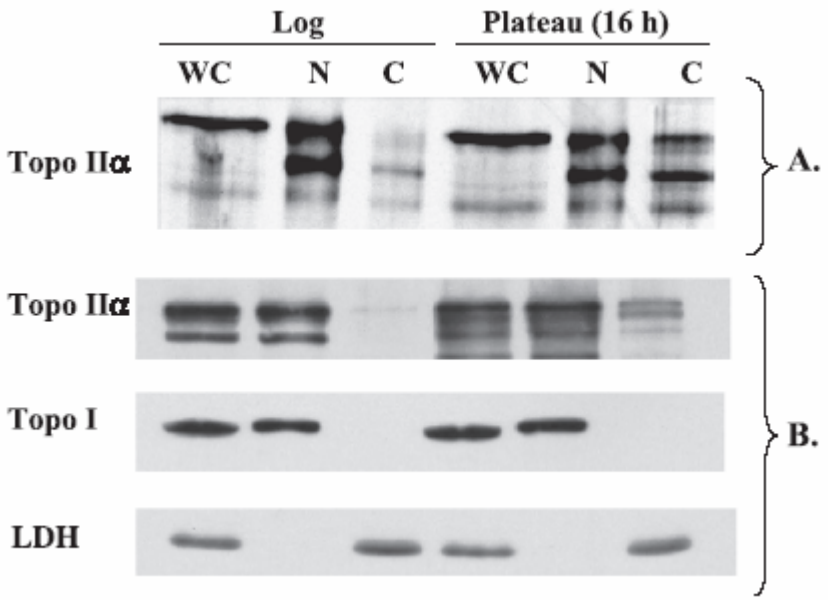


Figure 32. Nuclear-cytoplasmic Separation and Western Blot Analysis of DNA Topoisomerase I and II and LDH. Nuclear-cytoplasmic separation of log and plateau (16h) H929 cells followed by Western blot analysis for topo IIα, topo I, and LDH. Experiment A demonstrates that in plateau phase there is approximately one-half of the cellular topo IIα (partially degraded) in each of the nuclear and cytoplasmic compartments. In experiment B, the amount of total cellular topo IIα is distributed as approximately 75% to the nucleus and 25% to the cytoplasm in plateau phase growth (determined by densitometry). The subcellular locations of topo I and LDH were used as nuclear and cytoplasmic controls, respectively. WC, whole cells; N, nucleus; C, cytoplasm.

12.8 Topoisomerase II Enzyme Activity.

Although the total amount of topo IIα protein does not change between log and accelerated-plateau cells, a redistribution of topo IIα from the nucleus to the cytoplasm would be expected to decrease the amount of drug target in the nucleus. If the nuclear-cytoplasmic trafficking of topo IIα results in attenuation of nuclear content, then there would be less drug-induced DNA damage and fewer cleavable complexes expected. Thus, we hypothesized that a cytoplasmic distribution of topo IIα would protect cells from drug induced DNA damage by attenuating the amount of lethal drug-enzyme-DNA

complexes formed. To test this hypothesis, the amount of VP-16 induced DNA damage was compared in log and accelerated-plateau phase (16 h) H929 cells by the alkaline comet assay (Figure 33). The mean of three experiments showed that the average comet moment was higher in log-phase cells than accelerated-plateau cells at each drug concentration. For example, the average comet moment for log-phase cells was 80 ± 7 and 50 ± 5 for accelerated-plateau cells in the presence of 10 μM VP-16. The results indicate that there are less drug-induced double strand DNA breaks in cells seeded at the super-confluent cell density.

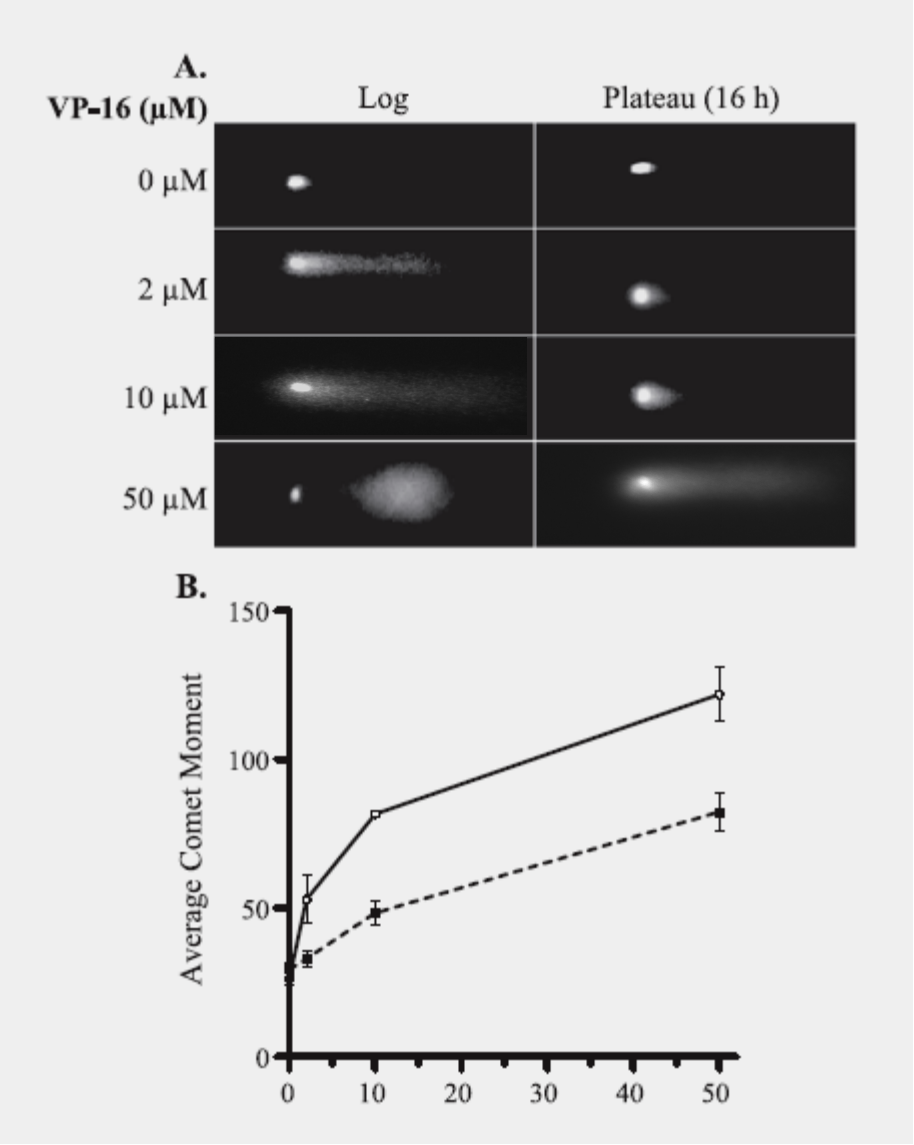


Figure 33 . Determination of VP-16 Induced Double-strand DNA Breaks. Measurement of VP-16 induced double-strand DNA breaks in log and accelerated-plateau H929 cells at 16 h using the comet assay. Cells were treated with 0-50 μM VP-16 as described in "Materials and Methods". The average comet moment of 50 cells at each drug concentration was calculated from three experiments and is shown in panel B. A representative comet is shown in A. (o), log-phase cells; (•), accelerated-plateau cells.

To determine if the decrease in DNA damage can be explained by a reduction in the number of drug-induced enzyme-DNA complexes formed, the amount of topo IIα drug-stabilized cleavable complex formation was compared in log and accelerated-plateau (16 h) cells using a modification to the band depletion assay originally described for observing topo I cleavable complex formation in the presence of topotecan. We adapted the same procedure to observe topo IIα cleavable complex formation in the

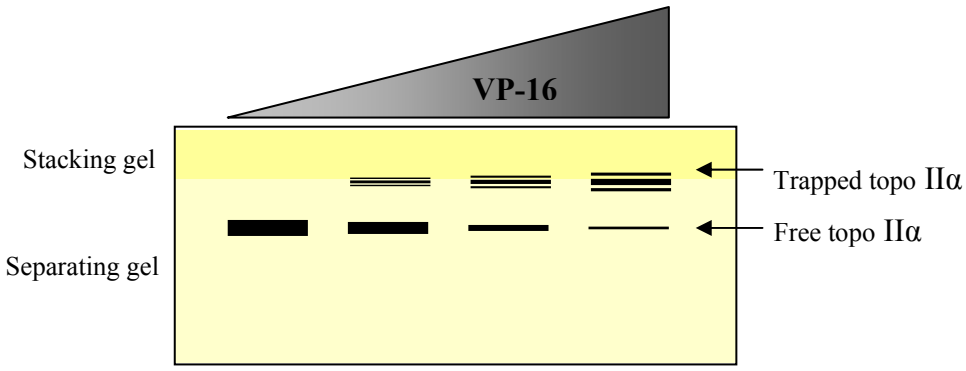


Figure 34 . Illustration of Band Depletion Assay. The band depletion assay was preformed as described in "materials and methods". Briefly, cells treated with increasing concentrations of etoposide (VP-16) are lysed in a buffer containing guanidine hydrochloride, which acts as a strong protein denaturant that traps preexisting covalent topo II-DNA complexes. Cell preparations are then electrophoresed on an SDS-polyacrylamide gel. Topo IIα-DNA complexes run more slowly than free topo IIα, which is observed as a decrease in the intensity of the topo IIα by Western blot analysis.

presence of etoposide. This assay is based on the observation that topo II covalently bound to DNA migrates more slowly than free topo II on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Figure 34). Accordingly, as topo II forms increasing amounts of cleavable complexes in the presence etoposide, there is a corresponding decrease in the

molecular weight signal of topo IIα by Western blot analysis (Figure 35) (Kaufmann et al., 1997). Results from the band depletion assays showed that a 7-fold higher concentration of etoposide was needed to deplete the topo IIα levels by 50% in accelerated-plateau H929 cells. The drug concentration to achieve 50% band depletion is 5 μM in log-phase cells and 35 μM in accelerated-plateau cells, suggesting that there are fewer enzyme-drug DNA complexes formed in accelerated-plateau cells relative to log-phase cells. These results were further confirmed by measuring the catalytic activity (kDNA decatenation) of topo II present in the nuclear extracts from log and accelerated-plateau H929 and HL-60 cell lines (data not shown). Approximately 4-times more nuclear extract protein was needed to decatenate 1 μg of kDNA in either accelerated-plateau H929 or HL-60 cells than in log-phase cells.

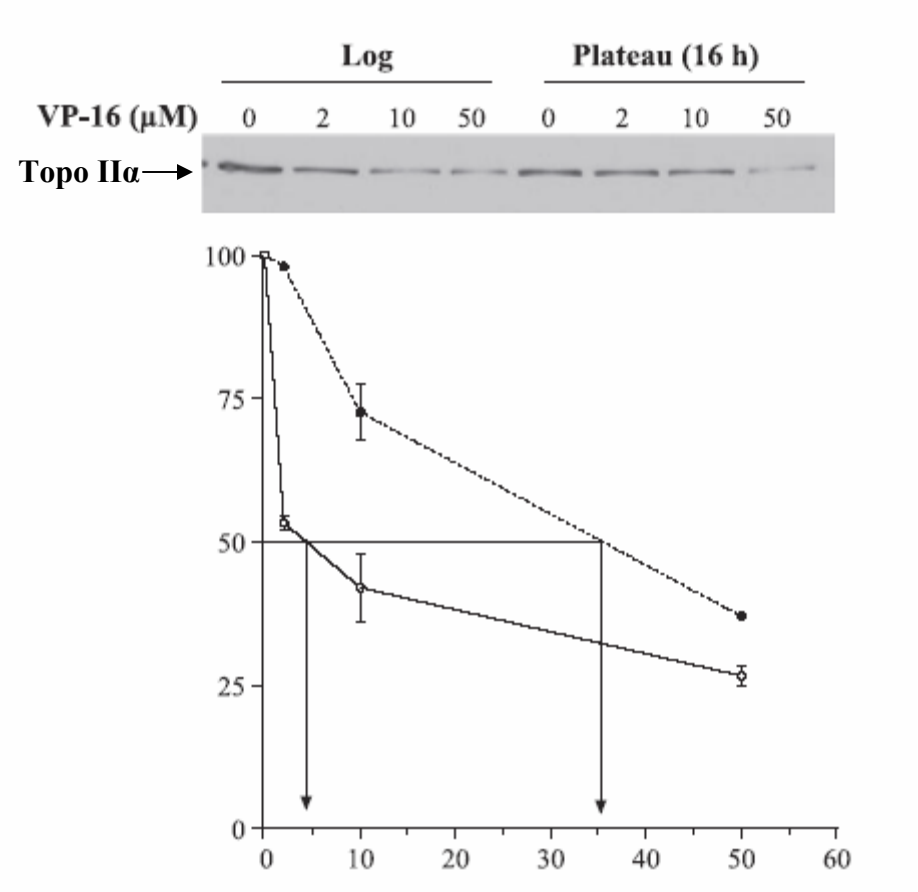


Figure 35. Measurement of Cleavable Complexes. Differences in the formation of VP-16-stablized enzyme-DNA complexes were examined in log and accelerated-plateau H929 cells using the band-depletion assay.These results show that a 7-fold increase in VP-16 was needed to achieve a 50% reduction in the topo IIα band in plateau-phase cells relative to log-phase cells. (o), log-phase cells; (•), accelerated-plateau cells. Topo IIα cleavable complexes become trapped in the stacking gel

Collectively, these data suggest that there are significantly fewer topo catalyzed double-strand DNA breaks in VP-16 treated accelerated-plateau cells than log H929 cells because there are fewer drug-induced DNA-protein complexes formed. Thus, a decrease in drug-induced DNA-protein complexes could explain in part the observed drug resistance to topo inhibitors; attenuation in the total amount of cleavable complexes formed can best be explained by the cytoplasmic trafficking of topo IIα enzyme. Also, nuclear topo IIα present in plateau H929 cells appears to be less drug sensitive.

12.9 Subcellular Distribution of Topo IIα in Malignant Plasma Cells.

We next studied the distribution of topo I, topo IIα, and topo IIβ in clinical samples: bone marrow aspirates from myeloma patients. Representative aspirates from two out of 10 patients examined are shown in Figure 20 and figure 21. The aspirates (both with >90% plasma cells) were obtained before high-dose chemotherapy but after standard-dose chemotherapy. Figure 37 shows the subcellular distribution of topo IIα in one untreated patient with multiple myeloma. In all patients, there was a considerable proportion of topo IIα in the cytoplasmic compartment of the plasma cells. The percentage of plasma cells that was brightly fluorescent and displayed a nuclear distribution of topo IIα was always in the range of 10-15% and reflected cells that were probably in the S-or G_2/M-phase of the cell cycle. Topo I was found to be strictly nuclear or nucleolar except for one patient (90% of cases). In the one patient who had relapsed after high-dose chemotherapy, topo I was found to be exclusively cytoplasmic. The topo IIβ subcellular distribution was mostly a mixed nuclear and cytoplasmic pattern, and in ~50% of the patients showed nucleolar staining as well. Generally, topo IIβ labeling

demonstrated a low expression by immunofluorescence and was nearly undetectable on Western blots. When the nuclear/cytoplasmic ratios of topoisomerases were examined in the malignant plasma cells from 10 myeloma patients by confocal laser scanning microscopy, only topo IIα was present with ratios below 1 in the majority of cells. Collectively, the data in this study suggests that a correlation may exist between the cytoplasmic location of topo IIα and decreased sensitivity of these cells to topo inhibitors.

12.10 Conclusions.

In this study, we have investigated the role of topoisomerase IIα trafficking in cellular drug resistance. We developed a cell model (called accelerated-plateau) using human myeloma H929 and 8226 cells that reproducibly translocates topo IIα to the cytoplasm. It was necessary to separate the influence of the cell cycle, drug uptake, topo protein levels, and enzyme trafficking of drug sensitivity in this cell model. Compared to log-phase cells, the cytoplasmic redistribution of topo IIα in the accelerated-plateau cells correlated with a 10-fold resistance to VP-16 and a 40-60% reduction in the number of drug-induced double strand DNA breaks. In addition, 7-fold more VP-16 was necessary to achieve 50% topo IIα band depletion, suggesting that there are fewer drug-induced topo-DNA complexes formed in the accelerated-plateau cells than the log-phase cells. The total cellular amount of topo IIα and topo IIβ protein in log- and accelerated-plateau was similar as determined by Western blot analysis. There was a 25% reduction in S-phase cell number in plateau cells (determined by bromodeoxyuridine (BrdU) incorporation).

The data from this study support the hypothesis that the nuclear-cytoplasmic trafficking of topo IIα mediates, at least in part, cellular drug resistance to topo inhibitors by reducing the amount of topo IIα in the nucleus and the subsequent number of enzyme drug-induced DNA complexes formed. Thus, we established a novel mechanism of *de novo* drug resistance that is independent of drug transport and cellular quantity of topo IIα. Furthermore, Western blot analysis demonstrates that the cytoplasmic location of topo IIα is not likely to be a result of expression of a truncated enzyme that has lost the C-terminal NLS (Wessel et al., 1997).

These data may have clinical implications in the treatment of human myeloma, as immunohistochemistry (Figures 36 and 38) and Western Blot analysis (Figure 37) demonstrates that topo IIα has a cytoplasmic distribution in malignant plasma cells obtained from the bone marrow aspirates of myeloma patients before (Figure 36) and during treatment (Figures 37 and 38). The data also suggests that topo IIβ and topo I may be more appropriate molecular targets in multiple myeloma because neither their amount nor their subcellular localization were found to be altered at plateau densities.

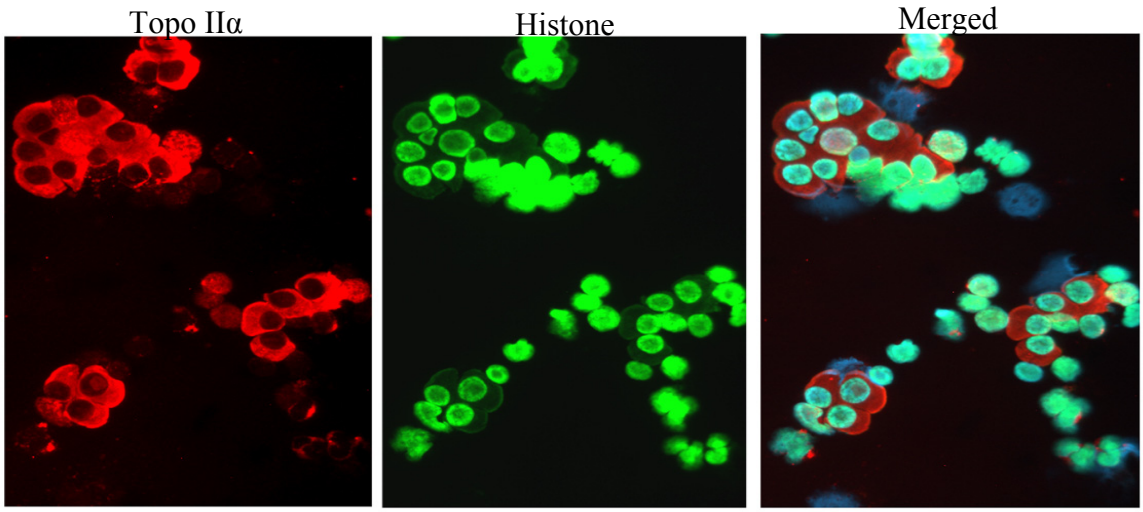


Figure 36. Subcellular Distribution of Topoisomerase IIα in Plasma Cells from One Untreated Multiple Myeloma Patient. Immunofluroescent staining for Topo IIα and histone. Goat anti-rabbit IgG-TRITC labeled antibody was used for detecting topo IIα (appears red). Goat anti-mouse IgG-FITC labeled secondary antibody was used to detect histone (appears green) as a nuclear control. Cells were mounted on microscope slides containing Vectashield mounting media containing DAPI, which binds DNA (appears blue).

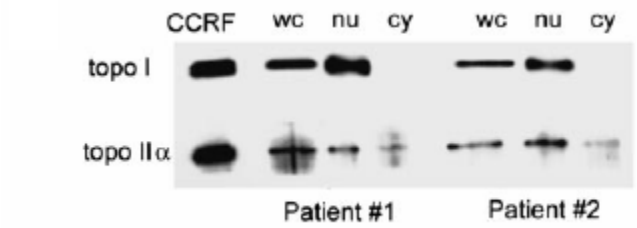


Figure 37 . Western Blot Analyses of Topoisomerase I and Topo IIα in Two Patients with Multiple Myeloma being Treated with Chemotherapy. The nuclear and cytoplasmic compartments were separated as described in "Materials and Methods" and the equivalent of 5.0 x 10^5 cells was loaded in each lane. The CCRF cells were loaded as a positive control for topo I and topo IIα immunoreactivity. WC, whole-cell lysates; nu, nuclear fraction; cy, cytoplasmic fraction.

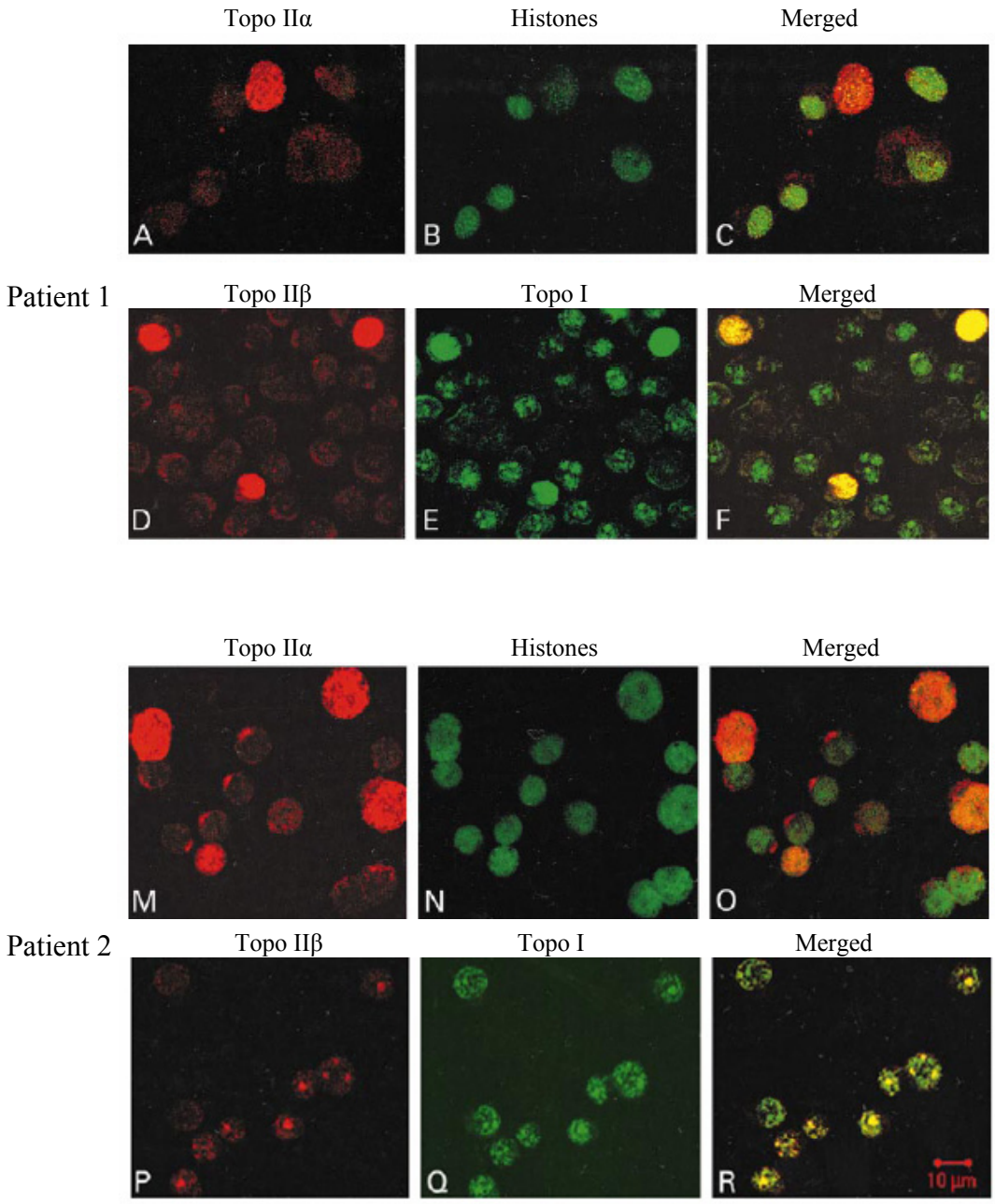


Figure 38. Subcellular Distribution of Topoisomerase in Two Multiple Myeloma Patients being Treated with Chemotherapy. Confocal microscopy was performed on plasma cells obtained from patient 1 (A-F) and Patient 2 (M-R). Cells were stained for topo IIα (A and M) with polyclonal antibody 454 and histones (B and N). The merged image (C and O) demonstrates that topo IIα is nuclear ~ 10% of the cells (proliferative fraction) and cytoplasmic or faintly nuclear in the remaining plasma cells. Histone staining was used to define the nucleus. Patient plasma cells were also stained for topo IIβ (D and P) with the polyclonal JB1 antibody, for topo I with the C-21 monoclonal antibody (E and Q) and the merged images (F and R). Topo IIβ was observed in the nucleus (D), cytoplasm (F) and nucleoli (P), but its amount was relatively low compared with topo IIα. Topo I distribution was typically nuclear (E) or nucleolar (Q).

Chapter Thirteen

Experimental Results

Part III: Human Topoisomerase IIα Contains Two Leptomycin B Sensitive Nuclear Export Signals

13.1 Introduction.

Resistance to chemotherapeutic drugs is a major obstacle in the treatment of leukemia and myeloma. We have previously found that myeloma and leukemic cells in transition from low-density log phase conditions to high-density plasteau phase conditions exhibit a cell density dependent translocation of topo IIα to the cytoplasm. The determination of the mechanism of topo IIα transport across the nuclear membrane could lead to a better understanding of how to regulate the levels of active enzyme in the nucleus, and thus sensitize cells to topo inhibitors. We questioned whether the cytoplasmic distribution of topo IIα was a result of decreased import or increased export. Initially, we tried to block the import of topo IIα in log and plateau density cells by using the general nuclear import inhibitor, lectin. Wheat germ agglutin (WGA) is a non-specific inhibitor of general nuclear import (Duveger et al., 1995). WGA is believed to inhibit nuclear import by binding nucleoporins and "clogging" the nuclear pore complexes. We did not observe a difference in the nuclear-cytoplasmic distribution of topo IIα in log or accelerated plateau 8226 or H929 cells treated with lectin as determined

by confocal microscopy (unpublished data). One possible explanation of these results is that in log-phase cells, topo IIα was already present in the nucleus prior to drug treatment and does not shuttle continuously between the nucleus and cytoplasm of log-growing cells. We also treated accelerated plateau 8226 and H929 cells with lectin to determine if a greater percentage of topo IIα protein would become trapped in the cytoplasmic compartment of lectin treated cells compared to untreated cells. We observed no significant differences in the nuclear-cytoplasmic distribution of topo IIα in the presence of lectin. These results suggest the possibility that topo IIα is exported from the nucleus to the cytoplasm, and thus is not inhibited by lectin. Therefore, we investigated the ability of the nuclear export inhibitor, Leptomycin B, to block the nuclear export of topo IIα in the accelerated-plateau cells.

13.2 LMB Modulation of Topoisomerase IIα Trafficking.

To determine the mechanism by which topo IIα accumulates in the cytoplasmic compartment, accelerated-plateau H929 cells were treated with the nuclear export inhibitor, LMB (Figure 39). This *Streptomyces* metabolite has been shown to inhibit CRM1-mediated nuclear export of proteins with leucine-rich nuclear export signals (Engel et al., 1998; Wolff, B. et al., 1997; Nishi et al., 1994; Hamamoto et al., 1985). Immunofluorescent microscopy demonstrates that the median N/C ratio of topo IIα is increased 58-fold in plateau H929 cells treated with 0.5 ng/ml of LMB for 16 h when compared to untreated cells. The median N/C ratio of untreated accelerated-plateau cells is 0.98 ± 2.0 and increases to 57.5± 170.2 in the presence of LMB. A total of 50 cells were examined per condition and the experiment was repeated three times. Similar results

were obtained in HL-60 cells (data not shown). The large standard deviation observed in the LMB-treated cells can be accounted for by the presence of several cells with N/C ratios > 300. Therefore, the median values have been reported as a more representative value of the true N/C ratio. These data indicate that the cytoplasmic translocation of topo IIα can be blocked by LMB and suggests that topo IIα is exported from the nucleus as opposed to retained in the cytoplasm in untreated cells. Furthermore, the average total pixel density of topo IIα in whole cells decreased from 113,929 ± 51,530 in control cells to 38, 053 ± 41, 040 in LMB-treated cells, a 67% reduction in pixel density of whole cells. These data suggest that topo IIα may be downregulated in the presence of LMB because there is a greater reduction in total pixel density of treated cells relative to untreated plateau cells. The pixel density of topo IIα immunofluorescence in the nuclear compartment is reduced from an average pixel density of 52,241 ± 21,716 to 868 ± 1226 pixels. One possible explanation is that LMB blocks the export of topo from the nucleus and topo becomes degraded by nuclear proteasomes. The degradation of topo has been shown to occur in the nucleus, but Western Blot analysis of topo IIα protein content in LMB treated plateau cells would be necessary to confirm these results. These results suggest that topo IIα trafficking in the log-plateau cell model could be a result of increased nuclear export.

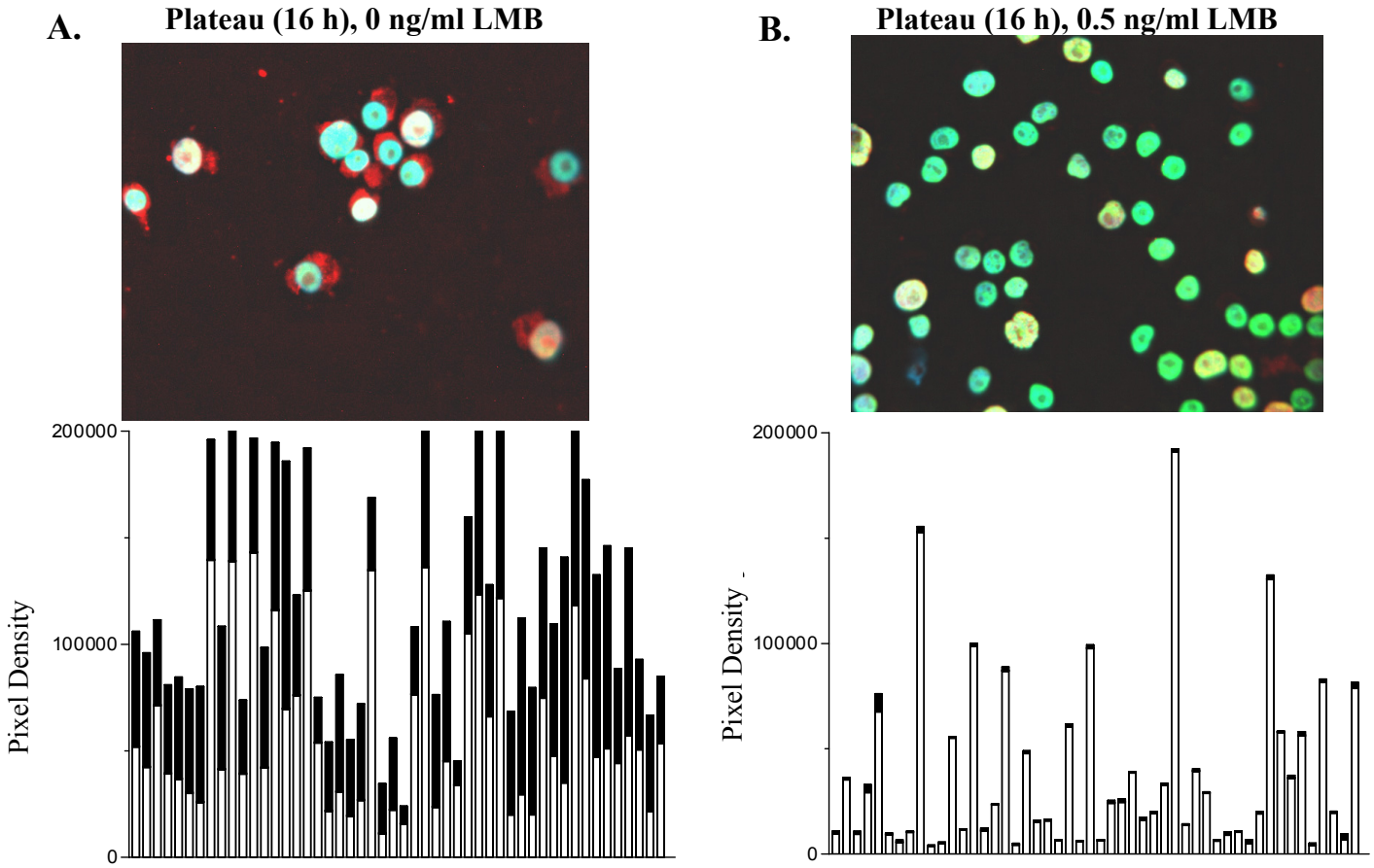


Figure 39. Confocal Microscopy and Nuclear-cytoplasmic Ratios of DNA Topoisomerased IIα Immunoflurescent Staining in the Presence or Absence of Leptomycin B. Subcellular localization of topo IIα and pan-histone in 16 h accelerated-plateau H929 cells ± 0.5 ng/ml LMB (panel A, 0 ng/ml LMB; panel B, 0.5 ng/ml LMB). The nuclear-cytoplasmic ratio per cell is shown for 50 plateau-phase cells (± LMB) in panels C and D. tetramethylrhodamine isothiocyanate (TRITC) staining (red) is for topo IIα and FITC staining (green) is for histones, in the immunofluorescence figures. The experiment was repeated three times in H929 cells and once in HL-60 cells (data not shown). Cytoplasm (■), nucleus (□).

Table 10. Determination of a Consensus Sequence from Established Nuclear Export Signals (NES)

α-actin (Wada et al., 1998)	A	**L***	P	H	A	**I**	M	R	**L**	D	**L**	A
β-actin (Wada et al., 1998)	A	**L**	P	H	A	**I**	L	R	**L**	D	**L**	A
TFIIIA (Fridell et al., 1996)		**L**		P	V	**L**	E	N	**L**	T	**L**	
PKIα (Wen et al., 1995)	E	**L**	A	L	K	**L**	A	G	**L**	D	**I**	N
MAPKK (Fukada et al., 1996)	A	**L**	Q	K	K	**L**	E	E	**L**	E	**L**	D
RevHIV (Fischer et al., 1995)	Q	**L**		P	P	**L**	E	R	**L**	T	**L**	D
Ran BP-1 (Zolotukhin and Felber, 1997)	K	**V**	A	R	K	**L**	E	A	**L**	S	**V**	R
C-Abl (Taageoera et al., 1998)		**L**	E	D	N	**L**	R	E	**L**	Q	**I**	C
hZyxin (Nix and Beckerle, 1997)	**L**	T	M	K	E	**L**	E	E	**L**	E	**L**	L
MdM2 (Roth et al., 1998)	S	**L**	S	G	D	**L**	S		**L**	A	**L**	C
P53 (Strommel et al., 1999)	**F**	R	E	**L**	N	E	A	**L**	E	**L**	K	D
Consensus	**H**X_{1-4}**H**X_{2-3}**H**X**H**(X = Leu, Ile, Val, or Phe)											
*Bold letters represent hydrophobic residues thought to be crucial for nuclear export												

13.3 Determination of Consensus Sequence for Nuclear Export Signals.

Since we observed a 58-fold increase in the nuclear-cytoplasmic ratio of topo IIα immunofluorescence in accelerated-plateau H929 cells treated with leptomycin B when compared to untreated cells, we hypothesized that topo IIα may contain a LMB sensitive nuclear export signal. Thus, we aligned several proteins with established nuclear export signals to determine a consensus sequence (table 10). We determined the consensus sequences as $HX_{1-4}HX_{2-3}HXH$ (where H represents the hydrophobic amino acids, leucine, isoleucine, valine, or phenylalanine). The hydrophobic amino acids are separated by a characteristic spacer region containing any amino acids (represented as the letter "X"). Using the consensus sequence we identified six matching nuclear export sequences in the amino acid sequence for human topo IIα (Table 11), which was downloaded from the National Center for Biotechnology Information database (accession number NP 001058). We initially expected to express exogenous topo IIα in human myeloma cells to investigate the nuclear cytoplasmic distribution between log and plateau cell-density.

13.4 Transfection of Topo IIα and Alternative Experiments.

For one year, I attempted to transfect a number of human myeloma and leukemia cell lines with a full-length topoisomerase IIα green fluorescent (GFP-topo IIα) fusion protein expression vector. The expression vector that I worked with was obtained from a laboratory that described a method for cloning the GFP-topo IIα gene that produced high levels of expression of the topo IIα-GFP chimera in the nucleus of human cells (Mo et al., 1998). During this time, I worked with the combined efforts of Dr. Terresita Muñoz and her laboratory personnel. However, we found that in most cases we were unable to

obtain a positive clone. The clones that were achieved often failed to grow exponentially, suggesting that the bacteria were resistant to the uptake of the large topo IIα gene. Thus, many of my initial experiments resulted in a low yield of plasmid with the topo IIα gene making subsequent transfection procedures unsuccessful. We used several different methods for transfecting human myeloma cell lines including cationic lipids and electroporation. We also attempted to express topo IIα-GFP under the regulation of an inducible promoter, but also had negative results. The most successful experiments demonstrated transient expression of the topo IIα-GFP chimera, but at very low transfection efficiency. For example, I tried to transfect human myeloma H929 cells, HL-60, and 8226 cells by electroporation. However, electroporation also produced negative results that included a high rate of cell death and low transfection efficiency. Of the cells that were transfected, I observed gross morphological changes in their appearance. In addition, translocation of GFP-topo IIα to the cytoplasm in plateau density cells was extremely limited when compared to endogenous topo IIα (data not shown). I found that expression of GFP-topo IIα recombinant protein was cytotoxic, inducing cell death in all cell lines tested (CCRF, H929, HeLa, HL-60, 8226, Flow fibroblast 2000) within 12-16 hours after transfection. This was a significant problem that impeded our ability to monitor the nuclear cytoplasmic trafficking of the topo IIα-GFP fusion protein because according to my accelerated-plateau cell model, the transfected cells would need to grow for 16-24 hours at a high-cell density.

Table 11. Putative Nuclear Export Signals in Human DNA Topoisomerase IIα

Topo IIα Amino Acids	**Consensus Sequence** $\mathbf{H}X_{1\text{-}4}\mathbf{H}X_{2\text{-}3}\mathbf{H}X\mathbf{H}$(X = Leu, Ile, Val, or Phe)												
80-90		G	**L***	Y	K		**I**	F	D	E	**I**	L	**V**
230-241		S	**L**	D	K	D	**I**	V	A	**L**	M	**V**	R
467-476		T	**L**	A			**V**	S	G	**L**	G	**V**	V
569-580		F	**L**	E	E	F	**I**	T	P	I	**V**	K	V
1017-1028		I	**L**	R	D		**F**	F	E	**L**	R	L	K
1054-1065	**F**	I	**L**	E	K		**I**	D	G	K	**I**	I	**I**

*Bold letters represent hydrophobic residues thought to be crucial for nuclear export

The lack of success with expressing exogenous topo IIα into human cells led us to look for an alternative method for defining a nuclear export signal. Different methods for defining a nuclear export signal have been described (Koster et al., 2005; Henderson and Eleftherion, 2000; Bogerd et al., 1996). We decided to crosslink the putative nuclear export signals found in topo IIα to bovine serum albumin (BSA), a large carrier molecule that is unable to be transported across the nucleus, and then microinject the NES-peptide BSA conjugates into the nucleus of cells. By labeling the NES-peptide BSA conjugates with a fluorescent molecule (ie FITC or TRIC) we were able to visualize the subcellular localization of the microinjected peptides. Nuclear export was observed when the NES-peptide was able to transport BSA from the nucleus to the cytoplasm. BSA is a suitable molecule for investigating nuclear-cytoplasmic transport pathways because it does not contain a functional NES. Therefore, putative nuclear export signals can be crosslinked to BSA to determine if they are sufficient to transport BSA across the nuclear membrane and into the cytoplasm. Although the objective of transfecting human myeloma cells with topo IIα was not abandoned, my specific efforts were redirected towards the microinjection experiments.

The six putative NES shown in table 11 were synthesized and their sequences confirmed as described in "materials and methods". The peptides were synthesized as native (nt) or mutated (Δ) sequences. The mutated peptides contained alanine in place of those hydrophobic residues suspected of being critical for Crm-1 mediated nuclear export. Crm-1 is a nuclear export receptor that recognized specific types of NES containing a characteristic spacing of hydrophobic amino acids. Although other export receptors are likely to exist, crm-1 is the only export receptor for NES containing proteins

that has been described to date. To facilitate conjugation with preactivated SMCC-BSA, the NES peptides were designed with a cysteine residue at the amino terminus. In this manner, SMCC-BSA forms a disulfide bond with the cysteine residue in the peptide sequences. We did an initial experiment to determine the optimal duration required to incubate the NES peptides with SMCC-BSA and the approximate quantity of total protein needed for gel electrophoresis. Figure 40 shows that after five minutes of reaction time, detection of NES peptides crosslinked with SMCC-BSA are noticeable as a shift in the apparent molecular weight of SMCC-BSA (compare lanes 4 and 5). We determined that 20-30 minutes are optimal for NES peptide crosslinking to SMCC-BSA. Proceeding one peptide at a time, the peptides were reacted with SMCC-BSA, and then the conjugated peptides were purified by size exclusion chromatography on a using a Pharmacia P500 FPLC system. As peptides passed through the LKB control Unit UV-1, the absorbance spectra was recorded. An example of the absorbance spectra obtained for peptide containing amino acids 80-90 is shown in figure 41. Three main peaks are evident and correspond with the size of the eluted fractions. The largest compounds elute first, whereas the smallest molecules become impeded within Superdex gel matrix beads and elute last. FPLC fractions 4-7 (Figure 41) are likely to be large SMCC-BSA molecules crosslinked with each other, fractions 16-18 represent conjugated BSA peptides, and fractions 30-32 represent unreacted peptide molecules. Peptide conjugation was confirmed by running FPLC eluted fractions on a 10% SDS-PAGE gel, and then visualizing the proteins by silver stain analysis (Figure 43 and 44). Similar conjugated BSA peptide fractions were pooled and concentrated before reacting with fluorescein isothiocyanate (FITC). FITC labeled BSA-peptides were again purified by gel filtration

chromatography (Figure 42) and a large single peak was noticeable on the absorbance spectra (data not shown). The type of cell to be microinjected was an additional experimental parameter that we had to determine before beginning our experiment. We initially wanted to microinject human myeloma or leukemia cell lines with the crosslinked peptide, but we ran into two problems. First, the myeloma and leukemia cell lines are suspension cells. To microinject suspension cells, they must be held in place by suction with one capillary while being pricked with a needle with the opposite hand. The human myeloma and leukemia cell lines proved to be too small, and instead became trapped inside the capillary (Figure 45). So, we tried using the adherent cell lines, HeLa and FlowFibroblasts2000. We found that pre-treating glass coverslips with HCl optimized the adhesion of HeLa cells to the coverslips. The HeLa cells spread out across the coverslips and gave us an easily identifiable nucleus to inject (Figure 45). Furthermore, microinjection of the HeLa cell directly onto the glass coverslips facilitated their visualization by fluorescence microscopy.

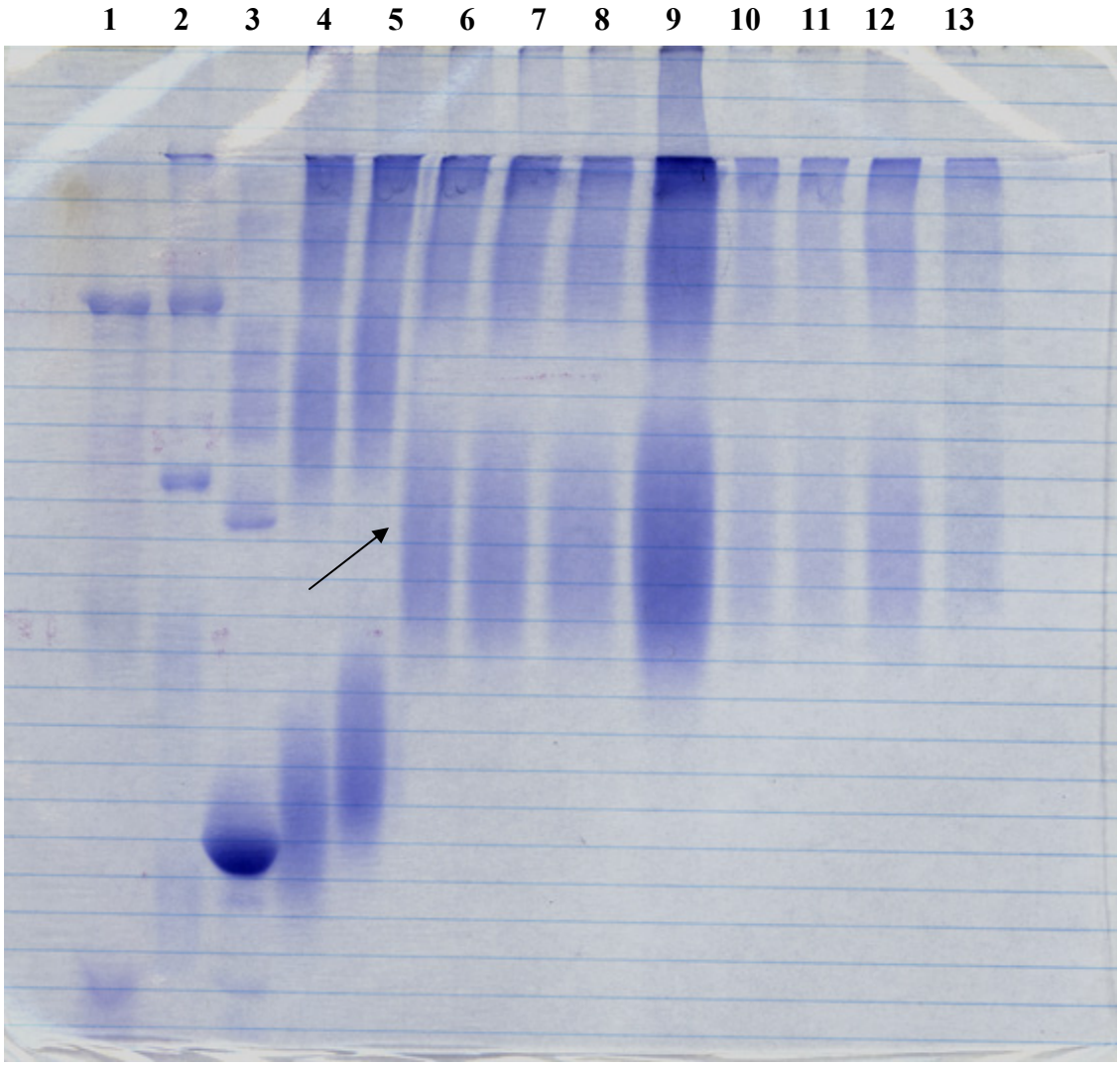


Figure 40. Coomassie Blue Stain of SDS PAGE gel with NES- peptide BSA Conjugates. Lanes 1 & 2 are molecular weight markers; lane 3, bovine serum albumin; lane 4, preactivated SMCC-BSA; lane 5, NES peptides incubated with SMCC-BSA for 5 minutes; lanes 6 and 7 are NES peptides incubated with SMCC-BSA for 20 minutes; lames 8-10 are NES-peptides conjugated with SMCC-BSA for 30 minutes; lanes 11-13 are NES peptides conjugated with SMCC-BSA for 1 h. Approximately 25µg of protein were loaded in lanes 4-8; 50 µg were loaded into lane 9, and 10 µg were loaded into lanes 10-13. Lane 4 represents free SMCC-BSA (lower smear) and SMCC-BSA crosslinked with itself (upper smear). Lane 5 shows that after 5 min, some peptide already began to crosslink with SMCC-BSA (compare lanes 4 and 5, notice upward shift). Arrow indicates conjugated peptides. We determined that 20-30 minutes was optimal for peptide conjugation to SMCC-BSA.

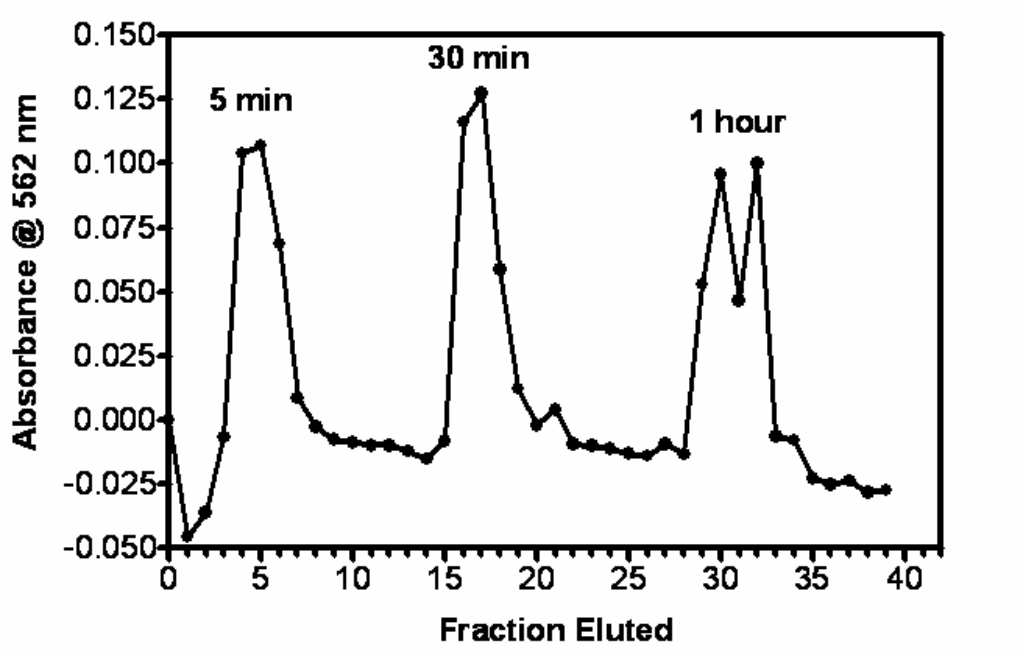


Figure 41. Absorbance Spectra of a BSA NES-peptide Conjugate at 562 nm. Three main peaks are evident and represent elution of different sized molecules. SMCC-BSA cross linked with itself is likely to be the largest complex to elute first (fractions 4-7) followed by BSA-peptide conjugates (fractions 16-18) and then unreacted peptides (fractions 30-34). An absorbance spectra was obtained for each BSA crosslinked peptide.

Figure 42. FITC-Labeled-BSA NES-peptide on Size Exlusion Coulmn. Arrows indicate the separation of FITC-conjugates from unreacted FITC.

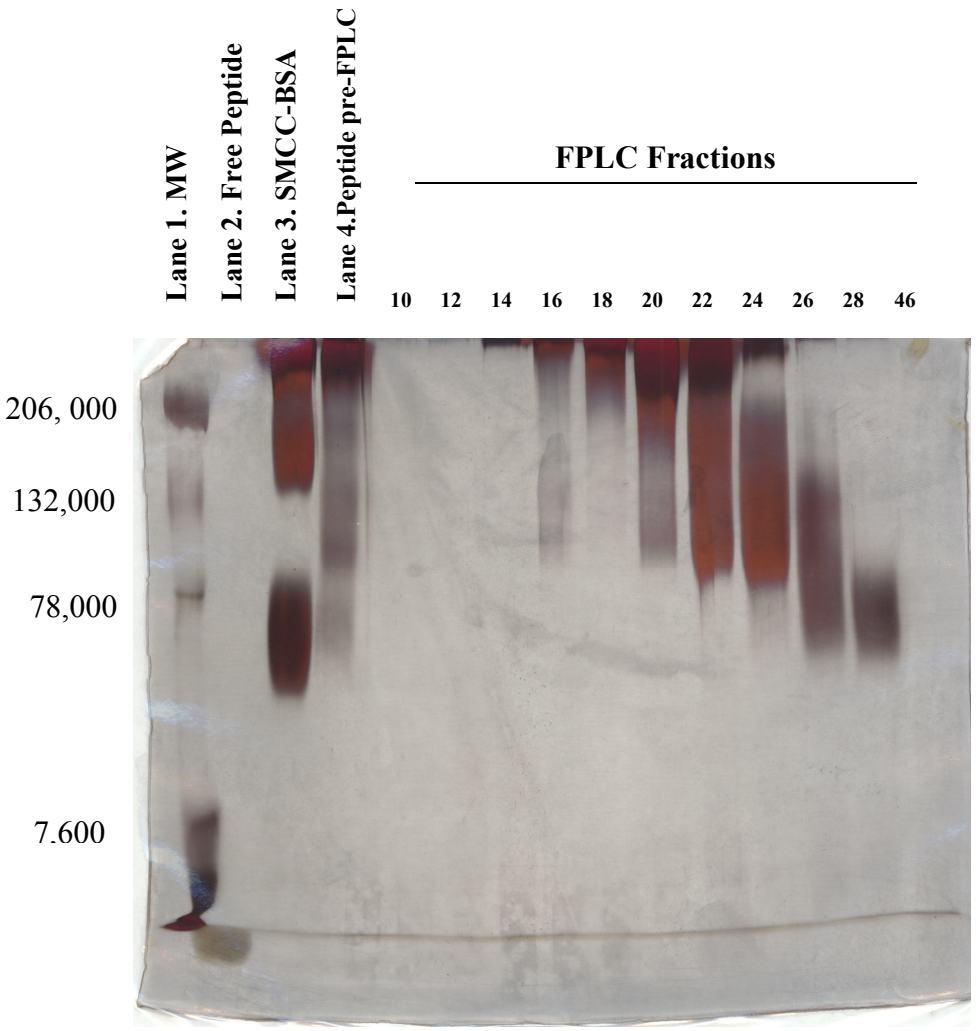


Figure 43. Silver-stain Analyses of SDS-Page BSA-NES-Peptide FPLC Fractions. FPLC Peptide fractions were collected and electrophoresed as described in Materials and Methods. Gels were stained with Silver staining. Lanes 20-28 show a shift in the apparent molecular weight of free peptide (lane 2) and free SMCC-BSA (lane 3). Lane 3 also shows that some SMCC-BSA crosslinks with itself.

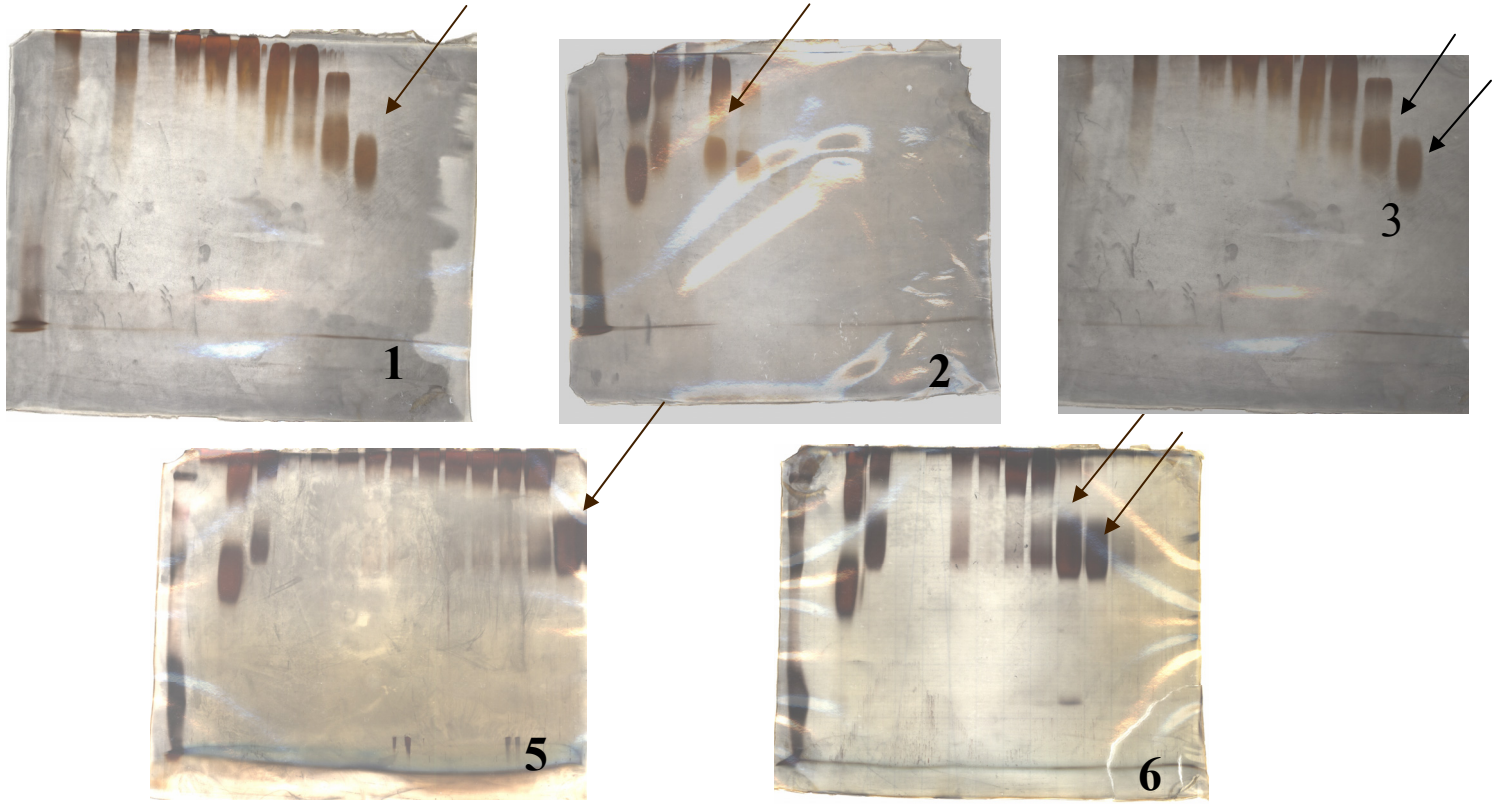


Figure 44. Silver Stain Analysis of each BSA Conjugated Peptide. SDS-PAGE gels were scanned into AdobePhoto Shop 6.0. (1) native peptide 80-91 (2) mutated peptide 80-91 (3) native peptide 230-241. Please note that mutated peptide 230-241 data not available because gel was shattered during the drying process. Since the native peptide did not export, SDS-PAGE was not repeated for the mutated peptide. (5) native pepide 569-580 (6) mutated peptide 569-580. Arrows indicate FPLC fractions containing SMCC-BSA crosslinked to peptides Figure 44 continued on next page.

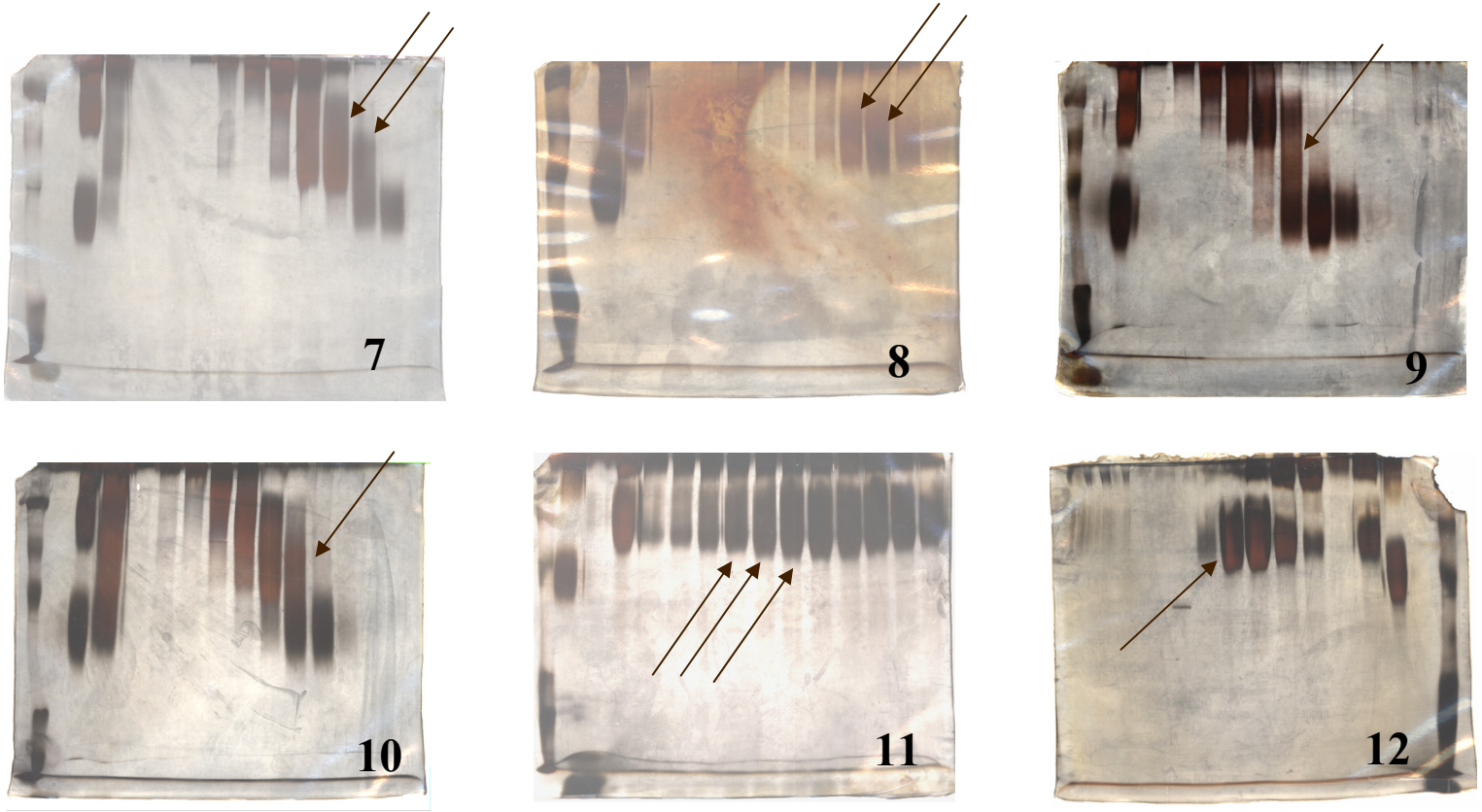


Figure 44 continued. (7) native peptide 569-580 (8) mutated peptide 569-580 (9) native peptide 1018-1024 (10) mutated peptide 1018-1024 (11) native peptide 1054-1065 (12) mutated peptide 1054-1065 Arrows indicate FPLC fractions containing SMCC-BSA crosslinked to peptides.

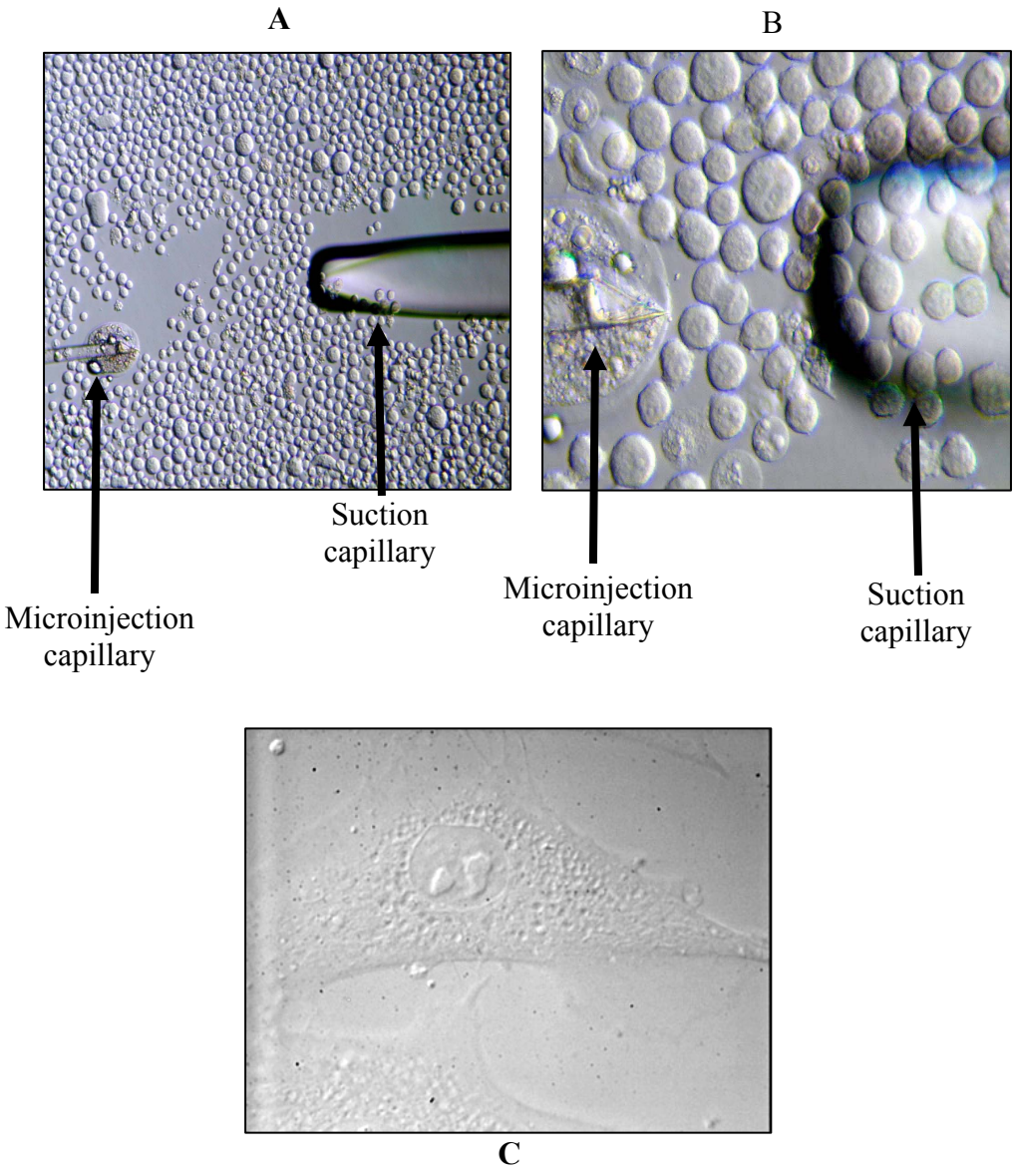


Figure 45. Comparison of H929 Cells with HeLa Cells for Microinjection. (A) H929 cells (low magnification) (B) H929 cells (High magnification) (C) HeLa cell. H929 cells are suspension cells, whereas the HeLa cells are adherent. The suction capillary is necessary to hold suspension cells in place while being microinjected.

13.5 Peptides $NES_{1054-1066}$ and $NES_{1017-1028}$ Signal the Nuclear Export of BSA-FITC.

The data suggest that topo IIα may be translocated from the nucleus to the cytoplasm under specific conditions, and this may result in altered drug sensitivity. Of the six putative NES identified in topo IIα, two peptides, $NES_{1054-1066}$ and $NES_{1017-1028,}$ exported BSA into the cytoplasm when microinjected into the nuclei of HeLa cells (Figures 46). BSA-$NES_{1054-1066}$ showed strong cytoplasmic staining and were seen in the cytoplasm within 15 minutes of microinjection, as compared to TRITC-BSA alone, (Figure 47), or the mutated BSA-$NES_{1054-1066}$ conjugate. BSA-$NES_{1017-1028}$ also appeared cytoplasmic within 15 min of being microinjected into the nucleus, but complete nuclear clearing (like the ntNES1054-1066) was not observed even after 90 min. The mutated BSA-$NES_{1054-1066}$ was nuclear in all cells observed even 90 min after microinjection. BSA-NES_{80-90} (Figure 47) mutated BSA-NES_{80-90} (Figure 47), BSA-$NES_{230-241}$, mutated BSA-$NES_{230-241,}$ and BSA-$NES_{467-476}$, BSA-$NES_{569-580}$, and mutated BSA-$NES_{569-580}$ all remained in the nucleus even 4 h after microinjection (data not shown).

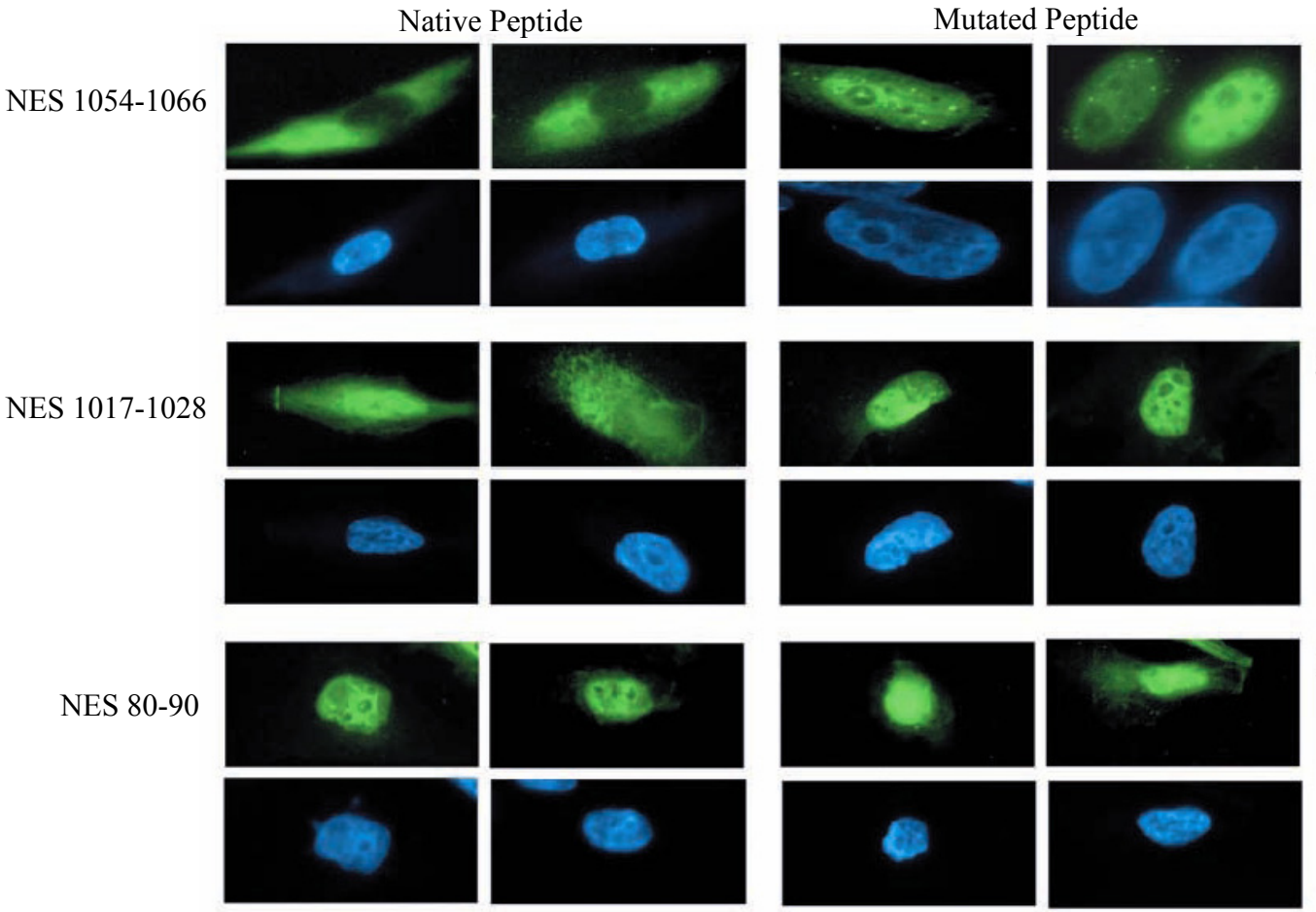


Figure 46. HeLa cells Microinjected with Peptide-BSA-FITC Conjugates. HeLa cells microinjected with either wild-type (left column) peptide-BSA-FITC conjugates (green), and then counterstained with DAPI (blue). A total of 20-50 cells were successfully microinjected per peptide and similar results were seen in all cells.

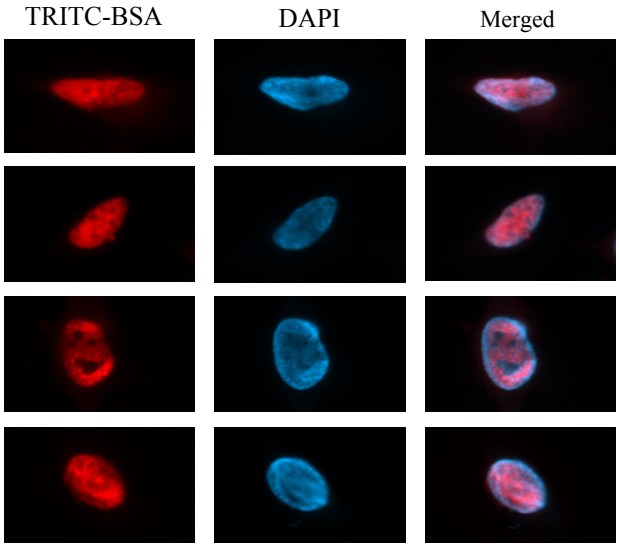


Figure 47. HeLa Cells Microinjected with TRITC-BSA. HeLa Cells were microinjected with BSA-TRITC in the nucleus and counterstained with DAPI.

13.6 LMB Blocks ntNES$_{1054-1066}$ and ntNES$_{1017-1028}$ Mediated Nuclear Export.

LMB is a specific inhibitor of Crm-1 mediated nuclear export of proteins. To determine if the nuclear export of BSA conjugated to peptides NES$_{1054-1066}$ and NES$_{1017-1028}$ was Crm-1 dependent, LMB pretreated HeLa cells were microinjected in the presence of 2 ng/ml LMB in ethanol. Fluorescence microscopy demonstrated that LMB blocked the export of BSA conjugated to peptides NES$_{1054-1066}$ and NES$_{1017-1028}$ (Figure 48). Peptide NES$_{1054-1066}$ had a strong perinuclear staining, suggesting that the protein cargo is docking at the NPC. Although the NES defined by microinjection are sufficient to transport a non-shuttling protein to the cytoplasm, these leucine rich sequences may or may not serve a role in exporting topo IIα.

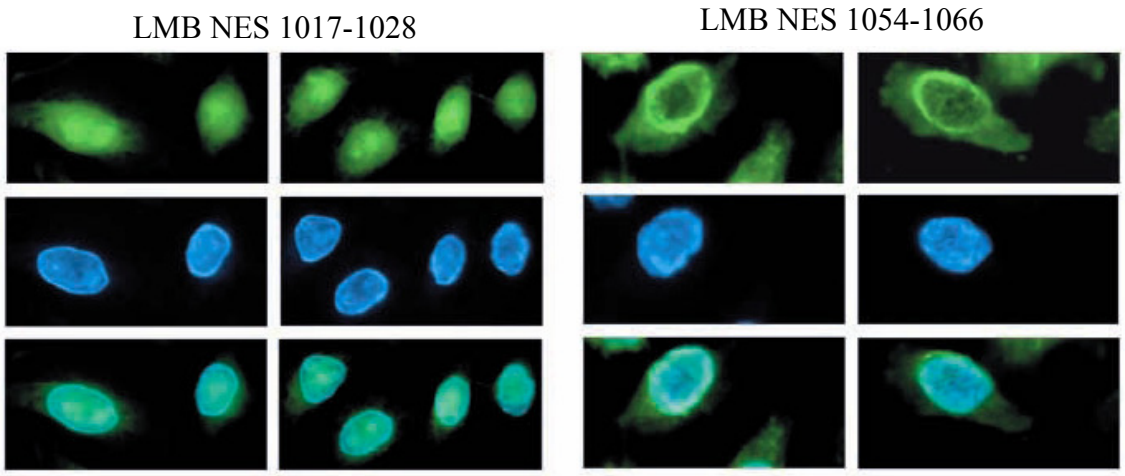


Figure 48. HeLa Cells were microinjected with wild-type peptide-BSA-FITC conjugates in the presence of 2 ng/ml LMB (leptomycin B).

13.7 Topoisomerase IIα Cloning, Site Directed Mutagenesis, and Gene Expression.

While obtaining results from the microinjection of the BSA-peptides, we began to obtain promising results with the transfection of a FLAG-epitope tagged topo IIα in HeLa cell lines. Although my focus in the laboratory was concerned with the microinjection of conjugated BSA-peptides, I had a vested interest in the project and maintained a significant involvement with the transfection studies. As such, I shared responsibility for certain experimental procedures such as, design of the experiments, selection of the mutated nucleotides (Table 12), and immunostaining of the FLAG-topo IIα chimeras. In the earlier stages of the work, I transformed bacteria and performed a number of plasmid preparations.

FLAG peptide is an eight amino acid protein (NYKNNNNK) that does not occur in nature. FLAG does not contain any putative nuclear export signal and its small size limits any secondary protein structure problems. Hela cells transfected with a FLAG-topo IIα plasmid vector expressed a full-length (170 kDa) topo IIα recombinant protein (Figure 49) Using a specific transfection protocol previously described by VandenHoff et al., 1992, we were able to transfect H929 and HL-60 cells by electroporation with a high degree of efficiency for these cell lines (2-20%). The success of the transfection experiments is attributed to the buffer described by Van den Hoff et al., 1992. The buffer contains ATP and glutathione to promote the rapid repair of cellular membranes.

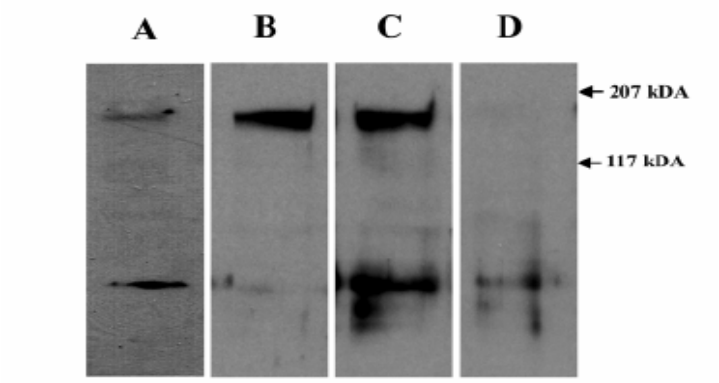


Figure 49. Western blot of full-length FLAG-topo IIα. HeLa cells were transfected with plasmid containing FLAG-topo IIα plasmid via cationic lipid, and harvested after 20 hours. Protein extracts from 2.0 x 10^6 cells per lane were separated on SDA-PAGE gel, blotted onto nitrocellulose and probed with FLAG M2 antibody. Lane A is protein from cells transfected with non-mutated FLAG-topo IIα plasmid, lane B is FLAG-topo IIα 1054-1066, lane C transfected with FLAG-topo IIα plasmid 1017-1028, and lane D is a non-transfected control.

13.8 FLAG-topoisomerase IIα Immunofluorescence.

BSA-peptide microinjection data indicated that the putative nuclear export sites at 1017-1028 and 1054-1066 may function to signal export of topo IIα. To confirm these data with a full- length topo IIα protein, H929 human myeloma cells were transfected with FLAG-topo IIα expression vectors possessing mutated hydrophobic residues in the nuclear export sites at 1017-1028 and 1054-1066 (Table 14). Twenty hours post-transfection, live cells were isolated by centrifugation on a ficoll-paque gradient and plated on glass microscope slides using cytospin funnels. After fixing and permeabilization, slides were stained with anti-FLAG M2 monoclonal antibody-FITC conjugate and counterstained with mounting media containing DAPI to show the location of the nuclei. Images were acquired using a fluorescent microscope (Figure 50), with quantitation of FITC fluorescence using Adobe Photoshop 7.0 (data expressed in Figure 51). Figure 50 establishes that the wild-type (non-mutated) FLAG-topo IIα protein is

present in the nucleus of the cells plated at log density, whereas FLAG-topo IIα protein is located in the cytoplasm in cells plated at plateau density. Quantitation of fluorescence revealed a statistically significant shift (P=0.00001) for log cells with a nuclear:cytoplasmic ratio of 5.9:1, to a ratio of 0.42:1 in plateau cells (Figure 51) when using the wild-type FLAG-topoIIα plasmid.

Table 12. Wild-type and Mutated Nucleotide Sequences of Putative Nuclear Export Signals.

Wild-type sequence 1017-1028														
TTG	GAT	ATT	CTA	AGA	GAC	**TTT**	TTT	GAA	**CTC**	AGA	CTT	AAA	TAT	TAT
L	D	I	L	R	D	**F**	F	E	**L**	R	L	K	Y	Y
Mutated sequence 1017-1028														
AGC	CTG	GAC	AAA	GAT	GCT	**GTT**	GCA	GCA	**ATG**	GTC	AGA	AGA	GCA	
	D	I	L	R	D	**A**	F	E	**A**	R	L	K	Y	Y
Wild-type sequence 1054-1065														
CGC	TTT	ATC	TTA	GAG	AAA	**ATA**	GAT	GGC	AAA	**ATA**	ATC	ATT	GAA	AAT
R	F	I	L	E	K	**I**	D	G	K	**I**	I	I	E	N
Mutated sequence 1054-1065														
CGC	TTT	ATC	TTA	GAG	AAA	**GCA**	GAT	GGC	AAA	**GCA**	ATC	ATT	GAA	AAT
R	F	I	L	E	K	**A**	D	G	K	**A**	I	I	E	N

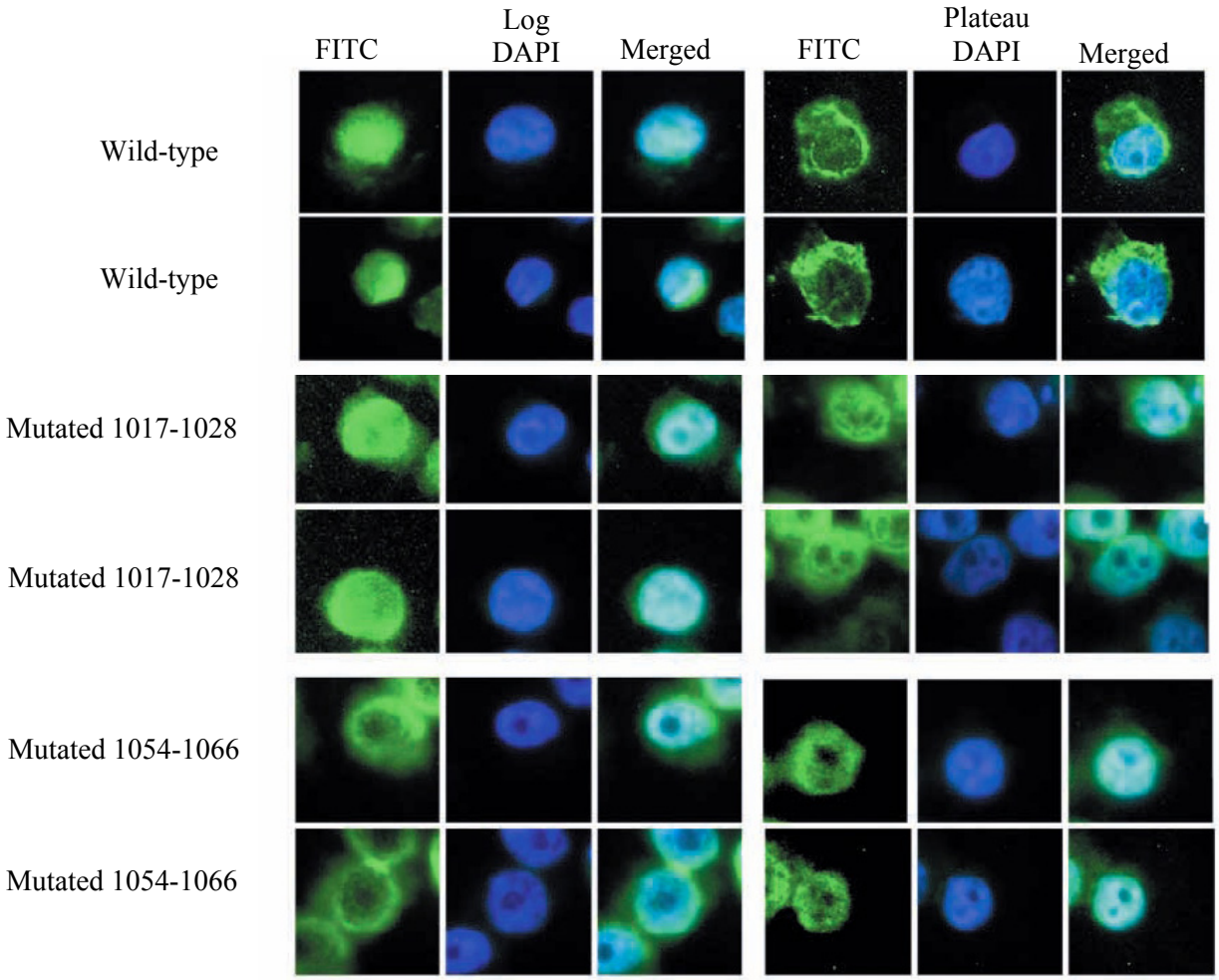


Figure 50. FLAG-topo IIα Immunofluorescence. Human multiple myeloma H929 cells were transfected by electroporation with full-length wild-type and mutated toto IIα and plated for 20 hours at log and plateau cell densities. Cytospins containing fixed cells were stained with FITC-labeled anti-FLAG M2 antibody (green), counterstained with DAPI for nuclear staining, and assayed by immunofluroescent microscopy..

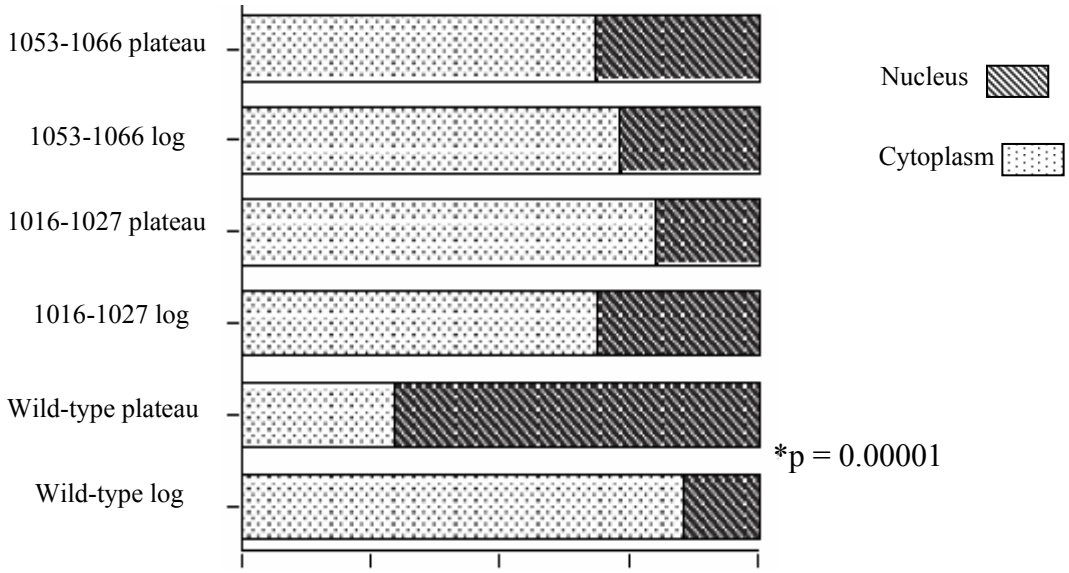


Figure 51. Quantitation of FLAG-topo IIα Immunofluoresence. Human myeloma H929 transfected cells (n = 20) stained with anti-FLAG M2 monoclonal antibody-FITC conjugate were assayed for nuclear and cytoplasmic immunofluorescence. Quantitation of FITC fluorescence was performed using Adobe Photshop 7.0 program. Wild-type FLAG-topo IIα was export to the cytoplasm in cells at plateau density (P = 0.00001), whereas topo IIα mutated at the putative export sites, 1016-1027 and 1053-1066, did not demonstrate statistically significant levels of export to the cytoplasm.

13.9 Peptide ntNES$_{1054\text{-}1066}$ and ntNES$_{1017\text{-}1028}$ are Conserved.

To determine if peptides NES$_{1054\text{-}1066}$ and NES$_{1017\text{-}1028}$ are conserved, a BLAST search of the SWISS PROT database was performed to identify homologous sequences in topo IIα. Tables 15 and 16 summarize a list of representative species containing homologous topo IIα sequences. The data show that the characteristic spacing of hydrophobic residues in peptides NES$_{1054\text{-}1066}$ and NES$_{1017\text{-}1028}$ are highly conserved in a broad range of species. For example, leucine residues appearing in human topo IIα NES are often substituted with the hydrophobic amino acids isoleucine or valine. Furthermore, Phe1054 and Ile1055 in peptide NES$_{1054\text{-}1066}$ are highly conserved from mammals to the most primitive eukaryotic organism, *Giardia lamblia*, unlike Leu1056. This suggests that the presence of phenylalanine and isoleucine are critical for nuclear export of this peptide, and thus an omission of these two hydrophobic amino acids from the peptide sequence could explain why a previous report failed to identify NES$_{1054\text{-}1066}$ as the dominant nuclear export signal (Mirski et al., 2003) .

Table 13. Sequence Alignment of DNA Topoisomerase IIα $NES_{1017\text{-}1028}$

Homo sapiens (human) $NES_{1017\text{-}1028}$	D	**I***	**L**	R	D	**F**	F	E	**L**	R	**L**	K
Sus scrofa (pig), *Mus musculus* (mouse), *Rattus norvegicus* (rat)	D	**I**	**L**	R	D	**F**	F	E	**L**	R	**L**	K
Cricetulus griseus (Chinese hamster)	D	**I**	**L**		D	**F**	F	E	**L**	R	**L**	K
Gallus gallus (chicken)	D	**I**	**L**			**F**	F	E	**L**	R	**L**	
Saccharomyces cerevisia (yeast)		**I**	**L**			**F**			**V**	R	**L**	
Aspergillus niger (fungus), *Penicillium citrinum* (fungus)	D	**I**	**L**			**F**	F		**L**	R	**L**	
Nicotiana tabacum (tobacco)	D	**I**	**L**			**F**			**V**	R	**L**	
Encephalitozoon cuniculi (protozoan)		**I**	**L**			**F**			**L**	R	**L**	
Bombyx mori (silkmoth)		**I**	**L**	R		**F**			**L**	R	**L**	

*Bold letters represent hydrophobic residues thought to be crucial for nuclear export.

Table 14. Sequence Alignment of DNA topo IIα $NES_{1054\text{-}1066}$

Homo sapiens (human) NES $_{1054\text{-}1066}$	**F**	**I**	L	E	K	**I**	D	G	K	**I**	I	**I**	E
Cricetulus griseus (Chinese hamster), *Mus musculus* (mouse), *Rattus norvegicus* (Rat)	**F**	**I**	L	E	K	**I**	D	G	K	**I**	I	**I**	E
Caenorhabditis elegans (nematode)	**F**	**I**	L	E	K	**I**	D	G	K	**I**	V	**I**	E
Saccharomyces cerevisiae (yeast)	**F**	**I**	L		K	**I**				**I**	V	**I**	E
Candida glabrata (yeast)	**F**	**I**			K	**I**				**L**		**I**	
Aspergillus candidus (fungus)	**F**	**I**			K	**I**				**L**	I	**I**	
Trichophyton rubrum (fungus)	**F**	**I**			K	**I**		G		**I**	V	**I**	
Giardia lamblia (protist)	**F**	**I**			K	**I**				**I**		**I**	
Bold letters represent hydrophobic residues thought to be crucial for nuclear export.													

13.10 $NES_{1054-1066}$ and $NES_{1017-1028}$ Reside within a Putative Coiled-coil Domain

We were interested in predicting the structural features of the region containing $NES_{1054-1066}$ and $NES_{1017-1028}$. Each topo II monomer can be divided into three domains, an N-terminal domain that contains the ATP-binding region, the central domain containing the active site tyrosine residue, and the C-terminal domain that contains the nuclear localization sequences. Both of the NES are situated upstream of the bipartite NLS (Figure 52) and downstream of the active-site tyrosine residue (Tyr805). Furthermore, several CK-2 phosphorylation sites downstream of both NES have been identified *in vitro* and could be important for regulating the subcellular localization of topo IIα. Although $NES_{1017-1028}$ alone is predicted to form an α-helix, we were interested in predicting the motif of the complete amino acid sequence stretching from $NES_{1017-1028}$ to $NES_{1054-1066}$. According to EMBOSS and Predict Protein, two programs designed to predict protein motifs, amino acids 1017-1066 are characterized by a high potential to form α -helices and also contain five 4-3 hydrophobic repeats, a typical feature of a coiled-coil motif. Such a repeating pattern of hydrophobic amino acids has been shown to form a hydrophobic core, which is critical for dimerization. In this manner, the hydrophobic amino acids are predicted to align on the same interface which facilitates DNA binding or protein-protein interactions. Interestingly, amino acids 1014-1057 in topo IIα have previously been shown to form a stable two-stranded α -helical coiled-coil in solution. Furthermore, the Advanced Chemistry Development ChemSketch 5.12 software was used to predict the 3-D structure of native and mutate peptides 1054-1066 and 1017-1028. The secondary structures of each peptide sequence was cleaned and then, visualized with the 3-D viewer. Although each mutated peptide differed from their

native peptides by just two amino acids, figure 53 establishes that each mutated peptide appears more globular than the native peptide. These results suggest that the native peptides may have more accessible binding sites available to bind CRM-1, whereas the mutated peptides are more compact with fewer extensions for CRM-1 binding. These results, however, are limited to the microinjection data and do not necessarily reflect the tertiary structure of full-length topo IIα in living cells. However, the results suggest that the larger amino acids such as isoleucine, leucine, and phenylalanine, have the potential to form extensions that would facilitate CRM-1 binding.

13.11 Conclusions.

In order for topoisomerase targeted chemotherapy to function, the topoisomerase target must have access to the nuclear DNA. Therefore, the nuclear export of topoisomerase IIα may contribute to drug resistance, and defining this mechanism may lead to methods to preclude this avenue of resistance. We have identified nuclear export signals for topo IIα at amino acids 101701028 and 1054-1066 using FITC-labeled BSA-export signal peptide conjugates microinjected into the nuclei of HeLa cells. Functional confirmation of both signals was provided by transfectin of human myeloma cells with plasmids containing the gene for a full-length human FLAG-topoiosmerase fusion protein. We futher showed that export by both signals was blocked by treatment of cells with leptomycin B, indicating that a CRM-1 dependent pathway mediates export.

MEVSPLQPVN ENMQVNKIKK NEDAKKRL**S**V ERIYQKKTQL EHILLRPDTY IGSVELVTQQ
MWVYDEDVGI NYREVTFVPG LYKIFDEILV NAADNKQRDP KMSCIRVTID PENNLISIWN
NGKGIPVVEH KVEKMYVPAL IFGQLLTSSN YDDDEKKVTG GRNGYGAKLC NIFSTKFTVE
TASREYKKMF KQTWMDNMGR AGEMELKPFN GEDYTCITFQ PDLSKFKMQS LDKDIVALMV
RRAYDIAGST KDVKVFLNGN KLPVKGFRSY VDMYLKDKLD ETGNSLKVIH EQVNHRWEVC
LTMSEKGFQQ ISFVNSIATS KGGRHVDYVA DQIVTKLVDV VKKKNKGGVA VKAHQVKNHM
WIFVNALIEN PTFDSQTKEN MTLQPKSFGS TCQLSEKFIK AAIGCGIVES ILNWVKFKAQ
VQLNKKCSAV KHNRIKGIPK LDDANDAGGR NSTECTLILT EGDSAKTLAV SGLGVVGRDK
YGVFPLRGKI LNVREASHKQ IMENAEINNI IKIVGLQYKK NYEDEDSLKT LRYGKIMIMT
DQDQDGSHIK GLLINFIHHN WPSLLRHRFL EEFITPIVKV SKNKQEMAFY SLPEFEEWKS
STPNHKKWKV KYYKGLGTST SKEAKEYFAD MKRHRIQFKY SGPEDDAAIS LAFSKKQIDD
RKEWLTNFME DRRQRKLLGL PEDYLYGQTT TYLTYNDFIN KELILFSNSD NERSIPSMVD
GLKPGQRKVL FTCFKRNDKR EVKVAQLAGS VAEMSSYHHG EMSLMMTIIN LAQNFVGSNN
LNLLQPIGQF GTRLHGGKDS ASPR**Y**IFTML SSLARLLFPP KDDHTLKFLY DDNQRVEPEW
YIPIIPMVLI NGAEGIGTGW SCKIPNFDVR EIVNNIRRLM DGEEPLPMLP SYKNFKGTIE
ELAPNQYVIS GEVAILNSTT IEISELPVRT WTQTYKEQVL EPMLNGTEKT PPLITDYREY
HTDTTVKFVV KMTEEKLAEA ERVGLHKVFK LQTSLTCNSM VLFDHVGCLK KYDTVLDILR
DFFELRLKYY GLRKEWLLGM LGAESAKLNN QARFILEKID GKIIIENKPK KELIKVLIQR
GYDSDPVKAW KEAQQKVPDE EENEESDNEK ETEK**S**DSVTD SGPTFNYLLD MPLWYLTKEK
KDELCRLRNE KEQELDTLKR KSPSDLWKED LATFIEELEA VEAKEKQDEQ VGLPGKGGKA
KGKKTQMAEV LP**S**PRGQRVI PRITIEMKAE AEKKNKKKIK NENTEG**S**PQE DGVELEGLKQ
RLEKKQKREP GTKTKKQTTL AFKPIKKGKK RNPWSDSESD RSSDESNFDV PPRETEPRRA
ATKTKFTMDL DSDEDFSDFD EK**T**DDEDFVP SDA**S**PPKTKT **S**PKLSNKELK PQKSVV**S**DLE
ADDVKGSVPL SS**S**PPATHFP DETEITNPVP KKNVTVKKTA AKSQSSTSTT GAKKRAAPKG
TKRDPALNSG VSQKPDPAKT KNRRKRKP**S**T SDD**S**DSNFEK IVSKAVTSKK SKGESDDFHM
DFDSAVAPRA KSVRAKKPIK YLEE**S**DEDDL

Figure 52.The Complete Amino Acid Sequence of Human DNA Topo IIα (accession number NP 001058). The active-site tyrosine resiude (805) is indicated (*). $NES_{1017-1028}$. ^{1017}DILRDFFELRLK^{1028} and $NES_{1054-1066}$ ^{1054}FILEKIDGKIIIE^{1066} are shaded; bipartite NLS is single underscored; Ser/Thr phosphorylation sites are **bold** and underscored ; the predicted coiled-coil region is double underscored; the active-site tyrosine residue is indicated with a star, (☆).

Wildtype sequence (1054-1066)
RFILEKIDGKIIIE

Mutant sequence (1054-1065)
RFILEK**A**DGK**A**IIE

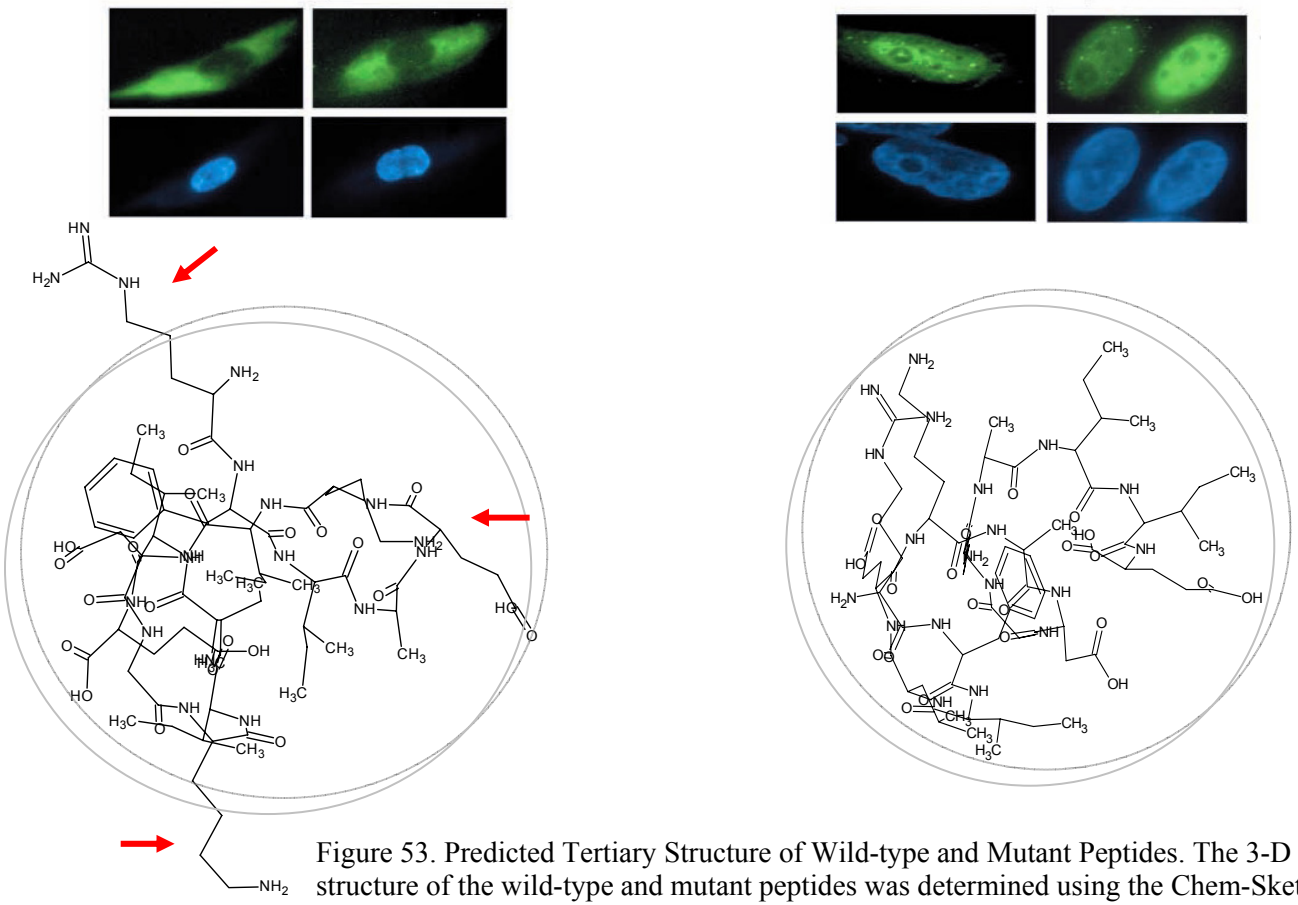


Figure 53. Predicted Tertiary Structure of Wild-type and Mutant Peptides. The 3-D structure of the wild-type and mutant peptides was determined using the Chem-Sketch 5.12 3-D viewer. Arrows indicated amino acids protruding at or away from the viewer. Figure 53 continue on next page.

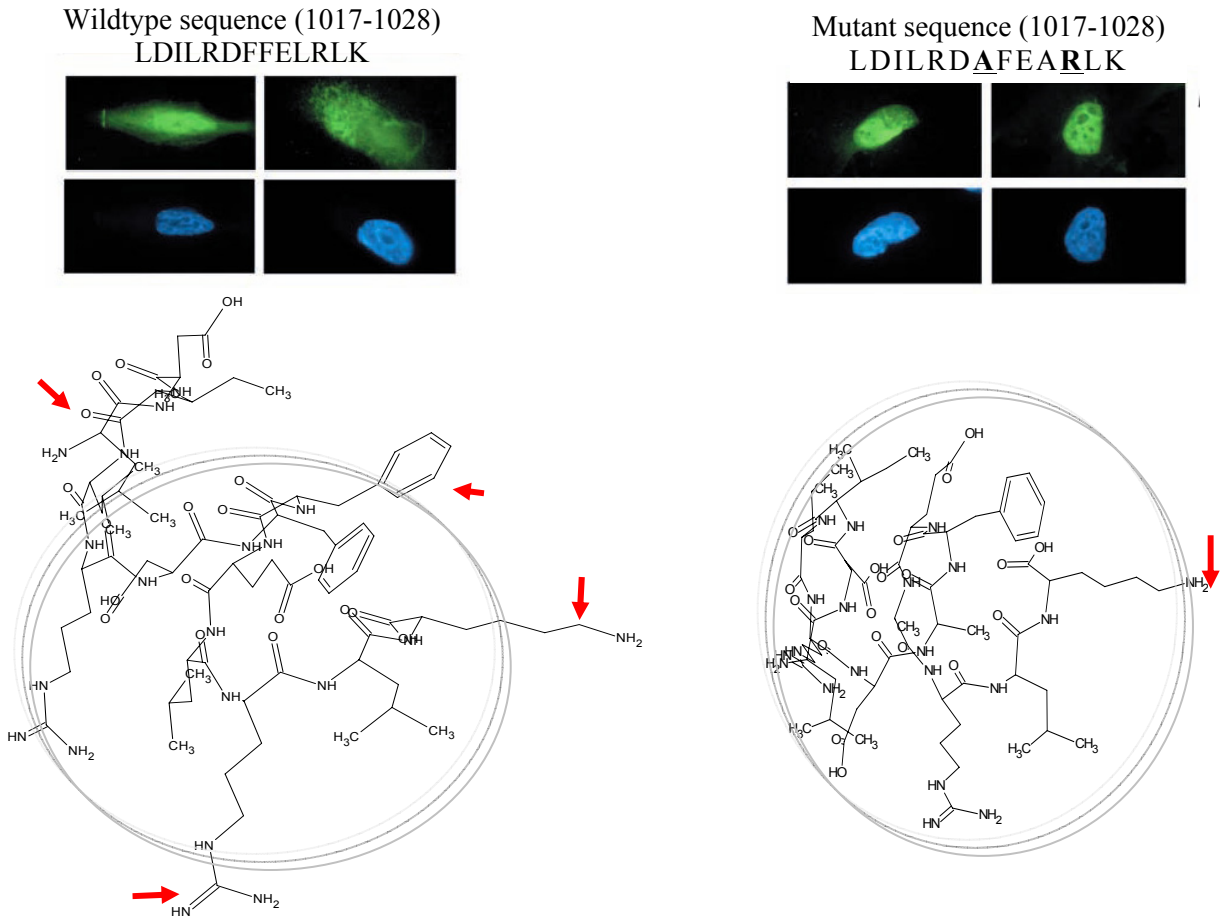


Figure 53. Continued. The 3-D structure of the wild-type and mutant peptides was determined using the Chem-Sketch 5.12 3-D viewer. Arrows indicated amino acids protruding at or away from the viewer.

Chapter Fourteen

Discussion

14.1 Previous Findings.

It is well established that most cell lines display a differential sensitivity to topo II inhibitors, which depends on cell density (Sullivan 1986, 1987; Chow and Ross, 1987, Markovits, 1987). When non-transformed cell-lines reach confluence there is a concomitant decrease in drug sensitivity to topo targeting cytotoxic agents that has been attributed to an attenuation in the amount of topo IIα content (Sullivan, 1987). However, in the tumor cell lines studied, confluence does not necessarily lead to a decrease in cellular topo II content, yet the cells are resistant (Sullivan 1986, 1987). We investigated several possible mechanisms of drug resistance in human myeloma and leukemia cell lines to explain the discrepancy in the cellular content of topo enzymes in transformed and non-transformed cell lines.

14.2 Log and Plateau Cell Lines Drug Sensitivity Phenotype.

Findings from this laboratory suggest that there are two general types of growth-dependent mechanisms of drug resistance that are unrelated to prior drug exposure. The first is demonstrated by non-transformed Chinese hamster ovary and human Flow 2000 fibroblasts cells lines which are 5-15 fold resistant to VP-16 in plateau-phase relative to log-phase cells (Table 7, page 122). This is due to an 80-90% reduction in total cellular

topo IIα protein (Figure 23, page 127), with a concomitant decrease in enzyme activity (data not shown). The second mechanism is seen in several hematological cell lines (mouse leukemia L1210, human myeloma RPMI-8226, human leukemia HL-60, human leukemia KG-1a, and human lymphoblastic leukemia CCRF cells), which show significant resistance to VP-16 at plateau phase (7.3-55-fold) (Table 7), but show no decrease in total cellular amounts of topo I, IIα, or IIβ protein when compared to log-phase cells (Figure 23).

Immunochemistry experiments demonstrated that in these cells, topo IIα is found in the nucleus of log-phase cells, but had a significant cytoplasmic distribution in plateau-phase cells (Figure 24, page 129). It appeared from this data that altered nucleocytoplasmic trafficking of topo IIα, but not topo I or topo IIβ, occurs in transformed cell lines at plateau-density. This was in contrast to other non-transformed cell lines that degrade topo IIα at plateau phase.

14.3 Novel Human Myeloma Cell Line Models.

We questioned whether the nuclear-cytoplasmic trafficking of topo IIα had a role in conferring altered drug sensitivity to topo targeting agents as well as cross-resistance to other non-topoisomerase associated cytotoxic agents. Alterations in the subcellular localization of topo enzymes have been previously associated with a decrease in drug sensitivity, but the weight of the data describe alterations in the amount of activity of drug resistant cell lines as a result of a truncated enzyme that has lost its C-terminal NLS (Wessel et al., 1997). There are two notable exceptions that correlate altered subcellular distribution of topo enzymes with altered drug sensitivity. First, multicellular spheroids

and Xenograft tumor cells are protected from etoposide-mediated DNA damage due to the cytoplasmic distribution of topo IIα (Oloumi et al., 2000). Secondly, adhesion of human myelomonocytoid U937 cells to fibronectin by means of β1 integrins protects cells against mitoxantrone and etoposide mediated DNA damage and is accompanied by an altered sub-nuclear relocalization of topo IIβ to the nucleolus. Thus, changes in the nuclear localization or binding properties of the nuclear pool of topo IIβ protein is suggested to have a role in cellular drug resistance to etoposide and mitoxantrone in these cells (Hazlehurst et al., 2001).

The lack of data to describe the subcellular distribution of topo IIα, especially the nuclear export of the enzyme, as a predictor of drug responsiveness could be attributed to the unavailability of *in vitro* cell models to study the nucleo-cytoplasmic distribution of topo enzymes, that would otherwise make it possible to thoroughly investigate the role of topo trafficking in drug sensitivity. Molecular analysis of the topo gene has been largely unachievable because overexpression of topo IIα is lethal to cells, as well as topo IIα gene knockouts. Most reports are successful at transfecting cells with truncated topo or topo peptides (Ernst et al., 2000). In either case, the exogenous full-length topo is not expressed in the nucleus because of a missing C-terminal NLS. These experiments fail to correlate a cytoplasmic distribution of topo IIα with drug resistance because endogenous topo is still present in the nucleus to cause drug-induced DNA damage. Furthermore, the expression of an exogenous cytoplasmic topo makes it ineffectual at studying the nuclear export of the protein (since it never enters the nucleus). In addition to these laboratory complications, it has proved very difficult to culture cells from a plasma cell dyscrasia, such as multiple myeloma. Much of our understanding of the biology of multiple

myeloma has been obtained by studying multiple myeloma derived cell lines. Unfortunately, human myeloma cell lines have proven difficult to transfect (refer to page 162).

We established an *in vitro* cell model system, referred to as accelerated-plateau, using established human myeloma cell-lines and overcame many of the experimental complications described above. Since log and plateau-phase cells represent low and high cell densities relative to one another, we postulated that cell-cell contacts may trigger a cytoplasmic relocation of topo enzymes. This model was used to describe the mechanism of growth dependent drug resistance described above (Table 9, page 135), and ultimately, to better characterize the mechanism of the nucleo-cytoplasmic trafficking of topo IIα (Figure 39, page 160).

We realize that the plateau density of cell cultures is far below the cell density existing in the tissues and biological fluids of patients. However, we consider that the changes that occur in the transition from the proliferative state in log culture to quiescent plateau densities are a reasonable model system to study the molecular mechanisms of acquiring drug resistance without a drug selection pressure.

14.4 Log and Accelerated-plateau Drug Sensitivity Phenotype.

Accelerated-plateau cells had a similar drug resistance phenotype as the natural-plateau cells described above, but the accelerated-plateau cells were also analyzed for cross-resistance to a range of drugs with different mechanisms of cytotoxicity (Figure 5 Table 11). The accelerated-plateau cells were 10-fold resistant to VP-16, but less than 1.5 fold resistant to Ara-c, taxol, BCNU, cis-platinum, and γ-irradiation. Similar results

were obtained in human myeloma RPMI-8226 cells. These data area not consistent with the multidrug resistance phenotype, but instead suggests that the observed drug resistance is specific for topo II interacting agents. Furthermore, drug resistance to DNA crosslinking agents, such as cisplatinum and γ-irradiation, has been found to be inversely proportional (Gornati, 1997; Barret et al., 1994) to topo II amount as a result of enhanced DNA repair processes by topo II. The drug resistance observed here, in the human myeloma cells, is not likely to be a result of enhanced DNA repair because there was minimal resistance to cisplatinum and γ-irradiation. Furthermore, cells were hypersensitive to both cisplatinum and γ-irradiation at 16 h, suggesting that there was a decrease in the total cellular content of topo II enzyme. However, Western blot analysis demonstrated minimal changes in the levels of topo protein after 16 hours, suggesting another mechanism exists for the observed hypersensitivity to the DNA crosslinking agents. The cells grown at plateau-phase for 24 h continued to be hypersensitive to cisplatinum but, there was a 4-fold decrease in sensitivity to γ-irradiation. Cell lines resistant to CDDP have not been found to be crossresistant to γ-irradiation, suggesting that there are different mechanism of resistance to γ-irradiation and cisplatinum (Caney et al., 2004). For example, there may be different DNA repair processes for radiation induced DNA damage as compared CDDP induced DNA damage (Caney et al., 1999; Wilkins et al., 1996). Furthermore, an association between the acetylation status of DNA histones and sensitivity to radiation has been previously reported (Zhang et al., 2004; Camphausen et al., 2004; Paoluzzi and Fig, 2004). This suggests that histones may be hypoacetlyated in plateau-phase myeloma cells as compared to the log-growing cells.

Exposure to an HDAC inhibitor could sensitize the plateau-cells to γ-irradiation, by maintaining the acetylation of histone proteins.

To elucidate other mechanisms conferring drug resistance to VP-16 and mitoxantrone, log and accelerated-plateau cells were examined for differences in cell cycle distribution, drug uptake, and subcellular distribution of topo protein. No considerable difference was observed in the uptake of [^{3}H-VP-16] between log and accelerated-plateau cells, suggesting that altered drug transport is unlikely to account for the observed drug resistance. Furthermore, cross-resistance to non-topoisomerase targeting agents would be expected, if plateau-phase human myeloma cells overexpressed MRP2 or MRP3 because both VP-16 and CDDP are substrates for MRP2 and MRP3. However, accelerated-plateau cells were not cross-resistant to CDDP. Plateau-phase cells were minimally resistant to the non-topoisomerase targeting agents, providing additional support of the results from the drug uptake studies. Since flow cytometric analysis indicate that there are minimal changes in the levels of topo protein and in the number of cells in S-phase after 16 hours respectively, these cellular events are also not likely to be the principle mechanisms of drug resistance.

14.5 Subcellular Distribution of Topo IIα.

Immunofluorescence microscopy demonstrated that topo IIα was nuclear in log-phase cells but had a cytoplasmic distribution in the accelerated-plateau cells at 16 h or 24 h. Cytoplasmic topo IIα was not a result of a C-terminally truncated protein that has lost the NLS, since there were single bands at the expected molecular weights for both topo IIα and topo IIβ by Western blot analysis. The cytoplasmic distribution of topo IIα

observed in the accelerated-plateau cell model was similar to that observed in the natural-plateau cells, but was observed in a shorter period of time (16 h for accelerated-plateau versus 4-5 days in natural-plateau cells). This suggests that the nuclear-cytoplasmic trafficking of topo IIα is a function of time and stimulated by cell-cell contact. The ratio of topo I and II β did not change over the same period of time. Topo I remained nuclear in log, plateau, and accelerated-plateau cells. Since Western blot analysis demonstrated a reduction of topo I content in the both 8226 and H929 accelerated-plateau cells (16-34% reduction; Figure 27, page 138), this suggests that topo I may be degraded by nuclear proteasomes or that its degradation in the cytoplasm is rapid, and thus its cytoplasmic location is below the threshold of immunofluorescence detection. Topo IIβ was distributed approximately equally between the nuclear and cytoplasmic compartments in log and plateau cells. Therefore, the subcellular distribution of topo I or topo IIβ did not correlate with drug responsiveness. Rather, it appeared that an alteration in the subcellular localization of topo IIα could be the principle mechanism contributing to the decrease in drug sensitivity to topo II inhibitors.

While it may appear unusual that DNA replication continues despite having a significant proportion of topo IIα in the cytoplasm, cells can survive with a predominantly cytoplasmic form of a catalytically active enzyme, which exists in several drug resistant cell lines. For example, in the drug resistant human small cell lung carcinoma H69-VP cell line, a catalytically active yet truncated topo IIα enzyme is located in the cytoplasm while topo IIβ is expressed in the nucleus (Grue et al., 1998). In these cells, chromosomal formation is dependent on cytosolic topo IIα entering the chromatin during mitosis when the nuclear membrane disappears rather than nuclear topo

IIβ performing the mitotic functions of topo IIα (Grue et al., 1998). Similar findings have been reported in drug resistant Chinese Hamster V511 cells that show minor alterations in cell growth despite having drastic reductions in the total amount topo IIα protein in both log growth and quiescence (Hashimoto et al., 1995). The expression of topo IIβ remained constant and topo IIβ was presumed to compensate for topo IIα activity in the V511 cells. This is supported by recent findings of HeLa cells transiently transfected with topo IIα or topo IIβ siRNA, which demonstrated that topo IIβ can partially substitute for topo IIα condensation and segregation in HeLa cells (Sakaguchi and Kikuchi, 2004). These findings could explain why there was only a minimal change in S-phase population of cells, but not a complete cessation of DNA replication. Western blot analysis of topo IIα protein in the nuclear and cytoplasmic compartments of log and accelerated-plateau H929 cells indicates that topo IIα was reduced by 25-50% (by densitometry) compared to the log cells (Figure 32, page 146). Part of the topo IIα content appears to be degraded in both the nuclear and cytoplasmic compartments and was attributed to experimental artifacts. Topo IIα degradation was likely to occur during the shearing of the nucleus from the cytoplasm as a similar degradation does not occur in the whole cells. Results of the comet assay confirmed that seeding cells at plateau density for 16 hours protects them from etoposide induced DNA damage and additional experiments demonstrated that etoposide treatment resulted in less DNA damage because there are fewer protein-drug-DNA complexes in the cells seeded at plateau density than log-phase cells. The data establishes that the nuclear-cytoplasmic trafficking of topo IIα mediates cellular drug resistance to topo inhibitors by reducing the amount of topo II in the nucleus and thus, reduces the number of enzyme-drug-DNA complexes formed. A redistribution of a

population of topo IIα to the cytoplasmic compartment would have a similar effect as to drug sensitivity as an overall decrease in topo IIα content, by decreasing the amount of drug target in the nucleus. Furthermore, these results may partially explain the hypersensitivity of accelerated-plateau cells to CDDP and γ-irradiation observed at 16 h. As previously mentioned, drug sensitivity to DNA crosslinking agents is inversely proportional to topo protein levels (Gornati, 1997; Barret et al., 1994). Although, the total cellular amount of topo IIα is not reduced, a shift from the nucleus to the cytoplasm could account for the observed drug sensitivity.

Several mechanisms of drug resistance to topo inhibitors have been defined *in vitro* and include: (1) over expression of drug transport proteins (P-glycoprotein, multidrug resistance-associated protein, lung resistance-related protein, or breast cancer resistance protein), that result in either increased drug efflux or decreased drug uptake (Shustik et al., 1995), (2) a cell-cycle-dependent decrease in the amount of topo II protein (Sullivan et al., 1986, 1987), (3) the expression of a truncated protein that has lost its C-terminal nuclear localization signal such that it remains in the cytoplasm (Wessel et al., 1997), and (4) mutations in the topo gene that result in an enzyme with altered catalytic or cleavage activity (Matsumoto et al., 2001). The data presented here demonstrates a fifth mechanism of drug resistance *in vitro*: (5) nucleocytoplasmic-trafficking of topo IIα is a mechanism of *de novo* drug resistance to topo inhibitors in human multiple myeloma cell lines (Engel and Turner et al., 2004; Engel et al., 2004; Valkov et al., 2000).

14.6 Clinical Relevance.

The data establishes that a measurable difference in the nuclear and cytoplasmic distribution of topo IIα, a chief molecular target of commonly used anti-cancer therapy, occurs in human myeloma cell-lines at plateau densities. The nuclear content of topo IIα is critical for topo targeting agents to be effective as cytotoxic agents because topo cleavage activity is necessary for drug-induced DNA damage to occur. The data presented here indicate that a cytoplasmic pool of topo IIα correlates with cellular drug resistance to etoposide (Engel et al., 2004; Valkov et al., 2000). This is also supported by evidence that multicell spheroids and xenograft tumor cells are protected from etoposide-mediated DNA damage due to the cytoplasmic distribution of topo IIα (Oloumi et al., 2000). These findings have potential clinical implications in the treatment of human myeloma as immunohistochemistry demonstrated that topo IIα has a cytoplasmic distribution in the malignant plasma cells obtained from the bone marrow aspirates of some multiple myeloma patients (Figure 36 and 38).

The magnitude of cytoplasmic immunofluorescent staining of topo IIα was significantly more pronounced in the malignant plasma cells found in the clinical preparation than that observed in the human myeloma cells lines at plateau density. Many of the tumor cells demonstrated complete nuclear clearing of topo IIα that was less frequently observed in the cell lines. The plateau and accelerated-plateau cell models showed a shift (decrease) in the nuclear: cytoplasmic ratio of topo IIα immunofluorescence in most cells, rather than complete nuclear clearing (ratios below 1.0). The data suggest that other physiological conditions besides cell-cell contact are involved in topo IIα trafficking that exist in the tumor microenvironment of the bone

marrow. For example, the cell line models demonstrate a cytoplasmic distribution of topo IIα *de novo*, but the cytoplasmic distribution observed in the clinical preparations could be influenced by prior exposure to DNA damaging agents. Multiple myeloma is characterized by karyotypic instability and many chromosomal aberrations are acquired after exposure to DNA damaging agents (refer to Appendix C on page 269 for a summary of hematological related chromosomal aberrations). The amount of chromosomal aberrations correlates with disease stage and prognosis. It is possible that certain chromosomes are susceptible to genetic translocations. For example, many chromosomal gene translocations that are observed in hematological malignancies involve the nucleoporins (Nup) (Arai et al., 2000, 1997; Nakamura et al., 1996; Boer et al., 1998). I have summarized a list of gene mutations that have been described in nucleoporins (refer to Table 8 on page 217 and all reference contained therein). Thus, exposure to various chemotherapeutic agents could somehow disrupt the homeostasis of nucleo-cytoplasmic protein trafficking. This is supported by evidence that multiple gene aberrations in Nup98 occur in some hematological disorders. For example, Nup98 gene translocations have been reported in treatment related AML and myelodysplastic syndrome (MDS). Also, the chromosomal translocation t(11;20)(p15;q11) results in a Nup98/Topo I fusion protein in *de novo* acute myeloid leukemia (AML) (Potenza et al., 2004; Iwase et al., 2003; Chen et al., 2003). These observations establish that a significant modulation occurs in topo I, which could theoretically alter topo enzyme stability, enzyme activity, and subcellular distribution. Similar chromosomal translocations involving topo II have not been found. Additional studies to analyze the subcellular distribution of topo II in

untreated patients compared to treated or relapsed patients combined with cytogenetic analysis are needed

14.7 Topoisomerase IIβ as an Alternative Molecular Target in Human Myeloma.

Another interpretation of the data is that topo IIβ could be an alternative molecular target in the treatment of human multiple myeloma. It is well documented that topo IIβ protein expression is rather constant over the cell cycle and less influenced by cell-cycle changes than topo IIα. Western blot analysis demonstrated no reduction in the total cellular content of topo IIβ in plateau-cells or accelerated-plateau phase cells when compared to log-phase cells. Topo IIβ was detectable in both the nuclear and cytoplasmic compartments of the plasma cell in clinical samples as well as, in the human myeloma cells lines. However, there was no measurable difference or shift in the amount of topo IIβ immunofluorescence detected in the cytoplasm at plateau density as compared to log density. This suggests that the balance of topo IIβ nuclear import is influenced in the same manner or by cell-cell contact as observed with topo IIα. Recently, microinjection studies identified an NES in topo IIβ (Mirski et al., 2003) and the data presented here demonstrates a possible shuttling of topo IIβ between the nucleus and cytoplasm. A determination of whether leptomycin B can shift the nuclear:cytoplasmic ratio of IIβ toward to the nucleus in plateau-phase cells could address the mechanism of topo IIβ trafficking. Nevertheless, the data suggests the factors resulting in cytoplasmic distribution of topo IIα in tumor cells do not alter topo IIβ net trafficking in the same manner.

Several new cytotoxic agents that are specific for topo IIβ are being investigated for the effectiveness in the treatment of solid tumors that are refractory to topo II containing therapy (Hazeldine et al., 2005, 2002; Barthelmes et al., 2001; Gao et al., 1999) Many of these drugs are still in development and are derivatives of XK469, a topoisomerase IIβ selective poison that is reaching phase I clinical trials (Gatto and Leo, 2003). Tumors with large populations of cells in the G_0/G_1 phase are believed to be more sensitive to topo IIβ specific inhibitors because, unlike topo IIα, topo IIβ protein expression is not cell-cycle dependent. Thus, the low proliferative index of malignant plasma cells observed in multiple myeloma makes topo IIβ an attractive molecular target. Furthermore, XK469 results in upregulation of the topo IIα protein (Mensah-Osman et al., 2002). Based on these findings a plausible regimen for multiple myeloma patients would contain a topo IIβ specific cytotoxic agent (ex. XK469) combined with a nuclear export inhibitor (leptomycin B) followed by a topo II targeting cytotoxic agent (i.e..., etoposide). Theoretically, the topo IIβ specific cytotoxic agent would induce cell death via topo IIβ activity while also elevating the cellular content of topo IIα protein. A nuclear export inhibitor would be necessary to inhibit the export of topo IIα from the nucleus and thus, provide a molecular target in the nucleus for etoposide mediated DNA damage to occur. This sequence could only be effective if topo IIα activity and levels remained sufficient to induce DNA damage. Inhibitors of the proteasome represent a new class of cytotoxic agents with promising results in the treatment of hematological malignancies. A proteasome inhibitor may be useful for maintaining the total cellular content of topo protein.

Sequestering proteins in the nucleus is gaining acceptance as a potential mechanism for inducing apoptosis in cancer cells (Yashiroda and Yoshida, 2003). One recent study treated human chronic myelogenous leukemia cells with a tyrosine kinase inhibitor (STI571) to induce the nuclear import of BCR-Abl, a chimeric oncoprotein that activates mitogenic and anti-apoptotic cell signaling pathways when it is in the cytoplasm of cells (Vigneri and Wang, 2001). The cells were then exposed to leptomycin B to block the export of BCR-Abl into the cytoplasm, thus trapping the protein in the nucleus. A general weakness of this drug combination is that leptomycin B inhibits all CRM-1 mediated nuclear protein trafficking, but *in vitro* the drug combination selectively killed transformed cells expressing BCR-Abl chimera. Furthermore, a phase I clinical trial of LMB demonstrated that the drug caused profound anorexia and malaise in patients (Newlands et al., 1996). The clinical trials with LMB were discontinued but, the discovery that CRM is the molecular target of LMB instigated new efforts to reevaluate LMB for potential use in chemotherapy. Safer leptomycin B analogues are currently being developed and investigated for their antitumor activity (Kau and Silver, 2003). Further investigations into the mechanisms of topo trafficking are needed to develop drugs specific for topo that may be less toxic in humans.

14.8 Nuclear Content of Topo IIα is a Determinate of Topo II Drug Cytotoxicity.

There has been one report to describe the role of the nuclear-cytoplasmic trafficking of topo IIα in *de no*vo drug resistance (Oloumi et al., 2000). The weight of the data has been limited to measuring the total cellular content of topo protein found either in drug-resistant cell lines (Mirski et al., 2000' Davis et al., 1998; Kusumoto et al.,

1996; Chen and Beck, 1995;) or in clinical specimens (Bauman et al., 1997). Traditional practice has relied heavily on *in vivo* topo quantification as a prognostic indicator (Schrader et al., 2004; Lohri et al., 1997). As such, there have been numerous reports to describe a correlation between topo protein expression with a positive response to topo inhibitors (Lohri et al., 1997; Kaufmann, et al., 1994). However, there is a growing awareness in both scientific and clinical forums that monitoring the subcellular distribution of topo is also important when predicting drug responsiveness (Oloumi et al., 1993). This is supported by several reports unable to explain alterations in drug sensitivity to topo targeting agents with alterations in either topo mRNA, topo protein expression or activity, or P-gp expression (Yamazaki et al., 1997; Davis et al., 1998; Yamazaki et al., 1997; Boege et al., 1993). For example, eight breast epithelial cell lines, including six derived from breast cancers, were analyzed for mechanisms of drug resistance to camptothecin and etoposide (Davis et al., 1998). Drug resistance to camptothecin was attributed to low levels of topoisomerase I and activity. However, topo II levels and activity did not correlate with drug sensitivity or resistance to etoposide. These cells were analyzed for alterations in S-phase, doubling time, expression of mdr-1, p53, Bcl-2 and Bax proteins, but none of these parameters correlated with drug resistance to etoposide (Davis et al., 1998). Only one cell line (BT47 cells) exhibited a high bcl-2/Bax ratio and mutant p53 protein that was believed to contribute to decreased sensitivity to etoposide. The BT47 cells also displayed slow growth characteristics similar to that observed in the human myeloma cell line models, but the subcellular distribution of topo enzyme was not investigated in any of the cells explored. A shift toward the cytoplasm in the nucleo-cytoplasmic shuttling of topo II could have similar

effects as an overall decrease in protein levels, by decreasing the amount of active enzyme in the nucleus. A similar study was performed that analyzed 14 unselected human lung cancer cell lines, including small-cell lung cancer and non-small cell lung cancer, for drug sensitivity to doxorubicin and etoposide, two topo II targeting agents (Yamazaki et al., 1997). There was variation in drug sensitivity to etoposide and doxorubicin in all cell lines, but neither topo IIα content nor enzyme activity correlated with observed drug resistance. Other cellular parameters were not investigated, including the subcellular distribution of topo IIα. This could be significant because a shift in the subcellular distribution of topo IIα would not be detectable by Western blot of topo II protein in whole cells. Furthermore, the observation of cytoplasmic topo IIα in patient samples described here suggests that there is a clinical role for the nucleo-cytoplasmic trafficking of topo that could explain *de novo* drug resistance previously described in other tumor types. Thus, examining the nuclear and cytoplasmic content of topo protein by immunocytochemistry, especially topo IIα, is an important factor to consider when attempting to explain cellular drug resistance in the laboratory and clinical preparations.

14.9 Possible Roles of Cytoplasmic Topo II.

One of the potential consequences of exporting a pool of topo IIα to the cytoplasm is a decrease in sensitivity to topo inhibitors. This could result from cytoplasmic topo IIα serving as a drug sink by trapping VP-16 in this compartment. This is supported by data demonstrating that the binding of VP-16 to topo II can occur in the absence of DNA (Burden et al., 1996). For this to occur, the amount of drug binding to

topo IIα would need to be sufficient to result in a decrease in drug- induced DNA damage.

One question that has not been addressed is whether topo IIα is recruited to the cytoplasm to perform a specific function. If topo II were acting on cytoplasmic substrates, there are at least two theoretical possibilities. First, topo II could be involved in maintaining the secondary structure of RNA or be involved in splicing RNA. To date, there has been no direct evidence of topo II binding RNA. A second possibility is that topo IIα could be recruited to the cytoplasm as an early apoptotic event. I have hypothesized that a link exists between the calcium/calmodulin pathway and nuclear-cytoplasmic trafficking of topoisomerase IIα during apoptosis in chapter 15, page 220..

14.10 Topoisomerase IIα Contains Two Functional Nuclear Export Signals.

One question that we addressed was whether the cytoplasmic distribution of topo IIα was a result of increased nuclear export or decreased nuclear import. Identification of the direction and mechanism of transport across the nuclear membrane could lead to a better understanding of how to regulate the levels of active enzyme in the nucleus and thus, sensitize cells to topo inhibitors. Human topo I (Mo et al., 2000), IIα (Mirski, et al., 1999), and IIβ (Mirski, et al., 1999) have NLS that target them into the nucleus, but only recently has the mechanism of nuclear-export of these proteins been investigated (Mirski et al., 2003). Sumoylation followed by redistribution of topo I from the nucleoli to the nucleoplasm in response to topotecan or camptothecin exposure has been reported (Mo et al., 2002; Rallabhandi et al., 2002). The lack of data describing the shuttling of topo enzymes may be because topo is usually reported to occur in the nucleus of cells and a

cytoplasmic distribution of topo IIα has usually been ascribed to the expression of a truncated protein that has lost its C-terminal NLS (Mirski, et al., 2000). However, proteins that appear predominately nuclear may still shuttle between the nucleus and the cytoplasm if the rate of nuclear import is greater than the rate of nuclear export. Thus, demonstrating that a protein shuttles between the nucleus and cytoplasm requires defining the specific conditions that will shift the steady state kinetics toward nuclear export (Table 15). Many conditions have been shown *in vitro* to alter the shuttling of proteins between the nucleus and cytoplasm, including changes in the cell-cycle and oxidative stress (Miyamoto et al., 2004; Kodiha et al., 2004; Greber and Gerace, 1995). I have created a list of molecular and biochemical agents that have been demonstrated to alter specific transport pathways for different proteins. This table demonstrates that certain cellular conditions or exogenous agents can induce a shift in the nuclear-cytoplasmic trafficking of specific proteins. In the accelerated-plateau cell model used in these experiments, it is likely that intensive cell-cell contact initiates a signal that induces the export of topo IIα to the cytoplasm. This is supported by the findings of others that a cytoplasmic distribution of topo IIα occurs in the outer-proliferating cells of multicell spheroids in Xenograft tumors when compared to monolayers formed by the same cells (Oloumi et al., 2000).

Table 15. Inhibitors of the Nuclear-cytoplasmic Transport Pathway

Biochemical Agent or MolecularFactor	Target	Mechanism
Wheat germ aggultinin (Duverger et al., 1995)	N-Acetylglucosamine moieties expressed on nucleoporins	Non-specifically blocks protein import by binding sugar moieties on nucleoporins
Anti-FG repeat antibodies (Snow et al., 1987)	Importins/Nucleoporins	Blocks importins from docking to the NPC
Trichostatin A (Chen et al., 2001)	NF-kB	Blocks NF-kB import
Cyclosporin A (Liu et al., 1991)	Cyclophilin, NFAT	Blocks NFAT import; cyclosporin-cyclophilin complex binds calcinuerin and inhibits dephosphorylation of NFAT
3, 5–Bistrifluoromethyl pyrazoles (Trevillyan et al., 2001)	NFAT	Blocks NFAT import independently of calcinuerin
Leptomycin B (Wolff et al., 1997) **Myxobacterial cytotoxins (Ratjadones)** (Koster et al., 2003)	Chromosome region maintenance protein 1 (Crm-1)	Blocks CRM1 mediated nuclear export
Arylene bis(methylketone) compounds (Dubrovsky et al., 1995)	Pre-integration complex (PIC)	Blocks import of HIV viral genome
Arginine-rich RNA molecules (ARM) (Fineberg et al., 2003)	HIV-1 Rev	BSA molecules with ARM peptides block HIV-1 Rev protein import
Transfected importin-β binding domain (Kutay et al., 1997); **importin-β P446L mutant protein** (Timinszky et al., 2002)	Importin-β	Dominant negative competitive inhibitor of importin α/β mediated import
Cellular Stress: UV radiation Oxidative stress Heat shock (Miyamoto et al., 2004; Kodiha et al., 2004)	Ran gradient	Blocks nuclear export of importin-α
Wortmannin (Arcaro and Wymann, 1993)	Phosphatidylinositl-3 kinas (PI3 kinase)	Blocks nuclear-cytoplasmic trafficking regulated by PI3kinase phosphorylation
Okadaic acid **Calyculin A** (Elrick and Docherty, 2001)	Phosphatase inhibitors	Blocks some phosphorylation dependent trafficking. .
Peptide Aptamers (Colas et al., 2000)	Peptide containing a NLS or NES directed against any protein; may also contain ubiquitin.	Redirects protein to the nucleus (NLS) or cytoplasm (NES); modify any protein with ubiquitin.
Energy Regenerating System and Ran Mix (Timinszky et al., 2002)	Ran gradient	Energy source for continued nuclear-cytoplasmic shuttling.
Thapsigargin (Greber and Gerace, 1995)	Calcium pump inhibitor	Blocks signal-mediated protein import

14.11 Nuclear Transport and Oncogenesis.

Alterations in nucleo-cytoplasmic protein transport are strongly implicated in a number of human diseases that include several autoimmune disorders (Wesierska-Gadek et al., 1996) and human cancers (Kau et al., 2004). The data presented in this investigation describes a mechanism of drug resistance observed in human myeloma and leukemia cell lines that involves alterations in the subcellular distribution of topo IIα. The data presented herein suggests that alterations in the nuclear-cytoplasmic trafficking of topo IIα may have a role in cellular drug resistance in human myeloma and leukemia cell lines. Several genetic abberations in the nuclear-cytolasmic transport machinery have been described in human leukemia and myeloma cells to date (Lam and Aplan, 2001). At least 17 chromosomal rearrangements in acute and chronic leukemia involve nucleoporin genes (Table 16) (Cronshaw and Matunis, 2004). Nucleoporin 98 (Nup98) is implicated in 15 of those chromosomal rearrangements (Slape and Aplan, 2004; Takeshita, et al., 2004). Furthermore, chromosomal translocations involving Nup98 and DNA topo I have been observed in some patients with therapy related acute myeloid leukemia, and in one patient with *de novo* AML (Iwase et al., 2003; Chen et al., 2003). Leukemias associated with nucleoporin gene rearrangements have been reported to be more refractory to therapeutic intervention. (Kakazu et al., 2001) suggesting that these gene rearrangements are involved in drug sensitivity. Nup98 gene rearrangements have also been implicated in therapy related myelodysplasia (MDS) (Ahuja, et al., 1999), a group of bone marrow disorders that resemble hematologic malignancies. MDS is characterized by the clonal expansion of stem cells and is most prevalent in individuals over 60 years of age. Nup gene rearrangements in multiple myeloma have not been

reported. In addition to the Nup family of proteins, the major vault protein (MVP) referred to as the lung resistance related protein (LRP), has been associated with decreased sensitivity to chemotherapy in multiple myeloma. The MVP is a ribonucleoprotein complex that localizes to the nuclear pore complexes (Chugani et al., 1993). Results of a clinical study showed that expression of MVP in myeloma patients was associated with the multidrug resistance phenotype not mediated by MDR-1/P-gp (Rimsza et al., 1999). Collectively, these studies illustrate the underlying role of nucleo-cytoplasmic trafficking and the NPC in oncogenesis. Furthermore, the significance of Nup98/topo I fusion proteins in conferring altered drug sensitivity represent one area of research yet to be explored

Table 16. Nucleoporin (NUP) Gene Rearrangements Associated with Hematological Malignancies*

Nucleoporin Gene Rearrangement	FUSION GENE	Protein Characteristics	Disease	References
t(7;11)(p15;p15)	NUP98/HOXA9	Homeodomain transcription factor	AML, CML, MDS	Nakamura et al., 1996
t(2;11)(q31;p15)	NUP98/HOXD13	Homeodomain transcription factor	AML	Arai et al., 2000
t(1;11)(q23;p15)	NUP98/PMX1	Homeodomain transcription factor	MDS/AML	Nakamura et al., 1999
t(7;11)(p15;p15)	NUP98/HOXA11	Homeodomain transcription factor	CML, AML	Suzuki et al., 2002
t(11;12(p15;q13)	NUP98/HOXC11	Homeodomain transcription factor	AML	Gu et al., 2003
t(7;11)(p15;p15)	NUP98/HOXA13	Homeodomain transcription factor	AML	Taketani et al., 2002
t(2;11)(q31;p15)	NUP98/HOXD11	Homeodomain transcription factor	AML	Terui et al., 2003
t(11;12)(p15;q13)	NUP98/HOXC13	Homeodomain transcription factor	AML	Panagopoulos et al., 2003
inv11(p15;q22)	NUP98/DDX10	RNA helicases	AML, MDS	Arai et al., 1997
t(11;20)(p15;q11)	NUP98/TOP1	DNA topo I	*de novo* AML; t-AML, t-MDS; polycethemia vera	Potenza et al., 2004 Iwase et al., 2003 Chen et al., 2003
t(10;20;11)(q24;q11;p15)	NUP98/TOP1	DNA topo I	t-MDS	Panagopoulos et al., 2002
t(4;11)(q21;p15)	NUP98/RAP1GDS1	Nucleotide exchange factor	T-ALL	Hussey et al., 1999
t(10;11)(q25;p15)	NUP98/ADD3	Membrane-cytoskeleton	T-ALL	Lahortiga et al., 2003
t(5;11)(q35;p15)	NUP98/NSD1	SET domain	AML	La Starza et al., 2004
t(8;11)(p11.2;p15)	NUP98/NSD3	SET domain	AML	Rosati et al., 2002
t(6;9)(p23;q34)	NUP214/DEK	DNA binding	AML, MDS	Boer et al., 1998
t(6;9)(p23;q34)	NUP214/SET	Nucleosome assembly protein family	AML	Saito et al., 2004

*Reviewed in reference Lam and Aplan, 2001.

Chapter Fifteen

Future Considerations

15.1 Phosphorylation

Phosphorylation has also been shown to be important in regulating the subcellular localization of many proteins and could have a role in topo IIα trafficking (Harreman et al., 2004). This is suggested by the finding that the outer proliferating cells of multi-cell spheroids contain a cytoplasmic pool of topo IIα and have a 10-fold decrease in the phosphorylation state of the enzyme when compared to monolayers containing nuclear topo IIα (Oloumie et al., 2000). Phosphorylation has been extensively investigated for yeast DNA topo IIα and to a lesser extent in topo IIβ. Phosphorylation

Table 17. Phosphorylation Sites in DNA Topoisomerase IIα Established *In vitro*

Kinase	Residue	Reference
PKC	Serine 29	Wells et al., 1995
Presumed to be Casein Kinase 1 or 2	Serine 1106	Chikamori et al., 2003
Casein Kinase 2	Serine 1377 Serine 1525	Wells et al., 1994
Casein Kinase 2	Threonine 1343	Daum and Gorbsky, 1998 Ishida et al., 1996
Casein Kinase 2	Serine 1469	Escarguiel et al., 2000
One or more proline directed kinases	Serine 1213 Serine 1247 Serine 1354 Serine 1361 Serine 1393	Wells and Hickson, 1995

events in topo II in human cells is less well characterized (Austin and Marsh, 1998) Several serine residues in topo IIα are phosphorylated *in vitro* by CK2 and PKC (Table 17). Many of the phosphorylation sites are located near the NLS in the carboxyl terminus (Figure 52, page 192). For topo IIα, the phosphorylation status at S29, S1213, S1247, S1354, S1361, S1393 vary throughout the cell cycle. In contrast, phosphorylation at S1377, S1525, and T1343 remains constant throughout the cell cycle (Austin and Marsh, 1998). To predict additional protein kinase recognition motifs, the complete amino acid sequence was entered into Scansite, a computer algorithm developed at Massachusetts Institute of Technology. Scansite 2.0 uses a library of phosphorylation data to predict phosphorylation sites and protein-protein binding domains in any protein (Obenauer et al., 2003).

Table 18. Predicted Phosphorylation Sites in DNA Topoisomerase IIα

Kinase Group	Amino Acid Residue	Recognition Sequence
Src SH3	Proline 887	MDGEEPLPMLPSYKN
Protein kinase C	Serine 1426	TAAKSQSSTSTTGAK
Protein kinase C	Threonine 193	YKKMFKQTWMDNMGR
Calmodulin dependent kinase IIγ	Serine 230	LSKFKMQSLDKDIVA
Calmodulin dependent kinase IIγ	Threonine1279	TKTKKQTTLAFKPIK
Casein kinase 2	Serine 1337	LDSDEDFSDFDEKTD
Casein kinase 2	Serine 1106	DEEENEESDNEKETE

The computer program can recognize 62 protein kinase or protein binding motifs that includes Ser/Thr or Tyr kinases and SH2 and SH3 binding domains. Figure 32 is the graphical output showing predicted phosphorylation sites that have not been reported in the literature. Table 18 summarizes the protein kinase recognition sequences identified under high stringency. As expected, topo IIα has recognition motifs for protein kinase C, casein kinase 2 and proline dependent Ser/Thr kinases. Several *in vitro* studies have reported similar phosphorylation occurrences by these kinases, albeit different amino acid residues

15.2 Calmodulin Dependent Kinases

Calmodulin dependent kinase-IIγ (CaMK-IIγ) is one unexpected result in the Scansite report. CamK-IIγ belongs to the family of calmodulin dependent kinases (CaMK). Calmodulin is a cytosolic protein that responds to intracellular calcium levels by activating various calcium-calmodulin dependent enzymes, such as calmodulin dependent kinases (CaMK) (Tombes et al., 1995). There are six different CaM kinases but, only CaMK I and CaMK II are consistently expressed in proliferative cells (Tombes and Krystal, 1997). CaMK II was originally believed to be encoded by a single gene. For example, CaMK-IIγ has for different isozymes, IIγB, IIγC, IIγG, and IIγH, generated by alternative splice sites. In one study, panels of human tumor and non-tumor cell lines were examined for expression of all CaMK-II protein isoforms (Tombes and Krystal, 1997). The tumor cell lines included several human breast cancer cell lines, colon adenocarcinoma, human neuroblastoma, and CCRF-CEM lymphoblastic leukemia cells, whereas NIH 3T3 mouse fibroblasts, mammary epithelia, and mouse embryo fibroblasts

were the non transformed cell lines examined. The tumor cell lines expressed an entirely different spectrum of CaMK-II isozymes than adult neuronal tissue. All tumor cell lines (including the neural derived cells) lacked CamK-IIα and expressed several alternative splice variants. CaMK- IIγG and IIγH were preferentially expressed in tumor cell lines as compared to undifferentiated fibroblasts. This data establishes that CaMK-IIγ is expressed in a variety of human cancer cell lines, including the hematological cell line, CCRF-CEM. Thus, it is possible that CaMK-IIγ is expressed in other hematological cell lines such as, human myeloma H929 cells. Expression of CaMK-IIγ in H929 cells would be significant if, topo IIα is regulated by CaMK-IIγ phosphorylation.

Identification of a CaMK phosphorylation site in topo IIα could be significant factor in regulating topo IIα subcellular distribution for several reasons. First, topo IIα has been shown to be hypophosphorylated in drug resistant cell lines in a calcium dependent manner (Grabowski et al., 1998; Aoyama et al., 1998; Ganapathi et al., 1996). Secondly, the complete amino acid sequence for topo IIβ was entered into Scansite but, does not have a calmodulin dependent kinase recognition motif even when the protein was scanned under low stringency. This is significant because topo IIβ subcellular localization is not altered by cell-density in the same manner as topo IIα suggesting that different cellular conditions can regulate the differential localization of the two enzymes. Thirdly, calcium levels present in the ER cisternae have been reported to regulate nuclear-cytoplasmic trafficking of proteins such that, when calcium levels are low, the NPC restricts the nuclear cytoplasmic trafficking of proteins (Greber and Gerace, 1995). It has been suggested that calcium stores signal the NPC when the cell senses stress such

as, oxidative stress, DNA damage, or proteins unfolding in the ER. Thus, there could be a link between NPC trafficking and early apoptotic events.

There is increasing evidence that calcium signaling pathways are intimately involved with apoptotic regulatory pathways. BCL-2, Bax, Bak, and Bid are apoptotic regulatory proteins that exert their effect in part by regulating calcium mobilization between the ER and mitochondria. Bax, Bid, and Bcl-2 localize in the ER, the central intracellular calcium stores of the cells. BCL-2 mediates the release of ER calcium pools, which results in a concomitant increase in mitochondrial calcium uptake (Oakes et al., 2003; Foyouzi-Youssefi et al., 2000). Bax and Bak, promote apoptosis in part by increasing calcium transfer from the ER to the mitochondria, which results in cytochrome c release during apoptosis (Nutt et al., 2002). Bax and Bid have also been shown to localize on the nuclear envelope and move from the cytoplasm to the mitochondria in response to camptothecin exposure (Gajkowska et al., 2004). This data suggests that camptothecin may initiate apoptosis in a via Bax and Bid in a calcium dependent manner.

Calcium release from the ER may be linked with oxidative stress, which has been one physiological condition demonstrated to modulate NPC mediated protein trafficking. This is significant because the tumor microenvironment can contribute to hypoxia. For example, solid tumors that are resistant to topo II containing chemotherapy usually have regions of low oxygen, which are not observed in normal tissue (Tomida and Tsuruo, 1999). Although multiple myeloma is not considered a solid tumor, the disease is characterized by anemia, which has been shown to induce or hasten hypoxia (Sordet et al., 2004; Mittelma, 2003). Furthermore, when Bid is expressed in hypoxic cells, it results in increased sensitivity to the topoisomerase II inhibitor etoposide (Erler et al.,

2004). Downregulation of Bid was attributed to the decrease sensitivity to etoposide; however, the subcellular localization of topo II enzymes was not investigated. Likewise, the total amount of Bcl2, Bid and Bax were not investigated in the natural-plateau or accelerated-plateau human myeloma cell line models. Thus, topo IIα could be regulated by calmodulin dependent kinase activity such that, when calcium is released from the ER under conditions of stress, topo becomes phosphorylated and degraded. In another study, proteasomes were found to accumulate in the nucleus of cells grown under stress conditions (glucose starvation) (Ogiso et al., 2002). This could partially explain how tumor cells evade etoposide toxicity and yet, maintain topo II content for cell division- by sequestering topo IIα in the cytoplasm away from the nuclear proteasomes.

Furthermore, repetitive hypoxia, which could be prevalent in multiple myeloma patients who are chronically exposed to DNA damaging agents, could select for drug resistant cells (Weinmann et al., 2004).

Chapter Sixteen

Concluding Remarks

Resistance to chemotherapeutic drugs is a major obstacle in the treatment of leukemia and myeloma. We have reported that resistance to topo IIα poisons, such as VP-16 is found to increase dramatically with concurrent increases in cell density. The high-cell density of myeloma cells in the bone marrow may mimic the high-density plateau phase conditions that we established in vitro, and thus these cells may export endogenous topo IIα. from the nucleus to the cytoplasm *in vivo*. In order for topo targeted chemotherapy to function, topoisomerase must have access to the nuclear DNA. Thus, the nuclear export of topo IIα must be added to the list of potential mechanisms of resistance to topo poisons. It is unique in that it does not require drug exposure and may mimic the high cell density microenvironment seen in the bone marrow of patients with multiple myeloma. Further defining this mechanism, and possibly modulating export, may lead to methods to preclude this avenue of resistance. Clinical drug resistance is likely to be a multifactorial phenomeon and enhancing drug activity of available agents will likely require a multifaceted approach.

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Appendices

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   1 MSGDHLHNDS QIEADFRLND SHKHKDKHKD REHRHKEHKK EKDREKSKHS NSEHKDSEKK
  61 HKEKEKTKHK DGSSEKHKDK HKDRDKEKRK EEKVRASGDA KIKKEKENGF SSPPQIKDEP
 121 EDDGYFVPPK EDIKPLKRPR DEDDADYKPK KIKTEDTKKE KKRKLEEEED GKLKKPKNKD
 181 KDKKVPEPDN KKKKPKKEEE QKWKWWEEER YPEGIKWKFL EHKGPVFAPP YEPLPENVKF
 241 YYDGKVMKLS PKAEEVATFF AKMLDHEYTT KEIFRKNFFK DWRKEMTNEE KNIITNLSKC
 301 DFTQMSQYFK AQTEARKQMS KEEKLKIKEE NEKLLKEYGF CIMDNHKERI ANFKIEPPGL
 361 FRGRGNHPKM GMLKRRIMPE DIIINCSKDA KVPSPPPGHK WKEVRHDNKV TWLVSWTENI
 421 QGSIKYIMLN PSSRIKGEKD WQKYETARRL KKCVDKIRNQ YREDWKSKEM KVRQRAVALY
 481 FIDKLALRAG NEKEEGETAD TVGCCSLRVE HINLHPELDG QEYVVEFDFL GKDSIRYYNK
 541 VPVEKRVFKN LQLFMENKQP EDDLFDRLNT GILNKHLQDL MEGLTAKVFR TYNASITLQQ
 601 QLKELTAPDE NIPAKILSYN RANRAVAILC NHQRAPPKTF EKSMMNLQTK IDAKKEQLAD
 661 ARRDLKSAKA DAKVMKDAKT KKVVESKKKA VQRLEEQLMK LEVQATDREE NKQIALGTSK
 721 LNYLDPRITV AWCKKWGVPI EKIYNKTQRE KFAWAIDMAD EDYEF
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Figure 54. The Complete Amino Acid Sequence for Human DNA Topoisomerase I. (accession number NP 001059). The NLS is underlined.

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   1 MAKSGGCGAG AGVGGGNGAL TWVNNAAKKE ESETANKNDS SKKLSVERVY QKKTQLEHIL
  61 LRPDTYIGSV EPLTQFMWVY DEDVGMNCRE VTFVPGLYKI FDEILVNAAD NKQRDKNMTC
 121 IKVSIDPESN IISIWNNGKG IPVVEHKVEK VYVPALIFGQ LLTSSNYDDD EKKVTGGRNG
 181 YGAKLCNIFS TKFTVETACK EYKHSFKQTW MNNMMKTSEA KIKHFDGEDY TCITFQPDLS
 241 KFKMEKLDKD IVALMTRRAY DLAGSCRGVK VMFNGKKLPV NGFRSYVDLY VKDKLDETGV
 301 ALKVIHELAN ERWDVCLTLS EKGFQQISFV NSIATTKGGR HVDYVVDQVV GKLIEVVKKK
 361 NKAGVSVKPF QVKNHIWVFI NCLIENPTFD SQTKENMTLQ PKSFGSKCQL SEKFFKAASN
 421 CGIVESILNW VKFKAQTQLN KKCSSVKYSK IKGIPKLDDA NDAGGKHSLE CTLILTEGDS
 481 AKSLAVSGLG VIGRDRYGVF PLRGKILNVR EASHKQIMEN AEINNIIKIV GLQYKKSYDD
 541 AESLKTLRYG KIMIMTDQDQ DGSHIKGLLI NFIHHNWPSL LKHGFLEEFI TPIVKASKNK
 601 QELSFYSIPE FDEWKKHIEN QKAWKIKYYK GLGTSTAKEA KEYFADMERH RILFRYAGPE
 661 DDAAITLAFS KKKIDDRKEW LTNFMEDRRQ RRLHGLPEQF LYGTATKHLT YNDFINKELI
 721 LFSNSDNERS IPSLVDGFKP GQRKVLFTCF KRNDKREVKV AQLAGSVAEM SAYHHGEQAL
 781 MMTIVNLAQN FVGSNNINLL QPIGQFGTRL HGGKDAASPR YIFTMLSTLA RLLFPAVDDN
 841 LLKFLYDDNQ RVEPEWYIPI IPMVLINGAE GIGTGWACKL PNYDAREIVN NVRRMLDGLD
 901 PHPMLPNYKN FKGTIQELGQ NQYAVSGEIF VVDRNTVEIT ELPVRTWTQV YKEQVLEPML
 961 NGTDKTPALI SDYKEYHTDT TVKFVVKMTE EKLAQAEAAG LHKVFKLQTT LTCNSMVLFD
1021 HMGCLKKYET VQDILKEFFD LRLSYYGLRK EWLVGMLGAE STKLNNQARF ILEKIQGKIT
1081 IENRSKKDLI QMLVQRGYES DPVKAWKEAQ EKAAEEDETQ NQHDDSSSDS GTPSGPDFNY
1141 ILNMSLWSLT KEKVEELIKQ RDAKGREVND LKRKSPSDLW KEDLAAFVEE LDKVESQERE
1201 DVLAGMSGKA IKGKVGKPKV KKLQLEETMP SPYGRRIIPE ITAMKADASK KLLKKKKGDL
1261 DTAAVKVEFD EEFSGAPVEG AGEEALTPSV PINKGPKPKR EKKEPGTRVR KTPTSSGKPS
1321 AKKVKKRNPW SDDESKSESD LEETEPVVIP RDSLLRRAAA ERPKYTFDFS EEEDDDADDD
1381 DDDNNDLEEL KVKASPITND GEDEFVPSDG LDKDEYTFSP GKSKATPEKS LHDKKSQDFG
1441 NLFSFPSYSQ KSEDDSAKFD SNEEDSASVF SPSFGLKQTD KVPSKTVAAK KGKPSSDTVP
1501 KPKRAPKQKK VVEAVNSDSD SEFGIPKKTT TPKGKGRGAK KRKASGSENE GDYNPGRKTS
1561 KTTSKKPKKT SFDQDSDVDI FPSDFPTEPP SLPRTGRARK EVKYFAESDE EEDDVDFAMF
1621 N
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Figure 55. The Complete Amino Acid Sequence for Human DNA Topoisomerase IIβ (accession Number NP 001059). Nuclear export signal (NES) is double-underscored. A specific nuclear localization signal has not been identified in topo IIβ, but the C-terminus is important for targeting the enzyme to the nucleus (Mirski e al., 2003).

Appendix C: Gene Rearrangements in Hematological Malignancies

Table 19.Oncogenes and Fusion Proteins in Hematological Malignacies*

Oncogene	Symbol	Functional Product	Diseases
Abelson Murine Leukemia Viral Oncogene Homolog 1	ABL1	A nuclear and cytoplasmic non-receptor tyrosine kinase activity implicated in cell differentiation, cell division, and cell adhesion.	When expressed as Philadelphia translocation its activity is associated with CML
Acute Myeloid Leukemia-1	AML-1	Transcription factor (activator) for various hematopoietic specific genes. Expressed during differentiation and hematopoesis.	Associated with acute non-lymphocytic leukemia.
Acute Myeloid Leukemia-1 / Myeloid Translocation Gene 8	AML1/MTG8	Fusion protein as a result of t(8;21). Proposed role in preventing cell differentiation and thus, propagating leukemic blast cells	Associated with *de novo* acute myeloid leukemias
Receptor Tyrosine Kinase	AXL	Receptor tyrosine kinase.	Hematopoietic cancers
B-cell CLL / Lymphoma 2	BCL2	B-cell leukemia/lymphoma protein is localized in the inner mitochondrial membrane. Blocks programmed cell death.	Associated with leukemias; linked with follicular lymphoma when overexpressed as a result of the t(14;18) translocation.
Breakpoint Cluster Region	BCR	GTPase activating protein for RAC1 and CDC42; exhibits serine/threonine kinase activity	Translocations t(9;22) BCR-ABL and t(4;22) PDGFRA-BCR associated with CML
Breakpoint Cluster Region / Abelson Murine Leukemia Viral Oncogene Homolog 1	BCR/ABL	Fusion protein with constitutive tyrosine kinas activity as a result of 9:22 chromosomal translocation.	CML, ALL
Cyclin D1	CCND1	Important for cell cycle regulation; Dysregulation of cyclin D1 associated with t(11;14)	Multiple myeloma

*Huret, J.-L. et al., 2003. Atlas of Genetics and Cytogenetics in Oncology and Haematology. [online] http://www.infobiogen.fr/services/chromcancer/ (accessed 2004).

Table 19 continued.

DEK Oncogene / Nucleoporin 214D	DEK/CAN	DEK is ubiquitous protein expressed in the nucleus of cells and interacts with histones.; fusion protein formed as a result of t(6;9) translocation.	Associated with poor prognosis in AML.
Transcription Factor 3 / pre-B-cell leukemia transcription factor 1	E2a/Pbx1	Chimeric transcription factor (activator) resulting from t(1;19) chromosomal translocation.	pre-B-cell Acute Lymphoblastic Leukemia
Erythroblastosis Virus E26 Homolog	ETS-1	Transcription factor involved in extracellular matrix remodeling; cell migration; tumor invasion	Lymphomas Monocytic leukemia
Fibroblast Growth Factor 3	FGFR3	Receptor for acidic and basic fibroblast growth factors.	Chromosomal translocation t(4;14) implicated in multiple myeloma
Homeobox-11	HOX-11	Homeobox protein; DNA binding protein	Found to be deregulated in acute lymphoblastic T-cell leukemia
Interluekin-3	IL-3	Cytokine involved in cell survival, proliferation, and differentiation.	Overexpression of protein associated with acute pre B-cell leukemia
Lymphoid Blast Crisis	LBC	guanine nucleotide exchange factor	Myeloid leukemias
Lymphocyte Specific Kinase	LCK	src family tyrosine kinase critical for T-cell development and activation	T-cell lymphoma

Table 19 continued.

Lymphoblastic Leukemia Derived Sequence 1	Lyl-1	Basic helix-loop-helix protein.	Involved in T-cell ALL through t(7:19) which involves Lyl-1 and T-cell receptor beta chain genes.
Mixed Lineage Leukemia	MLL	Transcription factor involved in maintenance of HOX gene expression by hematopoietic stem and progenitor cells	Associated with poor prognosis in acute myeloid leukemia
Mixed Lineage Leukemia / Lymphoid Nuclear Protein 4	MLL/LAF4	Fusion protein resulting from t(4;11) translocation.	70-80% of all ALL and 50-60% of AML infants; Reported in secondary leukemias after topo II inhibitors; poor prognosis
Musculoaponeurotic Fibrosarcoma	MAF	Transcription factor; overexpression implicated in cell proliferation and pathological interactions with bone marrow stroma.	Multiple myeloma
Myeloblastosis	Myb	A family of DNA binding proteins containing tandem repeats of a helix-turn-helix motif. Expression in hematopoietic cells is necessary fo proliferation.	Chromosomal aberrations at the 6q breakpoint linked with colon carcinoma, leukemias, and lymphomas
Myeloctyomatosis	c-Myc	Transcription factor involved in cell cycle regulation, apoptosis, metabolism, cell differentiation, and cell adhesion.	Overexpressed in most hematogenic malignancies. Most well characterized in Burkitts lymphoma.
Myosin, heavy polypeptide 11, smooth muscle / Core-binding factor beta subunit	MYH11/CBFB	CBFB is a transcription factor which master-regulates a host of genes specific to hemtopoesis and osteogenesis. Pericentric inversion Inv(16)(p13;q22) fuses CBFB with MYH11	Acute Myeloid Leukemia

Table 19 continued.

Nucleophosmin1 / Anaplasticlymphoma kinase	NPM/ALK	ALK is a receptor tyrosine kinase; The t(2;5) translocation results in a fusion protein with NPM; accumulates in the nucleoli of neoplastic cells.	Non-Hodgkin's lymphomas; Anaplastic lymphoma; Large cell lymphomas
pre-B cell Leukemia Transcription Factor 3	Pbx1/E2A	fusion protein that activates WNT-16 expression. WNT-16 expression contributes to t(1;19) pre-B ALL	Acute pre B-cell leukemia
PIM1 Oncogene	PIM-1	serine/threonine kinase	T-cell lymphoma
Promyelocytic Leukemia / Retinoic Acid Receptor alpha	PML/RAR	PML is a RING finger and nuclear matrix associated phosphoprotein. Functions in growth, transformation and tumor suppressor activity. Fusion with RAR may abrogate the corepressor function.	Acute premyelocytic leukemia
Reticuloendotheliosis	REL	Transcription factor; role in differentiation and lymphopoiesis.	Gene amplification associated with Hodgkin's lymphoma
Suppressor of variegation, Enhancer of zeste and Trithroax / Nucleoporin 214	SET/CAN	CAN is a nucleoporin that may serve as a docking site in receptor mediated nuclear import; Fusion protein resulting from t(6;9) translocation.	Acute myeloid leukemia
Stem Cell Leukemia	SCL	Transcription factor; t(1:14) in leukemia cell lines results in deletion between delta chain diversity regions	Acute T-cell leukemia
Transforming Acidic Coiled-coil Containing Gene 3	TACC3	Centrosomal proteins that can interact with microtubules.	t(4;14) implicated in Multiple myeloma.

About the Author

Roxane Engel was born in St. Petersburg, Florida and received her diploma from Boca Ciega High School. Roxane earned a Bachelors of Science Degree from the University of Tampa with a major in Biology and a minor in Chemistry. Roxane was an academic tutor and a teacher's assistant in the Genetics Laboratory. She completed a research internship at the V.A. Medical Center, graduated with Honors, and was recognized as the Most Outstanding Life Science Graduate.

Roxane enrolled in the doctoral program in Biochemistry and Molecular Biology at USF in the College of Medicine and worked at the Moffitt Cancer Center. She was a Graduate Student Representative, won a research travel award, received the Walter Trudeau Award, and presented research at several scientific meetings, including ones in New Orleans, San Francisco, New York City, and Amsterdam, Holland. Roxane completed one year at Quest Diagnostics as a Genomics and Esoteric Testing Specialist.