

2-18-2008

Drug Resistance to Topoisomerase Directed Chemotherapy in Human Multiple Myeloma

Joel G. Turner

University of South Florida

Follow this and additional works at: <https://scholarcommons.usf.edu/etd>



Part of the [American Studies Commons](#)

Scholar Commons Citation

Turner, Joel G., "Drug Resistance to Topoisomerase Directed Chemotherapy in Human Multiple Myeloma" (2008). *Graduate Theses and Dissertations*.

<https://scholarcommons.usf.edu/etd/539>

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.

Drug Resistance to Topoisomerase Directed Chemotherapy in
Human Multiple Myeloma

By

Joel G. Turner

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Biology
College of Arts and Sciences
University of South Florida

Co-Major Professor: Daniel M. Sullivan, M.D.
Co-Major Professor: James R. Garey, Ph.D.
Brian Livingston, Ph.D.
My Lien Dao, Ph.D.

Date of Approval:
February 18, 2008

Keywords: Nuclear Export, Tumor Suppressor Proteins, Cell Cycle Inhibitors,
ATP Binding Cassette Protein G2 (ABCG2), Chromosome Maintenance Protein
1 (CRM1)

Copyright 2008, Joel G. Turner

Table of Contents

List of Tables	iii
List of Figures	iv
Abstract	v
Chapter One: Review of nuclear export of proteins and chemotherapeutic resistance in cancer	1
Summary	1
Introduction	3
Nuclear export mechanisms	4
Export of drug targets	8
Topoisomerases	8
Topoisomerase II alpha	9
Topoisomerase I	10
Export of tumor suppressors	11
P53	11
APC/ β -catenin	13
FOXO family of transcription factors	14
Export of cell-cycle regulators	15
P21 ^{cip1}	15
P27 ^{kip1}	16
Nuclear export and potential drug targets	18
Chapter Two: Drug resistance to topoisomerase directed chemotherapy in human multiple myeloma	22
Summary	22
Introduction	23
Materials and Methods	26
Cell lines	25
Cell density and drug treatment	26
Immunofluorescent microscopy	27
Apoptosis assay	28
CRM1 siRNA knockdown and Western blot	28
Band depletion assay	30
Comet assay	30
Results	31
Log and plateau density myeloma cells	31

Intracellular trafficking of topo II α	33
CRM1 inhibitor and topo II α chemotherapeutics	35
Topo II α trafficking and CRM1 inhibition	37
CRM1 inhibitor and topo II α inhibitor synergy	37
CRM1 siRNA sensitizes myeloma cells to topo II α poisons	39
Topoisomerase Western blot	42
Increase in cleavable complex formation by CRM1 inhibition	42
Comet assay	45
Discussion	45
Chapter Three: ABCG2 expression, function and promoter methylation in human multiple myeloma	49
Summary	49
Introduction	50
Materials and Methods	53
Cell lines	53
Clinical trial with high-dose melphalan and topotecan	54
Real-time quantitative PCR (QPCR)	55
Western blot for cell lines and patient myeloma samples	56
Flow cytometry/ABCG2 functional assay	57
Immunofluorescent microscopy and quantitative measurement of ABCG2	59
Cell density and low dose drug treatment	59
Bisulfite sequencing and demethylation by 5-aza-2'deoxyctidine of the ABCG2 promoter in patient samples and myeloma cell lines	60
Methylation-specific quantitative PCR	61
Results	62
Quantitative PCR of ABCG2	62
ABCG2 protein expression determined by Western analysis, flow cytometry and immunofluorescence	65
ABCG2 functional assay: topotecan efflux	67
Effect of the microenvironment and topoisomerase inhibitors on ABCG2 expression	72
ABCG2 promoter methylation	75
Methylation-specific quantitative PCR of patient myeloma cells	77
Discussion	78
Dissertation Summary	84
References	86
About the Author	End Page

List of Tables

Table 1.1	Drug targets, tumor suppressors and cell-cycle inhibitors that undergo CRM1-mediated export in various cancers.	2
Table 3.1	ABCG2 mRNA expression determined by QPCR in human cancer cell lines	63
Table 3.2	ABCG2 mRNA expression determined by QPCR in CD138 selected human plasma cells from bone marrow aspirates obtained from patients with multiple myeloma prior to and during high-dose chemotherapy, and at the time of relapse	64

List of Figures

Figure 1.1 Nuclear export of proteins	5
Figure 2.1 Intracellular trafficking of topo II α in log and plateau density myeloma cells	32
Figure 2.2 Cellular localization of topo II α in 8226 and U266 cells under log and plateau-density growth conditions	34
Figure 2.3 CRM1 inhibitor and topo II α chemotherapeutics	36
Figure 2.4 H929 topo II α immunofluorescence	38
Figure 2.5 CRM1 inhibitor and topo II α inhibitor synergy	40
Figure 2.6 CRM1 knockdown using siRNA makes myeloma cells more sensitive to the topo II α poison doxorubicin	41
Figure 2.7 Log and plateau expression of topoisomerases in myeloma cell lines	43
Figure 2.8 Band depletion and comet assay	44
Figure 3.1 ABCG2 expression and function in myeloma cell lines	66
Figure 3.2 Functional assay in patient myeloma cells	68
Figure 3.3 ABCG2 expression increases in response to doxorubicin exposure	70
Figure 3.4 ABCG2 expression is elevated in log phase myeloma cells	71
Figure 3.5 ABCG2 expression increases in response to topotecan chemotherapy	73
Figure 3.6 ABCG2 increases in patient plasma cells after high-dose chemotherapy and at relapse	74
Figure 3.7 ABCG2 promoter methylation	76

Drug Resistance to Topoisomerase Directed Chemotherapy in Human Multiple Myeloma

Joel G. Turner

ABSTRACT

Human multiple myeloma is an incurable hematological malignancy characterized by the proliferation of plasma cells in the bone marrow. Myeloma represents approximately 20% of all blood cancers. In this research we have explored examples of both intrinsic and acquired drug resistance in myeloma.

Topoisomerases are enzymes that are critical for cell division, especially in rapidly dividing cells such as are found in cancer. Topoisomerase poisons are a common group of drugs that are used to treat cancer. Topoisomerase I and II poisons used in the treatment of multiple myeloma include topotecan, mitoxantrone, doxorubicin, and etoposide

In order for topoisomerase drugs to be effective, the enzyme must be in direct contact with the DNA. In chapters one and two we examined the export of topoisomerase II alpha from the nucleus as a mechanism of drug resistance. High density cells, similar to those found in the bone marrow, export topoisomerase II alpha from the nucleus to the cytoplasm, rendering the cell drug resistant. We found that blocking nuclear export using the CRM1 inhibitor ratjadone C, or CRM1 specific siRNA, could sensitize high density cells to topoisomerase drugs. Sensitization to topoisomerase inhibitors was correlated

with increased topoisomerase/DNA complexes and increased DNA strand breaks. This method of sensitizing human myeloma cells suggests a new therapeutic approach to this disease.

In chapter three we examined the role of the molecular transporter ABCG2 in drug resistance in multiple myeloma. We found that ABCG2 expression in myeloma cell lines increased after exposure to topotecan or doxorubicin. Myeloma patients treated with topotecan had an increase in ABCG2 mRNA and protein expression after drug treatment and at relapse. We found that expression of ABCG2 is regulated, at least in part, by promoter methylation both in cell lines and in patient plasma cells. Demethylation of the promoter increased ABCG2 mRNA and protein expression. These findings suggest that ABCG2 is expressed and functional in human myeloma cells, regulated by promoter methylation, affected by cell density, upregulated in response to chemotherapy, and may contribute to drug resistance.

Chapter 1

Review of nuclear export of proteins and chemotherapeutic resistance in cancer

SUMMARY

Expression levels of intact tumor suppressor proteins and molecular targets of antineoplastic agents are critical in defining cancer cell drug sensitivity; however, the intracellular location of a specific protein may be equally as important. Many tumor suppressive proteins must be present in the cell nucleus in order to perform their policing activities or for the cell to respond to chemotherapeutic agents. Examples of nuclear proteins needed to prevent cancer initiation or progression, or to optimize chemotherapeutic response, include: tumor suppressor proteins p53, APC/ β -catenin and FOXO family genes, negative regulators of cell cycle progression and survival such as p21 and p27, and chemotherapeutic targets such as DNA topoisomerase I and topoisomerase II α . (Table 1). Mislocalization of a nuclear protein into the cytoplasm can render it ineffective as a tumor suppressor or as a target for chemotherapy. It is possible that blocking the nuclear export of any or all of these proteins may restore tumor suppression, apoptosis, or in the case of topoisomerase I and topoisomerase II α , reversal of drug resistance to inhibitors of these enzymes. During the course of

Protein	Functional significance	Export receptor	Modification for nuclear export.	Cancer type where protein is exported to the cytoplasm
Topoisomerase II α	DNA topology, drug target	CRM1	Phosphorylation by Casein Kinase 2.	Multiple myeloma
Topoisomerase I	DNA topology, drug target	CRM1	Unknown	Anaplastic astrocytoma Neuroblastoma
p53	Tumor suppressor	CRM1	Ubiquitinylation by MDM2 E3 ubiquitin ligase.	Colorectal cancer Breast cancer
APC	Tumor suppressor	CRM1	Mutation causing frame-shift or premature termination.	Colorectal cancer
FOXO	Tumor suppressor	CRM1	Phosphorylation by Akt kinase.	Breast, prostate, and thyroid cancer Glioblastoma Melanoma
p21 ^{Cip1}	Cell-cycle inhibitor	CRM1	HER2/neu mutation and phosphorylation by Akt. BCR-ABL translocation and phosphorylation by Akt. Phosphorylation by PKC	Ovarian and breast cancer Chronic myeloid leukemia (CML)
p27 ^{Kip1}	Cell-cycle inhibitor	CRM1	Phosphorylation by human kinase-interacting stathmin (hKIS)	Breast cancer Acute myelogenous leukemia (AML)

Table 1.1: Drug targets, tumor suppressors and cell-cycle inhibitors that undergo CRM1-mediated export in various cancers.

disease progression or in response to the tumor environment, cancer cells appear to acquire intracellular mechanisms to export anti-cancer nuclear proteins. These export mechanisms generally involve modification of nuclear proteins that cause the proteins to reveal leucine-rich nuclear export signal protein sequences, with subsequent export mediated by CRM1. In this review we will define the general processes involved in nuclear export mediated by CRM1/RanGTP (Exportin/XPO1), examine the functions of individual tumor suppressor nuclear proteins and nuclear targets of chemotherapy, and explore the potential mechanisms that are used by the cancer cell to induce export of these proteins. In addition, we will briefly discuss experimental therapeutics that could potentially counteract nuclear export of specific proteins.

INTRODUCTION

Drug resistance is the single greatest impediment to the treatment of cancer. Cancer cells can be intrinsically drug resistance due to the breakdown of many normal cellular processes during cancer development or cancers may acquire drug resistance in response to selection by chemotherapeutic treatment. Acquired resistance may develop into cross-resistance to many other drugs that have very different mechanisms of action (Gottesman, 2002; Longley and Johnston, 2005). Specific types of drug resistance include, chemical inactivation of the drug(s) such as *cis*-platinum, irinotecan and methlytrexate (Ishikawa and Ali-Osman, 1993; Xu and Villalona-Calero, 2002), alteration of repair

mechanisms for drug induced damage to DNA in response to platinum drugs (Dabholkar et al., 1994; Durant et al., 1999; Fink et al., 1998), evasion of apoptosis induced by 5-fluorouracil (Longley and Johnston, 2005), drug efflux of mitoxantrone, VP-16, doxorubicin, vinblastine, anthrilmide and flavopiridol by ATP binding cassette (ABC) transporters (Ambudkar et al., 1999; Goldman, 2003; Gottesman et al., 2002; Krishna and Mayer, 2000; Roe et al., 1999; Thomas and Coley, 2003), down-regulation of pro-apoptotic factors or drug targets to vinca alkaloids and other microtubule inhibitors (Dumontet and Sikic, 1999; Kavallaris et al., 2001; Longley et al., 2003; Xu and Villalona-Calero, 2002), modification of specific drug targets to 5-fluorouracil, oxaplatin, irinotecan, and camptothecin (Boyer et al., 2004; Li et al., 1996), and mislocalization of either drug targets or tumor suppressive proteins (Fabbro and Henderson, 2003; Kau and Silver, 2003; Yashiroda and Yoshida, 2003). This latter mechanism, the export of drug targets, tumor suppressors, and cell-cycle inhibitors from the nucleus, is the primary focus of this review.

NUCLEAR EXPORT MECHANISMS

Once an mRNA is translated into a protein, the protein is directed to a specific intracellular compartment either for further modification or to perform its function within the cell. Movement of proteins is governed by specific amino acid signaling sequences contained within the particular protein.

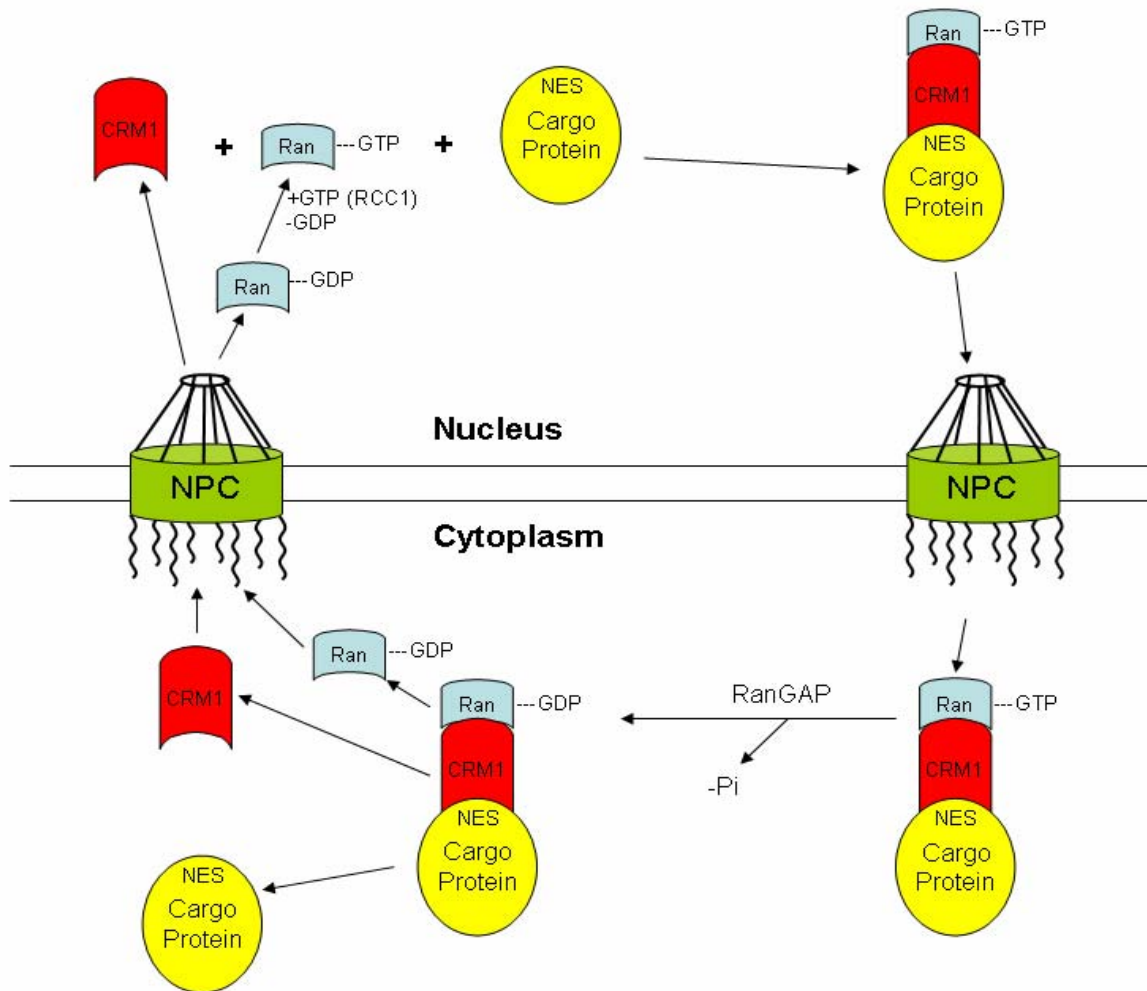


Figure 1.1, *Nuclear export of proteins*. Nuclear export of a cargo protein by association of the nuclear export signal (NES) with CRM1 and Ran-GTP and transport through the nuclear pore complex.

Signals exist for trafficking to the nucleolus (Guo et al., 2003; Liu et al., 2006; Nakamura et al., 2003) nuclear localization (Hodel et al., 2001) and nuclear export signals (Bogerd et al., 1996; Henderson and Eleftheriou, 2000; Ikuta et al., 1998; Kanwal et al., 2002), in addition to cytoplasmic compartment localization signals to the endoplasmic reticulum (Andres et al., 1990; Munro and Pelham, 1987), golgi (Zeng et al., 2003), mitochondria (Anandatheerthavarada et al., 2003), lysosomes (Bonifacino and Traub, 2003), and peroxisomes (Gould et al., 1989). Nuclear export signals (NES) in particular are comprised of hydrophobic amino acid residues including leucine and isoleucine-rich sequences (Kutay and Guttinger, 2005).

Proteins larger than 40-60 kDa cannot enter or exit the nucleus through the nuclear pore complex without the assistance of soluble transport receptors called karyopherin proteins, which bind to the export or import signal peptides (Bednenko et al., 2003). The nuclear pore of a cell is a very large (125 MDa) protein made of approximately 100 nucleoporins (Lim et al., 2008). The nuclear pore has a central iris-like transporter region through which the proteins are transported. In addition, the nuclear pore has eight large cytoplasmic filaments and a nuclear basket structure. The nuclear pore complex is imbedded in the nuclear envelop bilayers (Terry et al., 2007). The exact mechanism of transport of proteins through the central channel is not known. However, it is known that for nuclear export of a cargo protein to occur, it must bind to a transport receptor protein called an exportin which in turn is regulated by a small GTPase molecule

referred to as Ran-GTP (Arnaoutov et al., 2005). Nuclear export is controlled by the concentration gradient of Ran-GTP and Ran-GDP; Ran-GTP is highly concentrated in the nucleus and therefore drives nuclear export, whereas Ran-GDP is concentrated outside the nuclear membrane in the cytoplasm (Arnaoutov et al., 2005). Stable export complexes are formed in the nucleus with Ran-GTP, the exportin transport receptor and the cargo protein (Weis, 2007). After the protein complex is exported into the cytoplasm, Ran-GTP is hydrolyzed to Ran-GDP by Ran-GAP and the export complex is dissociated, releasing the cargo protein into the cytoplasm. The exportin receptor protein and Ran-GDP are subsequently recycled back into the nucleus through the nuclear pore complex to undergo another round of export (Figure 1). The primary export receptor protein is chromosome maintenance protein 1 or CRM1 (Daelemans et al., 2005; Yoneda et al., 1999). CRM1, with the assistance of Ran-GTP, binds to the nuclear export signal peptide (NES) of the cargo protein. However, the NES must be present and accessible to CRM1 (Arnaoutov et al., 2005; Black et al., 2001). The NES of a cargo protein can be exposed by changes in the three-dimensional conformation of the protein which are generally caused by protein phosphorylation or dephosphorylation (Vogt et al., 2005), although other protein modifications such as acetylation (Vogt et al., 2005), sumoylation (Pichler and Melchior, 2002), ubiquitination (Bonifacino and Traub, 2003; Vogt et al., 2005) or the binding of protein-specific co-factors can reveal export signals and induce export (Kutay and Guttinger, 2005; Poon and Jans, 2005; Yoneda et al., 1999).

EXPORT OF DRUG TARGETS

Topoisomerases

Topoisomerases (topo) are major targets in cancer chemotherapy due to their specific cellular activities. Topoisomerases that are useful as cancer drug targets in human cells are primarily of two types, type IB (topo I) and type II enzymes (topo II α and topo II β).

Topo I is a 100 kDa protein which produces a transient nick in one strand of the DNA double helix in an ATP independent manner and essentially allows the supercoiled DNA to unwind in a controlled manner. Topo II isoforms form a homodimer that makes a transient DNA double strand break, which requires the hydrolysis of an ATP molecule and allows the passage of an entire DNA strand through the strand break, thus making topological isomers of the DNA.

Topo molecules of both types are essential to organize the approximately two meters of DNA contained within each cell during transcription, DNA replication and recombination. There are two isoforms of topo II, topo II α (170kDa), and topo II β (180 kDa). Topo II β is constitutively expressed, present in the nucleus and nucleolus and may allow localized unwinding of DNA during DNA repair (Emmons et al., 2006). Topo II α is highly expressed in proliferating cells and is essential for transcription, DNA replication, chromatin condensation and chromatid separation. Topo II α $-/-$ mice are embryonic lethals that cannot progress past the 4 to 8 cell stage, whereas topo II β $-/-$ mice die at birth due to neurological defects (Lyu and Wang, 2003; Sakaguchi and Kikuchi, 2004).

Topoisomerase II alpha

Previous studies have revealed that topo II α has a bipartite nuclear localization signal (NLS) in the carboxyl terminus of the protein (Mirski et al., 1997; Wessel et al., 1997). Drug resistance to the topo II poison etoposide has been described in cells that express low levels of topo II α or which were truncated in the carboxyl terminal region of topo II α (Feldhoff et al., 1994; Wessel et al., 1997; Yu et al., 1997). Truncated topo II α , lacking a functional NLS, remains in the cytoplasm and therefore cannot produce DNA damage.

Two nuclear export signals for topo II α have been described in the C-terminal domain of the topo II α molecule (Mirski et al., 2003; Turner et al., 2004). This was determined by site-directed mutagenesis of export signal motifs of full-length topo II α protein (Turner et al., 2004). Topo II α nuclear export is mediated by CRM1, and is likely to be controlled by phosphorylation (unpublished data). In multiple myeloma cell lines, topo II α is actively exported into the cytoplasm in cells grown at high densities; consistent with patient bone marrow cell densities. In high density myeloma cell cultures, where topo II has been exported into the cytoplasm, the cells were found to be approximately 10-fold more resistant to topoisomerase poisons such as doxorubicin and etoposide (Engel et al., 2004). Drug sensitivity was not due to differences in cell cycle, drug uptake or topo protein levels, but was due primarily to enzyme trafficking to the cytoplasm (Engel et al., 2004). Blocking CRM1 nuclear export of topo II using leptomycin B

(Engel et al., 2004), ratjadone C, or a CRM1 specific siRNA knockdown was found to make drug-resistant human myeloma cells sensitive to the topo II poisons doxorubicin and VP-16.

Topoisomerase I

Topo I contains two motifs that are nearly identical to the published NES in topo II α (Turner et al., 2004), and it is likely that topo I may be exported by a CRM1 mediated mechanism. Topo I is normally located in the nucleus and the nucleoli of cells, but has been found to be exported to the cytoplasm in specific cancer cell lines. In addition, cytoplasmic localization of topo I occurs in direct response to the topo I inhibitory drugs topotecan and CPT-11. After treatment of anaplastic astrocytoma cells with the topo I inhibitor topotecan for one hour, the amount of cytoplasmic topo increased in the cytoplasm 50-100% and nuclear topo I decreased by 25% after topotecan treatment (Danks et al., 1996). Post topo I export the anaplastic astrocytoma cells became more resistant to the effects of topotecan. Cytoplasmic topo I was found to be fully active enzymatically, however the protein was smaller (68 kDa) than nuclear topo I (100 kDa) and therefore may be a degraded form of the enzyme.

Xenograft models of neuroblastoma treated with CPT-11 (Irinotecan) caused the cells to export topo I from the nucleus to the cytoplasm (Santos et al., 2004). Neuroblastoma xenograft cells attained an initial response rate to CPT-11 treatment of nearly 100% based on histological differentiation, however the

cells were completely refractory to any subsequent treatment by CPT-11. This was true even after *in vivo* passage of the cells into a different host animal. The drug-resistant cells demonstrated an overall decrease in topo I expression as well as in redistribution to the cytoplasm. In this model the cytoplasmic topo I were of two types, an enzymatically active 68 kDa topo I and an inactive 48 kDa form.

EXPORT OF TUMOR SUPPRESSORS

P53

Additional examples of the importance of nuclear export in cancer can be found in the tumor suppressor proteins p53, APC/ β -catenin and FOXO family genes. The critical function of p53 is to monitor the fidelity of genomic replication at the G₁/S phase cell cycle checkpoint. When wild-type p53 is activated it relocates from the cytoplasm to the nucleus where it functions as a transcription factor that induces expression of genes involved in DNA repair, cell cycle arrest, and apoptosis. P53 is a very important tumor suppressor that must be inactivated for cancer progression. Most of the literature has focused on p53 inactivation by mutation or deletion, including dominant-negative p53 mutants. Several recent reviews have described a litany of genetic alterations found in the p53 gene in various cancers (Bourdon, 2007; Soussi, 2007; Soussi and Wiman, 2007; Valkov and Sullivan, 2003). Genetic mutation of p53 accounts for 70% of colon cancers and 50% of breast cancers. Therefore, in the remaining percentage of these

cancers p53 must be inactivated by some additional means. There is a growing body of literature that describes p53 being kept out of the nucleus by cytoplasmic sequestration (Nikolaev et al., 2003; Wadhwa et al., 2002) or hyperactive nuclear export by MDM2 (Cuny et al., 2000). P53 contains four NES which bind CRM1 for nuclear export (Stommel et al., 1999; Zhang and Xiong, 2001), and three NLS which are involved in nuclear import. Once p53 is imported into the nucleus it forms a tetramer which blocks access to the nuclear export signals (Stommel et al., 1999). MDM2 protein is a negative-regulator of p53 but at the same time MDM2 expression is transactivated by the p53 tetramer. MDM2 possesses an E3 ubiquitin ligase which binds and ubiquitinates p53, changing its protein conformation and revealing the p53 NES's (Stommel et al., 1999). P53 is exported by the CRM1 nuclear receptor, and is degraded in the cytoplasm by proteasome proteolysis. MDM2 expression is controlled by p53 but its activity is modulated by the tumor suppressor protein p14ARF which binds to MDM2 and inactivates it, thereby stabilizing p53 function in the nucleus (O'Brate and Giannakakou, 2003). There are also studies indicating the contribution of P13K/AKT, c-ABL, and CBP/p300 to both MDM2 and p53 activity. In addition, p53 contains a number of phosphorylation events that prevent nuclear export and increase nuclear accumulation. Specific kinases that phosphorylate p53 include checkpoint kinase 1 (Chk1) and Chk2. Therefore alteration of p53 associated proteins, ubiquitination and phosphorylation could be used to maintain p53 in the nucleus and induce apoptosis in cancer cells.

APC / β -catenin

Human adenomatous polyposis coli (APC) is a tumor suppressor gene that is mutated in both inherited and sporadic human colorectal cancers (Powell et al., 1992). APC is a large (312 kDa) multi-domain protein which binds to and negatively regulates β -catenin, a Wnt signaling effector (Senda et al., 2007). APC forms a multiprotein complex with the scaffold protein axin, glycogen synthase kinase-3 β (GSK3 β) and β -catenin. GSK3 β phosphorylates β -catenin leading to its proteosomal degradation, and ultimately blocks the Wnt signaling pathway (reviewed in Senda et al 2007) (Senda et al., 2007). Mutations in APC lead to nuclear export and allow β -catenin mediated tumorigenesis. APC has been observed to translocate between the nucleus and cytoplasm in several cancer cell lines. APC contains both an NLS sequence in the central domain of APC, and multiple NES sequences in the N-terminus and central region of the protein (Neufeld et al., 2000). APC export is mediated by CRM1 binding as shown by site-directed mutation of the NES amino acid signal and also by inhibition of export by leptomycin B (Neufeld et al., 2000). Mutations usually occur only in particular regions of the APC gene causing frame-shift mutations and pre-mature termination of translation. Trafficking of nuclear APC into the cytoplasm has been directly correlated to an increase in nuclear β -catenin and subsequent oncogenic transcriptional activity. The rate of nuclear export of APC, not the rate of import, has been directly correlated to the rate of β -catenin transcriptional activity and tumor progression (Rosin-Arbesfeld et al., 2003). Maintaining APC in the nucleus

could potentially inhibit colorectal cancer progression.

FOXO transcription factors

The FOXO or forkhead family of transcription factors are protein regulators that control apoptosis, DNA repair, defense against oxidative molecular damage and cell cycle progression. FOXO proteins must be in contact with the DNA in the nucleus in order to perform their anti-neoplasm activities. Post-transcriptional modifications of FOXO proteins include multiple phosphorylations by various kinases, acetylation and ubiquitination (Vogt et al., 2005). However it is the phosphorylation by the serine/threonine kinase Akt (or protein kinase B) that disrupts binding to DNA and leads to nuclear export of FOXO. Once exported into the cytoplasm FOXO proteins undergo proteosomal degradation.

Akt kinase is inappropriately activated by phosphoinositide 3-kinase (PI3K) in cancers where the tumor suppressor PTEN is mutated. Therefore, mutation of PTEN in breast cancer, prostate cancer, thyroid cancer, glioblastoma, and melanoma leads to nuclear export of FOXO. In studies where PTEN activity was restored, FOXO proteins localized to the nucleus and induced cell-cycle arrest and apoptosis (Nakamura et al., 2000). In addition, recent studies to discover small molecule inhibitors of PI3K, Akt, and CRM1 have been performed to keep FOXO proteins in the nucleus as a potential anti-cancer treatment (Kau et al., 2003; Kau and Silver, 2003).

EXPORT OF CELL CYCLE REGULATORS

P21^{Cip1}

P21^{Cip1} (p21) is a cell cycle inhibitor which binds to cyclin-cyclin dependent kinase 2 (cdk2) or cdk4 molecular complexes and prevents cell cycle progression at G₁. P21 transcriptional expression is regulated by wild-type p53 in response to cellular stress, activated DNA-repair mechanisms, apoptosis or cellular senescence. Cellular growth is arrested when p21 binds to cyclin-cdk complexes in the cellular nucleus, therefore nuclear localization of unmodified p21 (phosphorylation or ubiquitination) is essential for its anti-cancer functions.

Nuclear export of p21 protein is induced by multiple ways. In some human breast and ovarian cancers the cell membrane receptor tyrosine kinase, HER-2/*neu*, is overexpressed. HER-2/*neu* activates the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway. After activation by HER-2/*neu*, Akt, a serine/threonine kinase, is released from the inner surface of the cell membrane, enters the cell nucleus and phosphorylates p21 at threonine 145 which results in cytoplasmic localization of p21 (Zhou et al., 2001a). Threonine residue 145 of p21 is in the NLS region, therefore phosphorylation inhibits nuclear localization and promotes cytoplasmic localization of p21 and allowing cell proliferation. In addition, cytoplasmic p21 has anti-apoptotic properties by associating with apoptosis-signal-regulating kinase 1 (ASK1) (Asada et al., 1999).

Two nuclear export signals are necessary for export of p21 from the nucleus to the cytoplasm (Henderson and Eleftheriou, 2000; Hwang et al., 2007).

Reactive oxygen species induce nucleo-cytoplasmic translocation of p21, along with ubiquitination and subsequent proteosomal degradation. This process is blocked by leptomycin B or site-directed mutation of the two nuclear export signals, therefore nuclear export is by a CRM1-mediated mechanism.

Phosphorylation of p21 at serine 153 in the carboxy-terminus by protein kinase C is also sufficient to induce nuclear export. When this site is blocked by calmodulin protein binding to p21, p21 accumulates in the cell nucleus (Rodriguez-Vilarrupla et al., 2005).

In chronic myeloid leukemia (CML), cells eventually develop an aggressive, drug resistant form of the disease during the "blast crisis" phase. Curiously, p21 protein expression is upregulated in these cells, however, it has been observed that the p21 is predominantly localized to the cellular cytoplasm (Keeshan et al., 2003). CML is characterized by the presence of the oncogenic chimeric protein, Bcr-Abl. Bcr-Abl has been shown to upregulate p21 expression and activate the serine/threonine kinase Akt in a PI3-K independent manner. Akt then phosphorylates p21 and promotes nuclear export of p21, ultimately resulting in an aggressive, drug-resistant CML phenotype.

P27^{Kip1}

P27^{Kip1} (p27) is a potent cell-cycle inhibitor which is expressed at its highest levels in the G₀-G₁ transition of the cell-cycle. P27 forms a heterotrimer complex with cyclin E-CDK2 and inhibits its activity, effectively preventing cell-

cycle progression out of the G₀ cell-cycle phase. P27 function is inactivated by several mechanism including nuclear export, cytoplasmic sequestration and proteosome-ubiquitin degradation (Susaki and Nakayama, 2007).

Nuclear export of p27 is mediated by the CRM1 export receptor and p27 must be phosphorylated at serine 10 (S10) for CRM1 binding to occur (Ishida et al., 2002). Substitution of S10 with alanine by site-directed mutagenesis prevented co-immunoprecipitation of p27 with CRM1 and prevented nuclear export of p27. In addition, nuclear export of p27 was blocked by incubation with the CRM1 inhibitor leptomycin B (Foster et al., 2003). Before the cells can leave G₀ and enter G₁ p27 must be depleted or exported from the nucleus. In G₀ nuclear import proceeds CRM1 mediated nuclear export after phosphorylation at S10. Once exported, p27 undergoes two additional phosphorylations at threonine 157 (T157) and threonine 187 (T187). Akt mediates phosphorylation of T157 in the cytosol which functions to prevent nuclear re-entry by blocking the nuclear localization signal (Shin et al., 2005). High-frequency of Akt-mediated phosphorylation of p27 with subsequent cytoplasmic localization has been found in breast cancer and may be elicited by estrogens (Foster et al., 2003). Cytoplasmic localization of p27 and phosphorylation by Akt is an indicator of poor prognosis in acute myelogenous leukemia (Min et al., 2004). Phosphorylation at T187 also occurs in the cytosol but is mediated by cyclin E-Cdk2. Cdk2 phosphorylation of p27 at T187 allows it to form a complex with a ubiquitin ligase, it is then degraded by proteolysis in the cytoplasm during G₁ to S phase (Connor

et al., 2003).

NUCLEAR EXPORT AND POTENTIAL DRUG TARGETS

Preventing nuclear export of tumor suppressors and drug targets could be clinically useful in the treatment of cancer. Protein trafficking can be modulated by phosphorylation which reveal NES amino acid sequences, therefore, blocking protein modification especially by phosphorylation could prove useful. In our lab we were able to block nuclear export of topo II α by preventing phosphorylation of a specific serine residue in the carboxyl terminal region. Using a specific inhibitor of casein kinase II, 4,5,6,7-tetrabromobenzotriazole (Calbiochem), we were able to prevent nuclear export of topo II α and sensitize multiple myeloma cells to the topo II poisons doxorubicin and VP-16. In addition, we were able to sensitize cells using siRNA knockdown of casein kinase II (data not shown). To prove that phosphorylation of a specific amino acid led to nuclear export we mutated serine 1524 in a FLAG-tagged topo II vector from a serine to an alanine which prevented nuclear export in transfected cells. Small molecule kinase inhibitors may provide similar effects for other tumor suppressor protein targets such as Akt phosphorylation of FOXO (Brunet et al., 1999) and p21^{Cip1} (Zhou et al., 2001a), β -catenin phosphorylation by GSK3 β (Rosin-Arbesfeld et al., 2003; Senda et al., 2007), and hKIS phosphorylation of P27^{Kip1} (Boehm et al., 2002).

To date no chemical agents have been shown to inhibit nuclear import receptors such as importin- α , however, a number of substances have been found

to inhibit the function of the nuclear export receptor CRM1.

A recent study performed on ovarian cancer biopsies, it was found that CRM1 protein expression was upregulated in aggressive, late-stage cancers (Noske et al., 2008). Increased CRM1 expression was found to be a negative prognostic indicator in ovarian cancer and may prove to be a good therapeutic target.

Leptomycin B was the first specific CRM1 inhibitor to be discovered. Leptomycin B was isolated from *Streptomyces* bacteria by investigators who were searching for new antibiotic reagents (Hamamoto et al., 1983). Leptomycin B modifies CRM1 at a reactive site cysteine residue (Cys-529) by a Michael-type covalent addition which blocks binding of the NES to CRM1 at nanomolar concentrations (Kudo et al., 1999). Leptomycin B was tested in a phase 1 clinical trial but was not found to be clinically useful due to severe toxicities (Newlands et al., 1996).

Additional CRM1 inhibitors have been found, including the ratjadone A-D compounds (Falini et al., 2006; Kalesse et al., 2001; Koster et al., 2003). Ratjadones, which have a different chemical structure than Leptomycin B, also modify CRM1 at Cys-529 (Meissner et al., 2004). We found that ratjadone C inhibited nuclear export of topo II α and sensitized myeloma cells to the topo II inhibitors doxorubicin and VP-16 when used at single nanomolar concentrations (data in press). Knockdown of topo II α by siRNA abrogated this effect, demonstrating that it was topo II specific. However, we found that ratjadone also

sensitized cells to several additional chemotherapeutic agents tested including topotecan and *cis*-platinum (unpublished data). This may indicate that blocking CRM1 may sensitize cancer cells by preventing export of additional tumor suppressors or cell-cycle inhibitors.

In a study performed by Kau et al, a biological screening regimen was used to identify additional inhibitors of CRM1. The original purpose of this study was to maintain FOXO family transcription factors in the nucleus as a potential cancer therapy (Kau et al., 2003). From a library of small molecules, 19 were identified as general nuclear export inhibitors. Cells were transfected with an HIV Rev/GFP fusion protein, treated with the CRM1 small molecule inhibitors and assayed by fluorescence microscopy for nuclear export. The HIV Rev/GFP fusion protein contains a very strong nuclear export motif which binds to and is exported by CRM1. Of the 19 small molecule inhibitors identified, 11 were found to covalently modify CRM1 at Cys-529 by a Michael-type reaction, similar to Leptomycin B and ratjadone. Others were found to modify Cys-529 by either nucleophilic attack, substitution by a good leaving group or because of an undetermined chemical rearrangement.

In conclusion, CRM1 inhibitors may or may not be useful as a single agent, but their usefulness could be to potentially enhance other drugs when used in combination. Sequestering a variety of drug targets, tumor suppressors, cell cycle inhibitors or apoptosis inducing proteins in the nucleus and restoring

their normal anti-proliferative cell activity may sensitize cells to a number of different anti-cancer agents or treatments.

Chapter 2

Density-dependent drug resistance to topoisomerase II inhibitors in human multiple myeloma cells is abrogated by CRM1 inhibition

SUMMARY

We have previously demonstrated that topoisomerase II α is exported from the nucleus of human multiple myeloma cells by a CRM1-dependent mechanism at densities similar to those in patient bone marrow. This results in resistance to topoisomerase II poisons since the enzyme is trafficked to the cytoplasm where it is not in contact with the DNA, and thus unable to produce DNA cleavable complexes and cell death.

We inhibited topoisomerase II α nuclear export to determine whether nuclear localization of this enzyme would sensitize cells to topoisomerase II α poisons. Three methods were used to block topoisomerase II α nuclear export, the CRM1-specific inhibitor ratjadone C, CRM1 targeted siRNA knockdown, and knockdown of CRM1 expression by anti-sense oligonucleotides.

Immunofluorescence microscopy showed that both ratjadone C and CRM1 siRNA effectively inhibited nuclear export of topoisomerase II α . CRM1 specific siRNA produced an 81.5% knockdown of CRM1 protein. Three human myeloma cell lines, (8226, H929 and U266), were treated with ratjadone C or CRM1 specific siRNA and exposed to either doxorubicin or etoposide at high cell

densities. CRM1 treated cells were four-fold more sensitive to topoisomerase II poisons as determined by an apoptosis assay. Cell death was correlated with increased DNA double strand breaks, as shown by the comet assay. Band depletion assays of myeloma cells exposed to the CRM1 inhibitor increased topoisomerase II α covalently bound to DNA.

These results suggest that blocking topoisomerase II α nuclear export sensitizes myeloma cells to topoisomerase II inhibitors. This method of sensitizing human myeloma cells suggests a new therapeutic approach in this disease.

INTRODUCTION

DNA topoisomerases are ubiquitous enzymes that function to relieve the torsional strain in DNA for several critical intracellular processes (Wang, 2002). Topoisomerase II α (topo II α) is an important anti-neoplastic drug target. Clinically useful topo II poisons include etoposide (VP-16), doxorubicin, daunomycin, epirubicin and mitoxantrone (Burden et al., 1993; Gatto and Leo, 2003; Nitiss, 2002; Pommier et al., 2003; Sordet et al., 2003). Topoisomerase II poisons are agents that stabilize the covalent DNA-topo II complexes. During DNA replication the stabilized cleavable complexes are converted into DNA strand breaks, the accumulation of which ultimately results in cell death (Bertrand et al., 1991).

Resistance to topo II α poisons is a major obstacle in the treatment of multiple myeloma (MM). Topo II α poisons that are used in the treatment of MM

include mitoxantrone, doxorubicin, and etoposide (Kraut et al., 1998). Several mechanisms of resistance to topo II inhibitors have been described (Oloumi et al., 2000; Rasheed and Rubin, 2003; Sullivan et al., 1989; Sullivan and Ross, 1991; Valkov and Sullivan, 1997).

One mechanism of drug resistance is overexpression of drug efflux pumps such as p-glycoprotein (PGP), multidrug resistance proteins (MRP) and major vault protein (MVP). Topo II poisons are known substrates for PGP, MRP, and MVP. Previous analyses of patient myeloma cells have demonstrated that PGP and MVP are often overexpressed in plasma cells, although MRP is infrequently overexpressed (Fishman and Sullivan, 2001; Rimsza et al., 1999; Schwarzenbach, 2002).

Other mechanisms of drug resistance include topo II mutations that alter enzyme activity and mutations that produce a loss of the topo II nuclear localization signal so that the molecule remains in the cytoplasm (Feldhoff et al., 1994; Yu et al., 1997) These mechanisms have been found to be limited to cell lines and have not been reproduced *in vivo*.

Cell adhesion mediated drug resistance (CAM-DR) and stromal cell adherence are important parameters in the local bone marrow environment in MM patients and appear to be major determinants of drug resistance. Data from several laboratories have shown that the microenvironment may play a significant role in the drug resistance to antineoplastic agents. Hazlehurst et al have shown that fibronectin-adherent human U937 leukemia cells were resistant

to mitoxantrone because of a redistribution of topoisomerase II β to the nucleolus (Hazlehurst et al., 2006; Hazlehurst et al., 2001). In addition, cell adhesion mediated drug resistance was found to block cell cycle progression, induce p27^{kip1} levels and ultimately result in cell cycle arrest and drug resistance (Hazlehurst and Dalton, 2001). Bone marrow stromal cell contact and stromal cell soluble factors have been reported to induce drug resistance to mitoxantrone in human myeloma cell lines (Nefedova et al., 2003).

Our laboratory in collaboration with others has shown that human MM cell density is a determinant of sensitivity to topo II α inhibitors (Engel et al., 2004; Turner et al., 2004; Valkov et al., 2000b). At increased cell densities, a significant fraction of nuclear topo II α is exported to the cytoplasm resulting in reduced sensitivity to etoposide, doxorubicin and mitoxantrone. This appears to occur both in human myeloma cell lines and in CD-138 positive cells isolated from patients with MM (Engel et al., 2004). We have previously shown that myeloma cells in transition from low-density, log phase conditions to high-density, plateau phase conditions exhibit a substantial export of endogenous topo II α from the nucleus to the cytoplasm (Valkov et al., 2000b). We have reported that nuclear export of topo II α may contribute to drug resistance (Engel et al., 2004) and our data suggest that resistance was not due to differences in drug uptake, cell cycle or cellular topo II α protein levels (Engel et al., 2004; Turner et al., 2004; Valkov et al., 2000b). In a recent report, we have defined nuclear export signals for topo II α at amino acids 1017-1028 and 1054-1066 (Turner et al., 2004). Export by both

signals was blocked by treatment of the cells with leptomycin B, indicating that a CRM1 dependent pathway mediates export (Turner et al., 2004). In this study we show that inhibiting CRM1 mediated export of topo II α may render myeloma cells more sensitive to topo II α targeted chemotherapy.

MATERIALS AND METHODS

Cell lines

Human myeloma cell lines, RPMI 8226 (8226) cells, U266B1 (U266) and NCI-H929 (H929) cells were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were grown in RPMI-1640 media containing 100 U/ml penicillin, 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C, 5% CO₂. H929 cell media required the addition of 0.025% β -mercaptoethanol (Sigma Chemical, St. Louis, MO).

Cell density and drug treatment

The model used to assay microenvironmental factors involved incubating cells at high and low-density culture conditions. We have shown previously that cells grown at different densities exhibit specific characteristics such as drug resistance and nuclear-cytoplasmic trafficking of topo II α (Engel et al., 2004; Turner et al., 2004; Valkov et al., 2000b). Human myeloma cell lines (8226,H929,U266) grown at 2×10^5 cells/ml were defined as low density (log-

phase), and cells grown at 2×10^6 cells/ml were defined as high density (plateau-phase). Cell lines were placed at log and plateau density conditions and cultured for 20 hours with and without the CRM1 inhibitor ratjadone C (5 nM) (HZI / Helmholtz Centre for Infection Research Department of Chemical Biology, Braunschweig, Germany) or transfected with CRM1 siRNA (200 nM) (Dharmacon, Lafayette, CO). Cells were then treated with doxorubicin (2 μ M) (Sigma Chemical), or etoposide (10 μ M) (Sigma Chemical, St. Louis, MO).

Immunofluorescent microscopy

Cell lines (1×10^5) were plated on double cytoslides (Shandon, Waltham, MA) by cyto-centrifugation at 500 rpm for 3 minutes and fixed with 1% para-formaldehyde (Fisher Scientific, Suwanee, GA) on ice for 30 minutes. Permeabilization of cells was performed with 0.5% Triton X-100 (Sigma Chemical) in PBS at room temperature for 60 minutes. Cells were stained with a polyclonal antibody against topo II β which was produced in this lab (PAB454). The topo II α antibody was diluted 1:100 in a buffer containing 1% BSA (Sigma Chemical), 0.1% NP-40 (Sigma Chemical) in PBS and incubated for 1 hour at room temperature or overnight at 4°C. After three washes with PBS, slides were incubated with a secondary anti-rabbit Alexa Fluor[®] 594 (Invitrogen) in addition to a cytoskeletal protein stain, phalloidin-Alexa Fluor[®] 488 conjugate (Invitrogen). Each were diluted 1:1000 in 1% BSA, 0.1% NP-40 in PBS and incubated for 40 minutes at room temperature. Slides were washed four times in PBS, once in

distilled water and the nuclei stained with diamindino-2-phenylindole dihydrochloride hydrate (DAPI, Vector laboratories, Burlingame, CA). Immunofluorescence was observed by a Zeiss microscope Axio Imager Z1 microscope (Carl Zeiss Microimaging, Thornwood, NY) with an Axiocam MRm camera (Carl Zeiss Microimaging).

Apoptosis assay

Apoptosis was assayed using four different assays, Annexin V-FITC/propidium iodide (BD Pharmingen, San Diego, CA), anti-caspase 3/PE (BD Pharmingen), TUNEL assay (BD Pharmingen), and mitochondrial membrane potential by DiI C1(5) (Invitrogen). Each apoptosis assay was used according to standard manufacturer's protocol. In all assays, apoptosis was analyzed using Flow Cytometry on a FACSCalibur bench top analyzer with FlowJo analysis software (Becton-Dickenson, Franklin Lakes, NJ).

CRM1 siRNA knockdown and Western blot

All electroporation transfections were performed in a freshly made transfection buffer containing 120 mM potassium chloride, 0.15 mM calcium chloride, 10 mM potassium phosphate (pH 7.6), 25 mM HEPES, 2 mM EGTA (pH 7.6), 5 mM magnesium chloride, 2 mM ATP (pH 7.6), 5 mM glutathione, 1.25% DMSO, and 50 mM trehalose (Sigma Chemical). Each transfection consisted of 3×10^6 myeloma cells. Cells were washed two times in phosphate

buffered saline and placed in a 200 µl volume of transfection buffer. CRM1 specific siRNA or a scramble control siRNA (Dharmacon) was added (200 nM), the sample placed in a 2 mm electroporation cuvette and transfected at 140 V and 975 µF in a Bio-Rad GenePulser Xcell electroporation unit (Biorad, Hercules, CA). Transfected cells were incubated in the cuvette for 15 minutes at 37°C in a 5% CO₂ incubator, transferred to a sterile T25 tissue culture flask, and 10 ml of fresh media added. After 48 hours the transfected cells were harvested by centrifugation, washed with cold PBS, and lysed by sonication in SDS buffer (2% SDS, 10% glycerol, 60 mM Tris, pH 6.8). Protein from 2x10⁵ cells per lane was separated on 8% SDS-PAGE gels and transferred to PVDF membranes (Amersham, Piscataway, NJ) using a Biorad Mini-Transblot Apparatus (Biorad). Membranes were blocked for one hour at ambient temperature in a blocking buffer containing 0.1 M Tris-HCl, 0.9% NaCl, 0.5% Tween-20 (TBST) and 5% non-fat dry milk. CRM1 was identified by incubation in a 1:1000 dilution of H-300 antibody (Santa Cruz Biotech, Santa Cruz, CA) in blocking buffer overnight at 4°C. Membranes were washed three times for 10 minutes in TBST, and incubated with for one hour with goat anti-rabbit polyclonal IgG antibody Horseradish Peroxidase linked antibody (Sigma Chemical) in blocking buffer at a 1:2000 dilution. Antibody binding was visualized by Enhanced Chemiluminescence (Amersham) on autoradiography film (Kodak, Rochester, NY).

Band depletion assay

Band depletion assays were performed as described by Xiao et al (Xiao et al., 2003). Briefly, 5×10^5 cells were lysed in 50 μ l alkaline lysis solution (200 mM NaOH, 2 mM EDTA) and the lysate neutralized by the addition of 4 μ l of both 1 M HCl and 1.2 M Tris (pH 8.0). The lysate was then mixed with 30 μ l 3X SDS sample buffer (150 mM Tris-HCl, pH 6.8, 6 mM EDTA, 45% sucrose, 9% SDS, 10% β -mercaptoethanol) and the lysates were separated on 8% SDS-PAGE gels.

Comet assay

Log density H929 myeloma cells were plated at a concentration of 2×10^5 cell/ml and plateau density cells were plated at 2×10^6 /ml. All cells were grown in a 24 well plate (Falcon) using 1 ml per well. Drug treatment groups were vehicle only (1 μ l/ml DMSO), 10 μ M etoposide, 5 nM ratjadone C, or a combination of 10 μ M etoposide and 5 nM ratjadone C. Cells that were treated with ratjadone C were first plated at log or plateau densities and incubated for 20 hours with ratjadone C or vehicle, after which etoposide was added for 1 hour. After a one hour etoposide exposure the comet assay was performed as described by Kent et al (Kent et al., 1995), and modified by Chen et al (Chen et al., 2005). To ensure random sampling, fifty images were captured per slide on a Vysis fluorescent microscope and quantified using ImagequantR software (Molecular Dynamics, Sunnyvale Ca). The average comet moment value obtained from

vehicle control samples was subtracted from the average comet moment of each drug treatment sample. The data shown are means and standard deviations of two separate experiments. An analysis of the data was performed by student's t-test.

RESULTS

Log and plateau density myeloma cells

The myeloma cell lines, 8226, H929, and U266, were grown at log-density (2×10^5 cells/ml media), and plateau-density (2×10^6 cells/ml) growth conditions for 20 hours. Cells were then treated with the topo II α inhibitor, doxorubicin (1 μ M) for four hours. Cells were then assayed for apoptosis by caspase 3 expression assay. We found that cells grown at plateau densities, and treated with 1 μ M doxorubicin had extremely low levels of apoptosis as compared to log-phase cells (figure 2.1A). This data confirmed previous publications where we have shown that cells grown at different densities exhibit specific characteristics such as drug resistance and nuclear-cytoplasmic trafficking of topo II α (Engel et al., 2004; Turner et al., 2004; Valkov et al., 2000b).

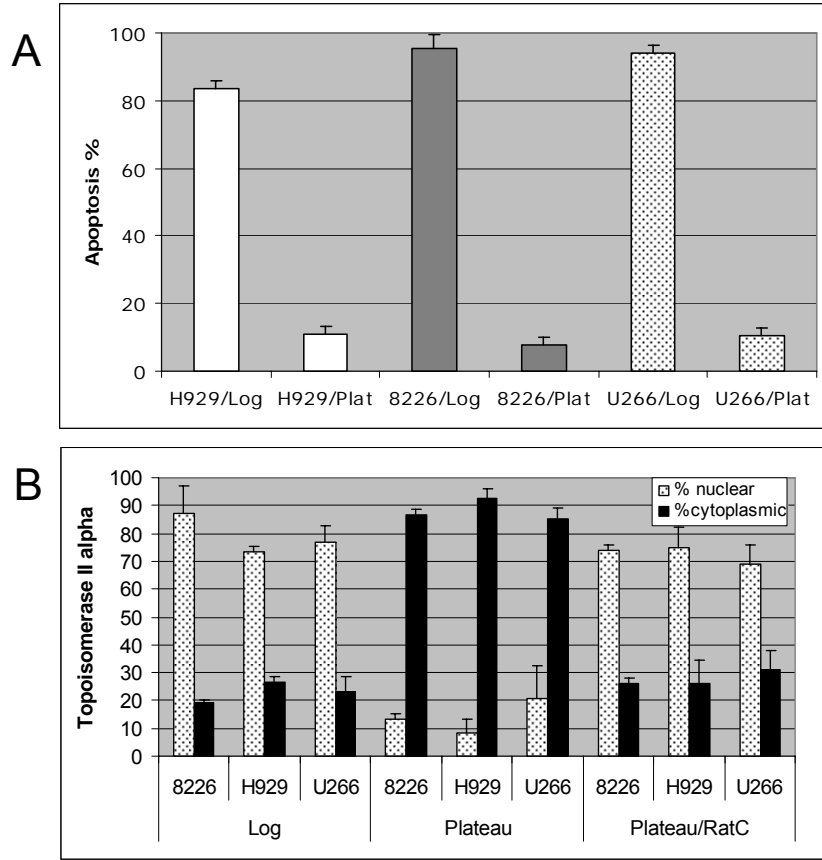


Figure 2.1: Intracellular trafficking of topo II α in log and plateau density myeloma cells. **2.1A:** H929 and 8226 human myeloma cells grown at plateau-phase (high-density) export topo II α , whereas log phase (low-density) cells maintain topo II α in the nucleus. Cells were grown 20 hours at log or plateau densities, treated with 1 μ M doxorubicin for 4 hours (n=2). Apoptosis was determined by caspase 3 staining using flow cytometry (10,000 cells). Cells which maintained nuclear topo II α were more sensitive to topo II α targeted chemotherapy. **2.1B:** Log, plateau, or ratjadone C treated cells (100 cells/experiment) were stained for topo II α by fluorescence microscopy (n=2). Myeloma cells grown in log-phase conditions had the majority (>90%) of the topo II α in the nucleus, whereas plateau-phase cells exported the topo II α into the cytoplasm. The CRM1 inhibitor ratjadone C was found to block export of topo II α in cells grown in plateau-phase conditions.

Intracellular trafficking of topo II α

Log, plateau, or ratjadone C treated cells (100 cells/experiment) were scored as “nuclear” or “cytoplasmic” if the majority (>90%) of topo II α was in that compartment as determined by fluorescence microscopy (figure 2.1B).

Myeloma cells grown in log-phase conditions had >90% of the topo II α in the nucleus, whereas plateau-phase cells exported >90% of the topo II α into the cytoplasm. CRM1 inhibition by ratjadone C was found to block export of topo II α in cells grown in plateau-phase conditions. As seen in figure 2.2 (8226/U266 cells) and figure 2.4 (H929 cells), topo II α (red) is exported into the cytoplasm in plateau-phase cells, but is maintained in the nucleus in log-phase cells. In these photomicrographs the nucleus is labeled using DAPI (blue). Log, plateau, or ratjadone C treated cells (100 cells/experiment) were scored as “nuclear” or “cytoplasmic” if the majority (>90%) of topo II α was in that compartment as determined by fluorescence microscopy (figure 2.1B). Myeloma cells grown in log-phase conditions had >90% of the topo II α in the nucleus, whereas plateau-phase cells exported >90% of the topo II α into the cytoplasm.

CRM1 inhibition by ratjadone C was found to block export of topo II α in cells grown in plateau-phase conditions. As seen in figure 2.2 (8226/U266 cells) and figure 2.4 (H929 cells), topo II α (red) is exported into the cytoplasm in plateau-phase cells, but is maintained in the nucleus in log-phase cells. In these photomicrographs the nucleus is labeled using DAPI (blue).

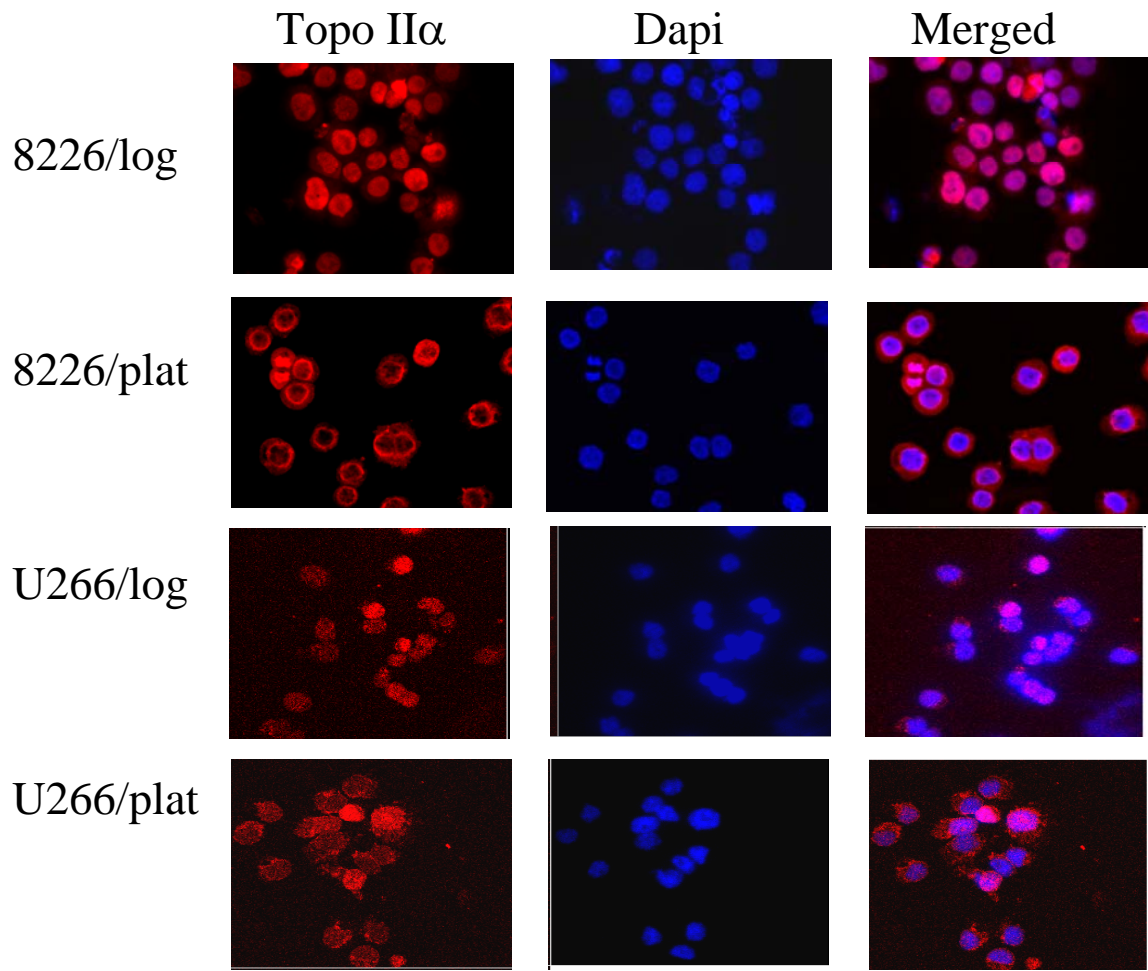


Figure 2.2: Cellular localization of topo II α in 8226 and U266 cells under log and plateau-density growth conditions. 8226 and U266 human myeloma cells were grown at log and plateau densities, fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and stained for topo II α (red), and DNA (DAPI-blue). Results indicate that topo II α is present in the nucleus of log density cells and is exported from the nucleus in plateau density cells.

CRM1 inhibitor and topo II α chemotherapeutics

Figure 2.3 illustrates that at high cell density (plateau) drug resistance was reversed by the CRM1 inhibitor, ratjadone C. The human myeloma cell lines 8226, H929 and U266 were incubated at high-densities (2×10^6 /ml) for 20 hours with CRM1 inhibitor ratjadone C (5 nM). Topo II α targeted agents etoposide (10 μ M) and doxorubicin (2 μ M), were then added, the cells further incubated an additional 7 hours and then assayed for apoptosis using the TUNEL assay (BD Pharmingen). Blocking CRM1-mediated nuclear export increased the effectiveness of the topo II targeted drugs to induce apoptosis in all three cell lines approximately four-fold, as compared to drug only controls. Additional apoptosis assays, caspase 3, annexin V staining, and mitochondrial membrane potential (DILC1(5)) staining demonstrated similar results (data not shown). In addition, to show whether the ratjadone C /doxorubicin and the ratjadone C /etoposide synergistic activity was due to topo II α nuclear localization, cells were transformed with a siRNA to knockdown topo II α expression. In all three cell lines and with both topo II inhibitors (doxorubicin and etoposide), we found that knock down of topo II α protein expression reversed the synergistic effect and reduced apoptosis to control (untreated) levels.

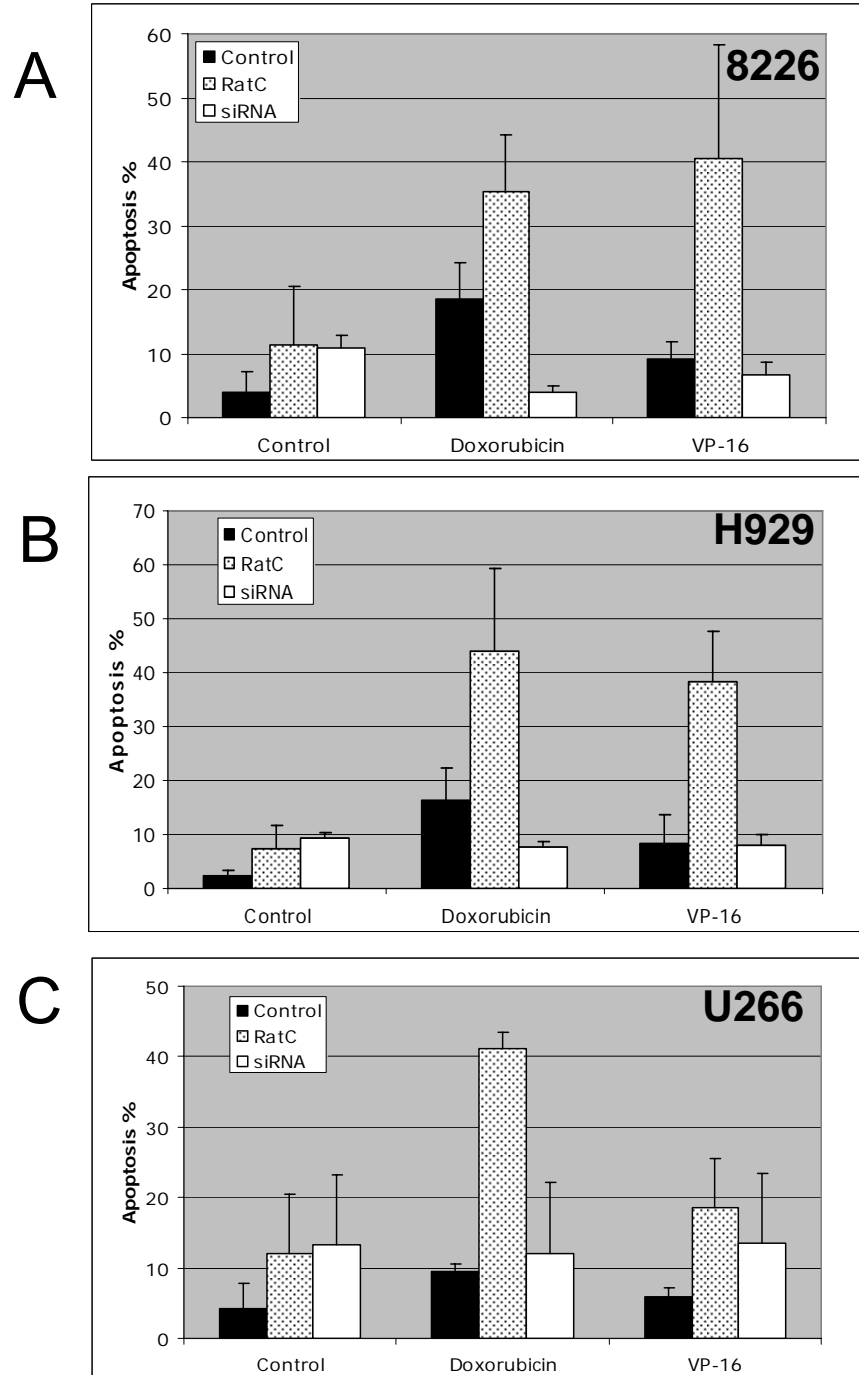


Figure 2.3: CRM1 inhibitor and topo II α chemotherapeutics. Human myeloma cells were incubated at high-densities (2×10^6 /ml) for 20 hours with the CRM1 inhibitor ratjadone C (5 nM). Cell cultures were then exposed to topo II α targeted agents etoposide (10 μ M) n=5, doxorubicin (2 μ M) n=9, for 7 hours and assayed for apoptosis using TUNEL assay (BD Pharmingen). Cells were also transfected with a siRNA specific to topo II α . CRM1 inhibition increased the effectiveness of DNA-damaging agents to induce apoptosis; this effect was reversed by topo II α siRNA knockdown and therefore is topo II α dependent.

Topo II α trafficking and CRM1 inhibition

H929 human myeloma cells were grown at log and plateau densities and stained for cytoskeletal protein (phalloidin-green), topo II α (red), and DNA (DAPI-blue). Results indicate that topo II α was present in the nucleus of log density cells and was exported from the nucleus in plateau density cells (figure 2.4). Nuclear export was blocked in plateau cells by a CRM1 inhibitor ratjadone C and by transfection with CRM1-specific siRNA. Under the conditions of this experiment, CRM1 siRNA knockdown was 69%. Ratjadone C treated plateau density cells are shown in figure 2.1B where the majority of topo II α was localized in the nucleus of each myeloma cell line.

CRM1 inhibitor and topo II α inhibitor synergy

Plateau myeloma cell lines 8226, H929 and U266 were grown for 20 hours, in the presence of ratjadone C (5 nM). The cells were then treated with increasing doses of doxorubicin (0, 0.5, 1, and 2 μ M) for four hours and assayed for apoptosis by Caspase 3 staining using flow cytometry. Data in figures 2.5A (8226), 2.5B (H929), 2.5C (U266), demonstrate that myeloma cells were rendered more sensitive to topo II α inhibitors in a dose-dependent manner by inhibiting CRM1 export with ratjadone C.

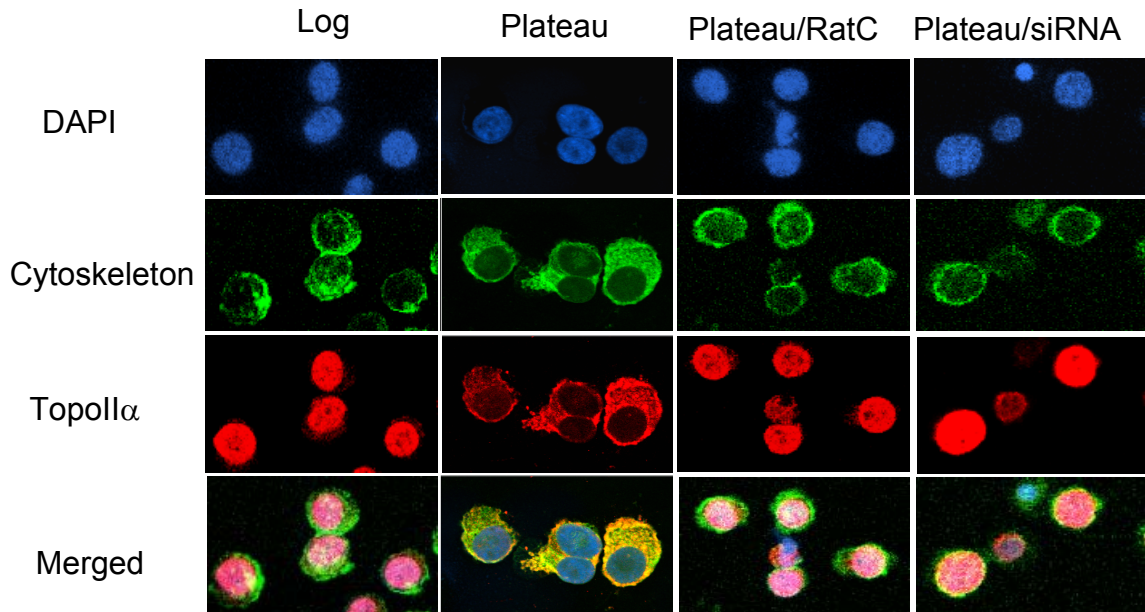


Figure 2.4: H929 topo II α immunofluorescence. H929 human myeloma cells were grown at log and plateau densities, fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and stained for cytoskeletal protein (phalloidin-green), topo II α (red), and DNA (DAPI-blue). Results indicate that topo II α is present in the nucleus of log density cells and is exported from the nucleus in plateau density cells. However, nuclear export is blocked in plateau cells by a CRM1 inhibitor ratjadone C and by transfection with CRM1 specific siRNA. Under the conditions of this experiment, CRM1 siRNA knockdown was 69%.

CRM1 siRNA sensitizes myeloma cells to topo II α poisons

In addition to CRM1 pharmacologic modification by a chemical agent, we used a CRM1 specific siRNA to determine if we could reproduce the topo II α inhibitor synergistic activity in another model system. H929 cells were transfected by electroporation with a CRM1 specific siRNA. After transfection, the cells were incubated at log-phase densities for 20 hours, and then concentrated at plateau-phase conditions. At 48 hours post-transfection the cells were treated with the topo II α inhibitor, doxorubicin (2 μ M) and assayed for apoptosis by Annexin V staining using flow cytometry, (figure 2.6A). CRM1 knockdown was found to increase the effectiveness of doxorubicin. To demonstrate that we were getting efficient siRNA knockdown, SDS lysates of equal cell numbers were assayed for CRM1 by Western blot (figure 2.6B). Percentage of knockdown, as compared to a control scramble siRNA, was assayed using Adobe Photoshop by pixel intensity of the CRM1 bands. A maximum knockdown of 81.5% occurred at 72 hours post transfection.

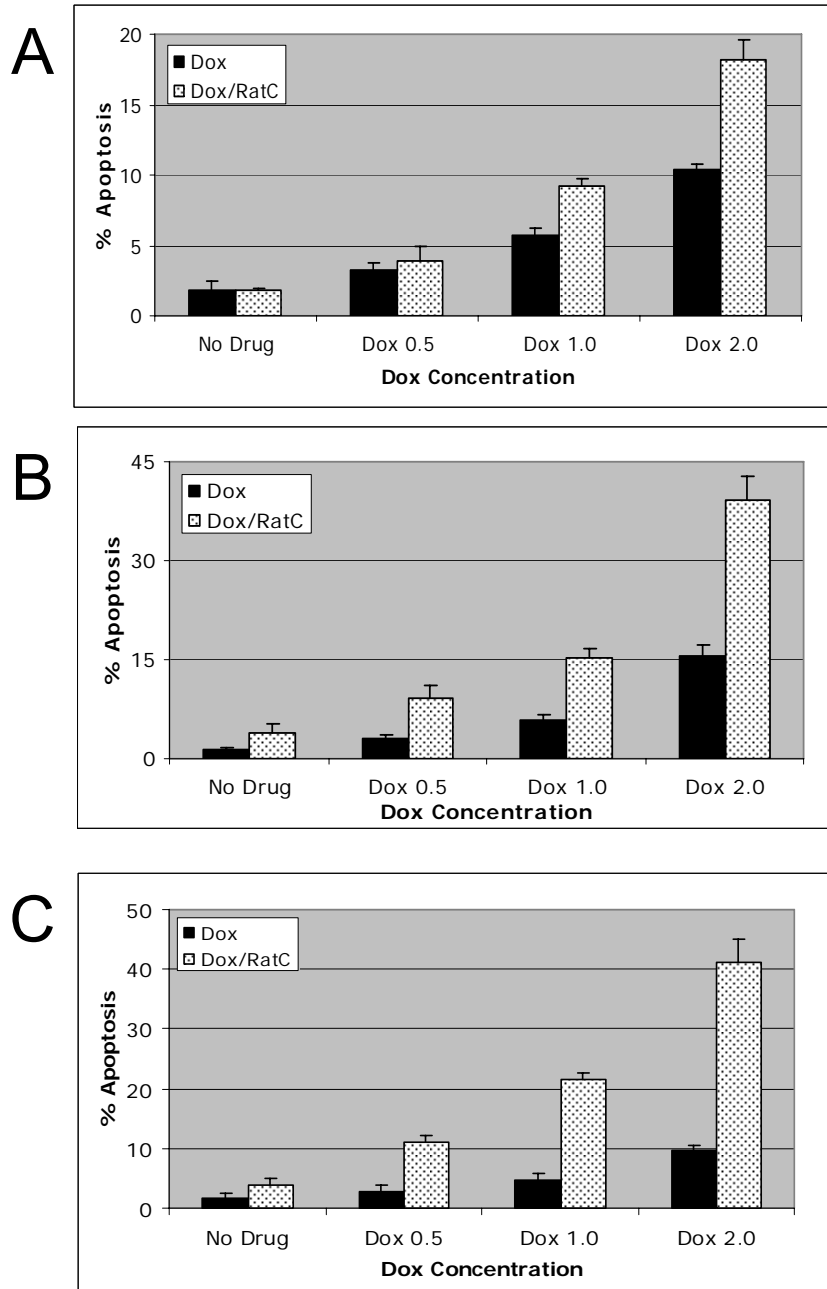


Figure 2.5: CRM1 inhibitor and topo II α inhibitor synergy. Myeloma cell lines 8226 (A), H929 (B), U266 (C), at 2×10^6 cells/ml were grown in culture for 20 hours. Cells were incubated with the CRM1 inhibitor ratjadone C (5 nM) for 20 hours. Cells were then treated with doxorubicin (0, 0.5, 1, and 2 μ M) for four hours and assayed for caspase 3 staining by flow cytometry. Myeloma cells are made more sensitive to topo II α inhibitors in a dose-dependent manner by inhibiting CRM1 export.

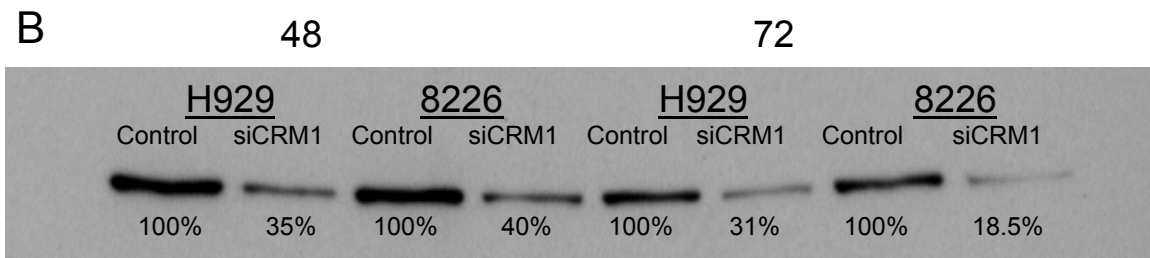
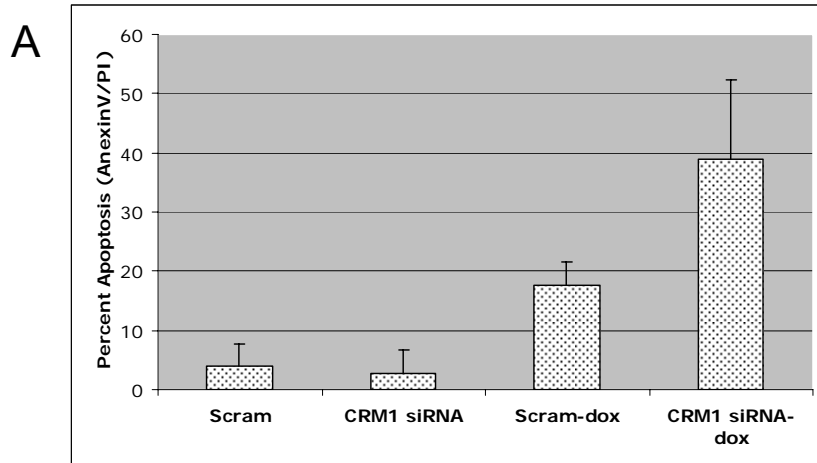


Figure 2.6: CRM1 knockdown using siRNA makes myeloma cells more sensitive to the top II α poison doxorubicin. **2.6A:** H929 cells were transfected with siRNA, incubated at log-phase for 20 hours, and concentrated at plateau-phase conditions. At 48 hours cells were treated with the top II inhibitor doxorubicin (2 μ M) and assayed for apoptosis by Annexin V staining using flow cytometry, n=2. **2.6B:** Western blot data for siRNA transfection. Percent knockdown was compared to control siRNA (scramble). CRM1 knockdown renders plateau density cells more sensitive to top II α inhibitors.

Topoisomerase Western blot

Cell nuclei contain three different topoisomerases; topoisomerase I, II α , and II β . To determine whether cell density conditions (log/plateau) affected the levels of endogenous topoisomerases, we assayed whole cell lysates by SDS-PAGE analysis. Topoisomerase Western blot assay based both on cell number and total protein were found to be nearly identical (data not shown). In all three myeloma cells lines topoisomerase I, II α and II β protein levels were relatively unchanged or changed very slightly in log and plateau density growth conditions (figure 2.7).

Increase in cleavable complex formation by CRM1 inhibition

The band depletion assay indicated that a combination of ratjadone C and etoposide produced more DNA/topo II α complexes, depleting the topo II α band in the Western blot analysis (figure 2.8A). These data indicates that blocking nuclear export of topo II α will increase the effectiveness of etoposide and induce apoptosis by increased cleavable complexes.

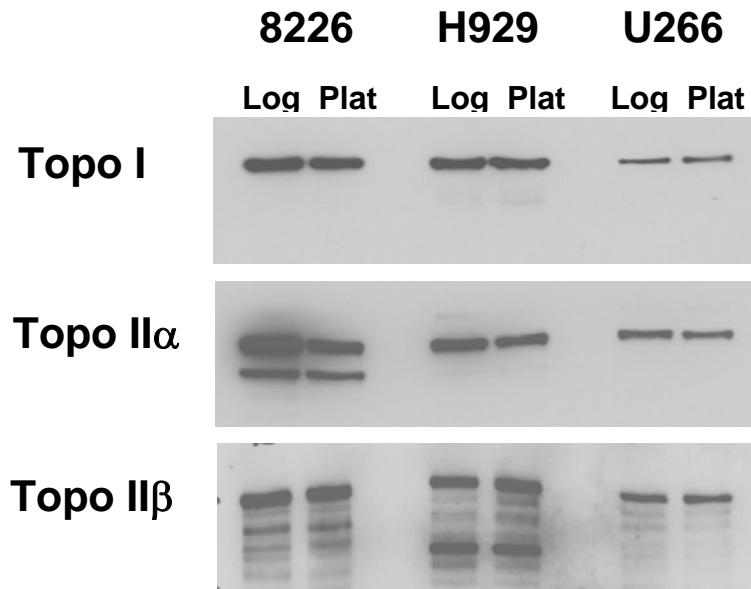


Figure 2.7: Log and plateau expression of topoisomerases in myeloma cell lines. All three human myeloma cell lines were assayed for endogenous topoisomerase protein expression by Western blot. Cells were compared in the same blots under log and plateau-phase growth conditions. No relative differences were found when equal protein loading was compared to equal cell numbers (data not shown). The data suggests that topoisomerases I, II α and II β did not change or changed very slightly in log vs. plateau cells.

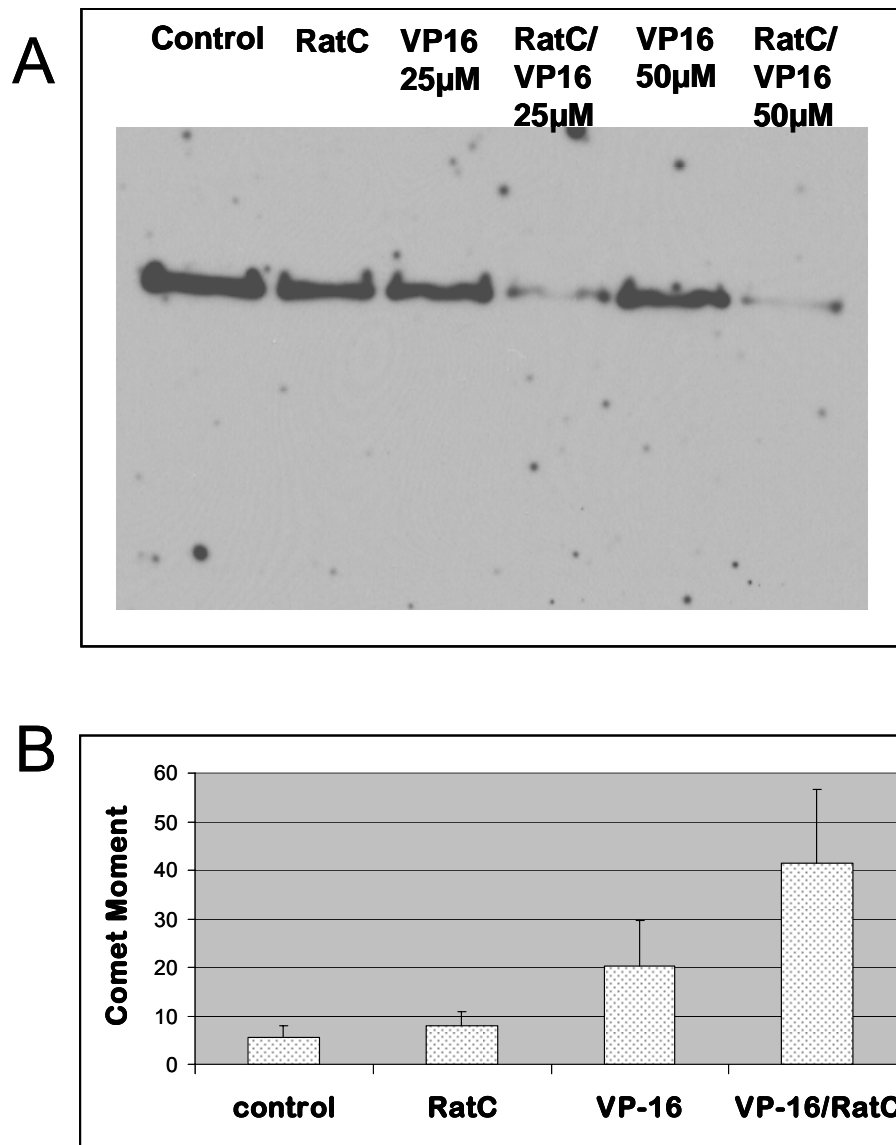


Figure 2.8: Band depletion and comet assay. **2.8A:** Band depletion. The combination of ratjadone C and etoposide produced more DNA/topo II α complexes, depleting the topo II α band in the Western blot analysis. This data indicates that blocking nuclear export of topo II α will increase the effectiveness of etoposide and induce apoptosis. **2.8B:** Comet assay. Plateau density H929 cells were treated with ratjadone C (5nM) 20 hours and then with etoposide (10 μ M) for 60 minutes. DNA fragmentation was measured by the comet assay. The CRM1 inhibitor ratjadone C, increased DNA fragmentation from the topo II α inhibitor etoposide.

Comet assay

Plateau density H929 cells were treated with 5 nM ratjadone C for 20 hours and then with 10 μ M etoposide for 60 minutes. DNA fragmentation was measured by neutral comet assay. The CRM1 inhibitor ratjadone C, increased DNA fragmentation induced by the topo II α inhibitor, etoposide (figure 2.8B). Increased DNA fragmentation led to increased apoptosis in etoposide/ ratjadone C treated cells.

DISCUSSION

Topo II α inhibitors function by stabilizing cleavable complexes resulting in DNA strand breaks. Accumulation of DNA strand breaks will eventually result in cell apoptosis. In order for DNA strand breaks to be produced, the enzyme must be in the nucleus and in contact with the genomic DNA. Therefore, preventing nuclear export of topo II α by CRM1 inhibition may improve the function of topo II α inhibitors.

The intracellular location of a protein may be at least as important as its expression. Diseases as dissimilar as cystic fibrosis (Welsh and Smith, 1993), schizophrenia (Karpa et al., 2000), nephrogenic diabetes insipidus (Edwards et al., 2000) and many types of cancers [reviewed in (Davis et al., 2007)] may be caused by intracellular mislocalization of individual proteins. Specific examples of proteins that must be in the nucleus to prevent cancer initiation, progression or chemotherapeutic response include, p53 [reviewed in (Fabbro and Henderson,

2003)], galectin-3 (Takenaka et al., 2004), FOXO (Nakamura et al., 2000), INI1/hSNPF5 (Craig et al., 2002), p27Kip1 (Min et al., 2004), p27WAF1 (Keeshan et al., 2003), and topo II α (Engel et al., 2004; Turner et al., 2004; Valkov et al., 2000b). Mislocalization of a protein can render it ineffective as a tumor suppressor or as a target for chemotherapy. However, it is possible that blocking nuclear export of any or all these proteins may induce tumor suppression, apoptosis, or in the case of topo II α may reverse drug resistance to topo II α inhibitors. This may be true in MM where the cells possess a CRM1-mediated mechanism whereby topo II α is exported from the nucleus and away from the DNA, rendering topo II α inhibitors ineffective to produce cleavable complexes and DNA strand breaks.

In previous reports, we have shown that myeloma cells, under high-density conditions, will export topo II α into the cytoplasm both *in vivo* and *in vitro* (Engel et al., 2004; Turner et al., 2004; Valkov et al., 2000b). We found that nuclear export of topo II α contributes to drug resistance (Engel et al., 2004) and the resistance was not due to differences in drug uptake, cell cycle or cellular topo II α protein levels. In addition, topo II α nuclear export has been shown to be CRM1-mediated and that topo II α protein contains two functional nuclear export signals at amino acids 1017-1028 and 1054-1066 (Turner et al., 2004). Export by both signals was blocked by treatment of the cells with leptomycin B indicating that a CRM1-dependent pathway mediates export (Turner et al., 2004).

In this study, we demonstrated that myeloma cells grown at high density are highly resistant to topo II α directed chemotherapeutic drugs (figure 2.1A) and that drug resistance correlated with nuclear export of topo II α (figures 2.1B, 2.2, and 2.4). Based on these data we proposed that blocking CRM1-mediated export of topo II α may make myeloma cells more sensitive to topo II directed chemotherapy. To evaluate whether the lack of topo II α export would sensitize cells, we were able to knockdown CRM1 mRNA and protein expression in cells transfected with CRM1 specific siRNAs, and by using the CRM1-inhibiting drug, ratjadone C. CRM1 inhibition by siRNA and ratjadone C in human myeloma cells was found to prevent nuclear export of topo II α in plateau density cell cultures (figures 2.1B and 2.4). Depletion or inhibition of CRM1 by siRNA or ratjadone C caused high-density myeloma cells to become four-fold more sensitive to the topo II inhibitors, doxorubicin and etoposide as measured by apoptosis (figures 2.3 and 2.5). Depletion of topo II α protein by specific topo II α siRNA knockdown reversed this synergistic effect, indicating that topo II α was the targeted molecule for CRM1 synergistic activity (figure 2.6).

In conclusion, maintaining topo II α in the nucleus by inhibiting CRM1 greatly enhanced the cytotoxic effect of the topo II α inhibitors, doxorubicin and etoposide in myeloma cells. Band depletion assays indicated that more DNA/topo II α complexes were stabilized in cells when CRM1 was inhibited because there was more topo II α present in the cell nuclei (figure 2.8A).

Increased cleavable complexes resulted in increased strand breaks (comet assay) (figure 2.8B) and subsequent apoptosis. These findings may have potential therapeutic value in the treatment of multiple myeloma.

Chapter 3

ABCG2 expression, function and promoter methylation in human multiple myeloma

SUMMARY

We investigated the role of the breast cancer resistance protein (BCRP/ABCG2) in drug resistance in multiple myeloma (MM). Human MM cell lines, and MM patient plasma cells isolated from bone marrow, were evaluated for ABCG2 mRNA expression by quantitative PCR, and ABCG2 protein by Western blot analysis, immunofluorescence microscopy and flow cytometry. ABCG2 function was determined by measuring topotecan and doxorubicin efflux using flow cytometry, in the presence and absence of the specific ABCG2 inhibitor, tryprostatin A. The methylation of the ABCG2 promoter was determined using bisulfite sequencing. We found that ABCG2 expression in myeloma cell lines increased after exposure to topotecan and doxorubicin, and was greater in log-phase cells when compared to quiescent cells. Myeloma patients treated with topotecan had an increase in ABCG2 mRNA and protein expression after treatment with topotecan, and at relapse.

Expression of ABCG2 is regulated, at least in part, by promoter methylation both in cell lines and in patient plasma cells. Demethylation of the promoter increased ABCG2 mRNA and protein expression.

These findings suggest that ABCG2 is expressed and functional in human myeloma cells, regulated by promoter methylation, affected by cell density, upregulated in response to chemotherapy, and may contribute to both intrinsic and acquired drug resistance.

INTRODUCTION

The development of drug resistance to chemotherapeutic agents remains one of the primary obstacles in cancer treatment. Membrane drug-efflux pumps such as P-glycoprotein (MDR1), multidrug resistance protein (MRP), and ABCG2 have been shown to produce resistance to several commonly used chemotherapeutic agents. Breast cancer resistance protein (BCRP) or ATP binding cassette protein G2 (ABCG2) is a 655 amino-acid polypeptide transporter that forms a homodimer (Doyle et al., 1998) and has been reported as a tetramer in plasma membranes (Xu et al., 2004). ABCG2 is a half-transporter, containing a single N-terminal ATP-binding cassette and six transmembrane segments. ABCG2 was first described in drug resistant MCF-7/AdrVp cells (Doyle et al., 1998) and has been the subject of recent reviews (Abbott, 2003; Allen and Schinkel, 2002; Doyle and Ross, 2003) Like other members of the ATP binding cassette family of membrane transporters, such as MDR1 and MRP1, ABCG2 is expressed in a variety of malignancies, where it may produce resistance to chemotherapeutic agents. Among cultured human cell lines that express high levels of ABCG2 are fibrosarcoma, ovarian cancer, breast cancer, and myeloma

cell lines (Allen et al., 1999). Human neoplasms frequently found to express ABCG2 protein include: adenocarcinomas arising from the digestive tract, the endometrium, and the lung; melanoma; soft tissue sarcomas (Candeil et al., 2004; Diestra et al., 2002) and hematological malignancies such as acute myeloid leukemia (AML) (Litman et al., 2000) and acute lymphoblastic leukemia (ALL) (Sauerbrey et al., 2002). Several studies have been performed to investigate potential correlations between ABCG2 expression and clinical outcomes. Studies from patients with AML demonstrated significantly increased expression of ABCG2 mRNA in the relapsed/refractory samples compared to pre-treatment (van den Heuvel-Eibrink et al., 2002). AML patients with high-levels of ABCG2 expression had significantly shorter overall survival rates (Ugglä et al., 2005) while decreased ABCG2 was found to be a prognostic factor in adult patients who achieved complete remission of AML (Benderra et al., 2004).

The substrate specificity of ABCG2 includes the anti-neoplastic drugs primarily targeting topoisomerases, including anthracyclines and camptothecins. Topoisomerase I and II inhibitors that are substrates of ABCG2 include topotecan, SN-38, CPT-11, mitoxantrone, daunomycin, doxorubicin, and epirubicin (Doyle et al., 1998; Litman et al., 2000). Topotecan in particular is an excellent substrate for ABCG2. In addition, flavopiridol resistance is mediated by ABCG2 (Honjo et al., 2001). Recently, several potent and specific inhibitors of ABCG2 have been developed, potentially opening the door to clinical applications of ABCG2 inhibition. These inhibitors include the targeted agents

gefitinib (Iressa) and imatinib mesylate (Gleevec) (Houghton et al., 2004) as well as the more specific inhibitors fumitremorgin C (Rabindran et al., 2000), tryprostatin A (Woehlecke et al., 2003; Zhao et al., 2002), and GF120918 (Glaxo) (Maliepaard et al., 2001a).

The normal tissue localization of ABCG2 is in hematological stem cells, placenta, bile canaliculi, colon, small bowel, and brain microvessel endothelium (Maliepaard et al., 2001a). Given the specific tissue localizations, the role of ABCG2 in healthy tissues may be to protect an organism or tissue from potentially harmful toxins. ABCG2 expression has been associated with Akt signaling (Mogi et al., 2003) and its promoter contains an estrogen-response element (Ee et al., 2004). However, regulation by the microenvironment or in direct response to chemotherapeutics has not been reported in multiple myeloma (MM). It has been shown that the ABCG2 promoter contains a potential CpG island, which may be regulated by methylation (Bailey-Dell et al., 2001). Another ABC family transporter, MDR1, has a promoter with a similar CpG island that has been shown to regulate gene expression via methylation of this site (Baker et al., 2005; David et al., 2004; Fryxell et al., 1999; Kusaba et al., 1999).

In the current study, we determined that ABCG2 is present and functional in human MM cells. Using quantitative PCR (QPCR), cytological staining, Western blot analyses, and functional efflux of chemotherapeutic drugs we found that ABCG2 may be involved in drug resistance. *In vitro*, we found that ABCG2 expression increased in response to exposure to the ABCG2 substrates,

doxorubicin and topotecan. Cell density also affected ABCG2 expression, as myeloma cells grown at log-phase densities had greater levels of ABCG2 than cells cultured at higher (plateau) densities. Myeloma patients treated with a high-dose chemotherapy (HDC) regimen that included topotecan had an increase in ABCG2 mRNA and protein expression after treatment and at relapse when compared to pre-treatment samples. Expression of ABCG2 is regulated at least in part by promoter methylation both in cell lines and in plasma cells from patients. Demethylation of the promoter using 5-aza-2'-deoxycytidine increased ABCG2 expression. Thus, ABCG2 may contribute to intrinsic drug resistance in human MM, and this may be augmented by exposure to chemotherapeutic agents that are substrates for ABCG2.

MATERIALS AND METHODS

Cell lines

Human MM cell lines, RPMI-8226 (8226) and NCI-H929 (H929) were obtained from the American Type Culture Collection, Manassas, VA, USA. Mitoxantrone resistant-8226 (8226MR) cells were isolated by Dr. William Dalton at the H. Lee Moffitt Cancer Center, Tampa, Florida (Hazlehurst et al., 1999). All cell lines were grown in RPMI-1640 media containing penicillin/streptomycin (Gibco, Gaithersburg, MD, USA), and 10% fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C and 5% CO₂.

Clinical trial with high-dose melphalan and topotecan

Human myeloma cells were obtained from patients enrolled in a phase I/II HDC protocol using melphalan, VP-16 phosphate and dose-escalated topotecan (MTV trial) followed by peripheral blood stem cell transplant. This protocol was approved by the University of South Florida Institutional Review Board, and signed informed consent was obtained from all patients prior to their participation in the study. Patients were infused for three consecutive days with melphalan ($50 \text{ mg/m}^2/\text{day}$ IV over 30 min), followed immediately by topotecan (from 0 to $9 \text{ mg/m}^2/\text{day}$ IV over 30 min), followed by VP-16 phosphate ($1200 \text{ mg/m}^2/\text{day}$ VP-16 equivalents IV over 4 hours) for two days. The dose escalation scheme for topotecan was: dose level (DL) 1, 0 mg/m^2 total dose over 3 days; DL 2, 10 mg/m^2 total dose; DL 3, 15 mg/m^2 total dose, DL 4, 20 mg/m^2 total dose; and DL 5, 27 mg/m^2 total dose over three days. Bone marrow aspirates were taken before the start of HDC (pre-HDC), on the day after completion of three days of melphalan/topotecan infusion (before the first of two days of VP-16), and in patients who had relapsed from this HDC protocol. Plasma cells were isolated from bone marrow aspirates by Ficoll gradient separation followed by CD138 antibody/magnetic bead (Miltenyi Biotech, Auburn, CA, USA) purification according to the manufacturer's instructions. Percent purity of CD138 selected cells for all patient samples was routinely between 80 and 99%. The analysis of patient plasma cells for ABCG2 mRNA expression was not an original endpoint

of the MTV trial. An IRB approved amendment allowed us to analyze aliquots of residual bone marrow aspirates for ABCG2 expression.

Real-time quantitative PCR (QPCR)

A quantitative primer/probe set was designed to evaluate and to quantify ABCG2 mRNA. Total RNA was extracted from human myeloma cell lines and patient CD138 selected cells by using the guanidine isothiocyanate and phenol/chloroform method (Chomczynski and Sacchi, 1987) (Trizol, Gibco) with the addition of 20 µg glycogen as a carrier for the RNA. Reverse transcription of RNA was performed using Omniscript reverse transcriptase (Qiagen, Germantown, MD, USA), according to the manufacturer's protocol.

Primers and probes for real time PCR were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Each primer set consisted of standard PCR primers (T_m 58-60°C) designed to span gene introns in order to exclude any possible genomic DNA contamination. Detection and quantitation of each gene was accomplished using an amplicon-specific fluorescent oligonucleotide probe (T_m 68-70°C), with a 5' reporter dye (carboxyfluorescein) and a downstream 3' quencher dye (carboxytetramethylrhodamine). The sequence of the primers used for ABCG2 detection were 5'-TTT CCA AGC GTT CAT TCA AAA A-3' (forward primer), 5'-TAC GAC TGT GAC AAT GAT CTG AGC-3' (reverse primer), and 5'-TTG CTG GGT AAT CCC CAG GCC TCT-3' (fluorescent probe) (Integrated DNA

Technologies). Two microliters of cDNA were assayed per well, and the QPCR performed as previously described (Turner et al., 2004). The ABCG2 expression data were found to be log normally distributed. Consequently, the geometric means were used to average both within and between patient data. Changes due to treatment were assessed by taking the logarithm of the ratio of the data compared to baselines level. Statistical significance was assessed using the Wilcoxon signed rank test. P-values below 0.05 were considered to be statistically significant.

Western blot for cell lines and patient myeloma samples

Human 8226 and H929 myeloma cells were harvested by centrifugation, washed with cold PBS, and lysed by sonication in 2% SDS buffer. Protein from 2×10^5 cells per lane was separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham) using a Biorad Mini-Transblot Apparatus (Biorad, Hercules, CA, USA). Membranes were blocked for one hour at ambient temperature in a blocking buffer containing 0.1 M Tris-HCl, 0.9% NaCl, 0.5% Tween-20 (TBST) and 5% non-fat dry milk. ABCG2 was identified by incubation in a 1:1000 dilution of BXP-21 antibody (Kamiya, Seattle, WA, USA) in blocking buffer overnight at 4°C. Membranes were washed three times for 10 minutes in TBST, and incubated for one hour with a goat anti-mouse IgG antibody linked to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) in blocking buffer at a 1:2000 dilution. Antibody binding was visualized by Enhanced

Chemiluminescence (Amersham, GE Healthcare, USA) on autoradiography film (Kodak, USA).

Protein loading on gels was assessed by Coomassie blue staining of the Western blots. Blots were incubated at room temperature in a shaker apparatus with 250 mg/L Coomassie blue in 50% methanol and 10% glacial acetic acid. Blots were then destained for 2 hours in a solution containing 50% methanol and 10% glacial acetic acid. Protein staining was compared visually to ensure equal loading in each lane, and unless otherwise noted was equivalent in each immunoblot.

Flow cytometry/ABCG2 functional assay

ABCG2 expression was assayed by flow cytometry using an antibody that specifically recognizes only membrane bound ABCG2 epitopes (Bcrp1-PE, Chemicon/Millipore, Billerica, MA, USA). Human myeloma patient cells and myeloma cell lines were fixed with 4% paraformaldehyde for 10 minutes and washed in phosphate buffered saline (PBS). Approximately 10^5 cells were labeled with 10 μ l of Bcrp1-PE antibody in 190 μ l of 1% bovine serum albumin (BSA) in PBS at 37°C for 30 minutes. Labeled cells were washed in PBS and assayed by flow cytometry on a FACScan (Becton Dickenson, Franklin Lakes, NJ, USA). ABCG2 function was assayed as the efflux of the ABCG2 substrates doxorubicin and topotecan or as the efflux of Hoechst 33342 (Sigma) (Kawabata et al., 2003). ABCG2 function was assayed in myeloma patient bone marrow

aspirates obtained before and after exposure to 1 μ M topotecan (aspirates obtained prior to HDC on the MTV protocol), and in H929, 8226 and 8226MR cell lines. The plasma cells were isolated from bone marrow aspirates (frozen in liquid nitrogen) using CD138 magnetic bead-antibody conjugates (Miltenyi Biotec) after separation by a Ficoll gradient. Topotecan is very fluorescent and accumulates in cells that do not express ABCG2. Myeloma cell lines and CD138 purified patient samples were incubated with 40 μ M topotecan or 1 μ M doxorubicin for 20 minutes at 37°C, washed twice in ice-cold PBS, and analyzed by flow cytometry for topotecan and doxorubicin fluorescence.

¹⁴C-Mitoxantrone uptake was also used to assess ABCG2 function. Cells were incubated for two hours with ¹⁴C-mitoxantrone with and without a large molar excess of unlabeled mitoxantrone. Radioactivity was measured by liquid scintillation counting (Perkin-Elmer, Wellesley, MA, USA). Controls used were identical cell samples without drug or co-incubated with the specific ABCG2 inhibitor tryprostatin A (Woehlecke et al., 2003; Zhao et al., 2002). Tryprostatin A was synthesized by Dr. Chunchun Zhang and Dr. James M. Cook, at the University of Wisconsin-Milwaukee. To determine if decreased drug uptake was due to ABCG2 activity, both patient samples and cell lines were co-incubated with the specific ABCG2 inhibitor tryprostatin A. This drug blocks the drug efflux function of ABCG2, resulting in increased fluorescence due to intracellular topotecan or doxorubicin accumulation. ABCG2 function was expressed as the

change in relative fluorescence in topotecan treated versus untreated control cells.

Immunofluorescent microscopy and quantitative measurement of ABCG2

Patient plasma cell samples and myeloma cell lines (1×10^5 cells) were plated on double cytoslides (Shandon) by cyto-centrifugation at 500 rpm, and fixed and stained with anti-ABCG2 (BXP-21, Kamiya Biomedical Labs) according to the protocol in Engel et al (Engel et al., 2004). Slides were washed with PBS, air-dried and the nuclei stained with 4',6-diamidino-phenylindole dihydrochloride hydrate (DAPI) (Vector laboratories, Burlingame, CA, USA). Cellular membrane ABCG2 staining was performed directly on paraformaldehyde fixed cells using the membrane specific antibody, Bcrp1-FITC (Chemicon). Immunofluorescence was observed by a Leitz Orthoplan microscope with a CCD camera.

Cell density and low dose drug treatment

The model used to assess possible microenvironmental effects involved incubating cells at high- and low-density culture conditions, assuming that high-density conditions mimic the *in vivo* bone marrow environment. We have shown previously that myeloma cells grown at different densities exhibit specific characteristics, including drug resistance to topoisomerase I and II inhibitors that depends on the nuclear to cytoplasmic trafficking of topoisomerases (Engel et al., 2004; Turner et al., 2004; Valkov et al., 2000a). Myeloma cell lines (8226, H929,

8226MR) grown at 2×10^5 cells/ml media were defined as low-density (log-phase), and cells grown at 2×10^6 cells/ml were defined as high-density (plateau-phase). Cell lines were placed at log and plateau density conditions and grown for 24 hours at 37°C in 5% CO₂. Cells were harvested and assayed for ABCG2 expression by flow cytometry, immunostaining, mRNA analysis (QPCR), and Western blot as described above. In addition, log- and plateau-phase cells were further incubated in the presence of 1 μM topotecan or 0.1 μM doxorubicin for 20 hours at 37°C in a 5% CO₂ incubator and harvested for the determination of ABCG2 expression.

Bisulfite sequencing and demethylation by 5-aza-2'-deoxycytidine of the ABCG2 promoter in patient samples and myeloma cell lines

Genomic DNA from patient biopsies or cell lines was extracted using the DNeasy Tissue Kit (Qiagen). Two micrograms of DNA were subjected to bisulfite conversion according to methods published in Warnecke et al (Warnecke et al., 2002). Primers were designed to clone the bisulfite-converted CpG island-rich portions of the human ABCG2 promoter using standard PCR conditions (ABCG2 forward, GGA TAA TAT TAG GTA AGG TTG AGT AA, ABCG2 reverse, TCA AAA TAA CTC CCT CCA AAC AAA AC).

ABCG2 low-expressing H929 cells, which had highly methylated promoter CpG islands, were treated with the demethylating agent, 5-aza-2'-deoxycytidine, to determine if ABCG2 promoter demethylation allowed increased ABCG2

expression. Cells were incubated for 72 hours in media containing 5-aza-2'-deoxycytidine (Sigma) at a concentration of 100 nM and harvested to detect ABCG2 expression by flow cytometry, immunostaining, QPCR, and Western analysis.

Methylation-specific quantitative PCR

Genomic DNA samples that were extracted from patient myeloma cells for bisulfite sequencing were further analyzed to determine the percentage of methylated alleles. Primers were designed to anneal specifically to methylated and non-methylated CpG dinucleotides in a region of the ABCG2 promoter. This area of the ABCG2 promoter was previously found to be methylated by bisulfite sequencing (data not shown). The primers used had the following sequences: 5'-TGA TTG GGT AAT TTG TGC GTT AGC G-3', methylated forward primer; 5'-TGA TTG GGT AAT TTG TGT GTT AGT GTT-3', un-methylated forward primer, and 5'-AAA TAA ACC AAA ATA ATT AAC TAC-3', reverse primer that was used for both PCR reactions. The PCR reaction was performed in a 96-well optical reaction plate. The reaction mixture consisted of 2 µl of bisulfite DNA, 0.2 µM each primer and 23 µl of SYBR green PCR mix (Biorad) according to the manufacturer's protocol. QPCR was performed in an ABI 5700 sequence detection system. For each sample, the exact number of alleles that were methylated and non-methylated were assayed, and the data expressed as the percentage of methylated alleles.

RESULTS

Quantitative PCR of ABCG2

Table 3.1 and Figure 3.1 show the relative expression of ABCG2 mRNA in several human cancer cell lines. High levels of expression were found in mitoxantrone resistant 8226MR cells (Hazlehurst et al., 1999) and MCF-7/mitox cells (Taylor et al., 1991) while parental 8226 cells (827.4 RU) and H929 cells (56.1 RU) had intermediate and low levels of expression, respectively. ABCG2 mRNA copy numbers were normalized to housekeeping gene GAPDH copy numbers and expressed as relative units (RU). Plasma cells obtained from patients prior to high-dose chemotherapy also had intermediate levels of expression, with a geometric mean of 118.4 RU (Table 3.2).

Patient bone marrow aspirates obtained prior to HDC, after three days of melphalan alone (DL 1) or after three days of melphalan and topotecan (DL 2-5), or at relapse from HDC, were analyzed for ABCG2 mRNA expression (Table 3.2). These were unused aliquots of bone marrow aspirates from MTV trial patients (Sullivan et al., 2001; Valkov et al., 2000a) for which we obtained IRB approval for ABCG2 analysis. All possible residual samples from this trial were analyzed; 42 paired

Cell/Tissue Type	ABCG2 mRNA (copies per cell normalized to GAPDH)
Normal PBMC	1.1 (1.0)
8226MR myeloma*	4402.7 (195.5)
8226 myeloma	827.4 (30.6)
H929 myeloma	56.1 (3.1)
CCRF leukemia	0.0
HL-60 leukemia	0.0
KG1A leukemia	0.6 (0.1)
MCF-7 breast cancer	37.9 (1.1)
MCF-7/Mitox [†]	5040.4 (589.3)
MDA 231 breast cancer	16.9 (0.2)
MDA 361 breast cancer	0.4 (0.1)
A375 melanoma	13.2 (0.4)
SK5 melanoma	19.7 (2.8)
SK28 melanoma	9.2 (0.3)
CRL 1974 melanoma	29.0 (1.8)

Table 3.1. ABCG2 mRNA expression determined by QPCR in human cancer cell lines. Results given as mean (SD). PBMC indicates peripheral blood mononuclear cells. *See Warnecke et al (Warnecke et al., 2002). [†]See Knutsen et al (Knutsen et al., 2000).

samples from 31 patients (paired either pre-HDC and during HDC, or pre-HDC and relapse). Ten patients had samples from all three time points. The frozen samples were thawed, selected using CD138 immunomagnetic beads and analyzed by QPCR.

Patients that had received three days of melphalan followed by two days of VP-16 (Table 3.2; 6 patients, DL 1) had no significant change in ABCG2 expression compared with pre-HDC plasma cells ($P = 0.56$), nor when relapse values were compared with pre-HDC

Patient	Dose Level MTV ¹	ABCG2 mRNA expression/cell ²		
		Pre-HDC	During HDC	Relapse from HDC
1	1	143.5	421.3	282.5
2	1	7.4	12.3	29.6
3	1	87.9	29.8	ND
4	1	140.8	67.5	38.9
5	1	20.6	49.0	ND
6	1	2.4	24.5	0.1
7	1	37.6	ND	40.4
8	2	8.6	18.7	12.4
9	2	8.7	111.8	62.3
10	3	33.2	86.4	422.6
11	3	30.0	35.6	38.9
12	3	253.2	154.7	ND
13	3	9.5	ND	44.1
14	3	10.2	ND	67.7
15	4	193.8	122.3	1234.8
16	4	882.6	1133.4	ND
17	4	443.9	1908.0	ND
18	4	202.8	1791.1	ND
19	4	813.6	1595.6	ND
20	4	446.0	852.5	ND
21	4	45.9	14.3	ND
22	4	342.2	164.0	ND
23	4	1604.1	1877.3	ND
24	4	558.1	316.2	ND
25	4	42.9	97.4	ND
26	4	176.3	ND	354.1
27	4	100.2	ND	140.6
28	5	44.2	183.1	ND
29	5	78.6	146.6	ND
30	5	53.9	31.6	103.0
31	5	24.7	2893.8	238.2
Pre-HDC versus During HDC³				
Dose Level 1 (n = 6)		29.3	48.2	p = 0.56 ⁴
Dose Levels 2-5 (n = 20)		118.4	232.5	p = 0.033
Pre-HDC versus Relapsed from HDC				
Dose Level 1 (n = 5)		26.6	p = 1.00	17.6
Dose Levels 2-5 (n = 11)		31.7	p = 0.001	117.1

Table 3.2. ABCG2 mRNA expression determined by QPCR in CD138 selected human plasma cells from bone marrow aspirates obtained from patients with multiple myeloma prior to and during high-dose chemotherapy, and at the time of relapse. The QPCR was repeated twice for each patient, and the value is the geometric mean of those two observations (see methods). ¹Patients on dose level 1 received three days of melphalan followed by two days of VP-16 phosphate. Those on dose levels 2-5 received three days of melphalan followed immediately by dose-escalated toptecan each day for three days, followed by two days of VP-16 phosphate. ²The expression of ABCG2 is normalized to that

of GAPDH. ³The analyses presented are for paired samples only, that is, pre + during and pre + relapse. ⁴p-values are from a Wilcoxon signed rank test. Abbreviations: MTV, melphalan + topotecan + VP-16 phosphate; HDC, high-dose chemotherapy; ND, not done either because of insufficient CD138 cells isolated or because the patient has not relapsed from HDC. (5 patients; P = 1.00).

In contrast, those patients that received melphalan and topotecan (Table 2; 20 patients, DL 2-5) had a significant increase in ABCG2 expression compared to pre-HDC samples (P = 0.033). In addition, patient samples from relapse in DL 2-5 (Table 2) also had a statistically significant increase in ABCG2 when relapse samples were compared with pre-HDC values (11 patients; P = 0.001). A statistical analysis comparing ABCG2 mRNA levels and patient clinical outcome (best response to high-dose chemotherapy) failed to find any statistically significant prognostic value of mRNA levels in this limited number of samples.

ABCG2 protein expression determined by Western analysis, flow cytometry and immunofluorescence

The protein expression of ABCG2 in human myeloma cell lines was assayed by Western blot, immunofluorescence staining, and flow cytometry, and found to correlate well with the QPCR data (Figure 3.1). Mitoxantrone-resistant 8226 (8226MR) cells expressed very high levels of ABCG2, while parental 8226 cells and H929 cells expressed intermediate and low levels, respectively. Flow cytometric analysis of human myeloma cell lines for ABCG2 membrane expression also showed high levels of expression.

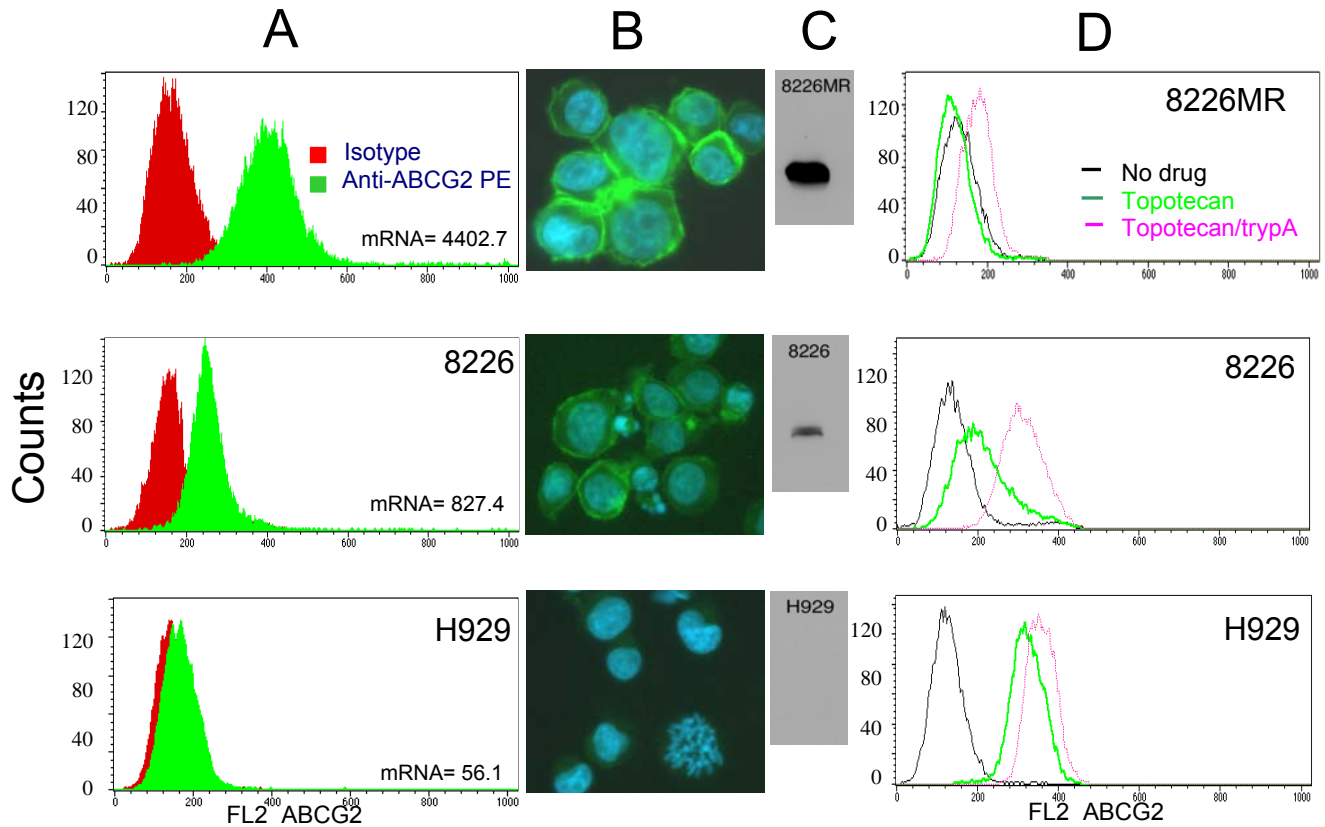


Figure 3.1. ABCG2 Expression and function in myeloma cell lines.

(A) Flow cytometric analysis of MM cell lines for ABCG2 membrane expression was performed. Mitoxantrone resistant 8226MR cells express more ABCG2 than wild-type 8226 cells, and H929 cells express very little, as shown by the shift in fluorescence. (B) Immunostaining of myeloma cell lines using an anti-ABCG2 FITC (Chemicon) labeled antibody (ABCG2 is green and DAPI is blue). (C) Western blot of protein (25 µg/lane) extracted from myeloma cell lines for ABCG2. (D) Functional analysis of ABCG2 using topotecan as a substrate and the ABCG2 specific inhibitor tryprostatin A. Topotecan, a very good ABCG2 substrate and a naturally fluorescent molecule, is effluxed in high (8226MR) and moderate (8226) ABCG2 expressers, but is accumulated by H929 cells (which do not express ABCG2). Tryprostatin A (TrypA) blocks the efflux of topotecan, demonstrating that topotecan efflux is ABCG2 dependent.

8226MR expressed more ABCG2 than wild-type 8226, and H929 expressed very little as shown by the shift in fluorescence (Figure 3.1A). Immunostaining of myeloma cell lines using an anti-ABCG2 Bcrp1-FITC labeled antibody (Chemicon) demonstrated high levels of ABCG2 expression in 8226MR and 8226 cells, but not in H929 cells (Figure 3.1B). A Western analysis of protein extracted from myeloma cell lines for ABCG2 also followed the same pattern of protein expression (Figure 3.1C).

ABCG2 functional assay: topotecan efflux

The purpose of the functional assay was to evaluate ABCG2 mediated drug efflux in myeloma cell lines. Topotecan, an exceptional ABCG2 substrate and a naturally fluorescent molecule, was effluxed in high (8226MR) and moderate (8226) ABCG2 expressers, but was not effluxed by H929 cells (low expressing cells). To show that efflux was specific to ABCG2 and not other transporters, the inhibitor tryprostatin A was used as a control (Figure 3.1D). Tryprostatin A efficiently blocked the efflux of topotecan in 8226 and 8226MR cells, demonstrating that in these cell lines topotecan efflux was ABCG2 dependent (Figure 3.1D) (Woehlecke et al., 2003; Zhao et al., 2002). ABCG2 function was expressed as the change in relative fluorescence in topotecan treated versus untreated control cells.

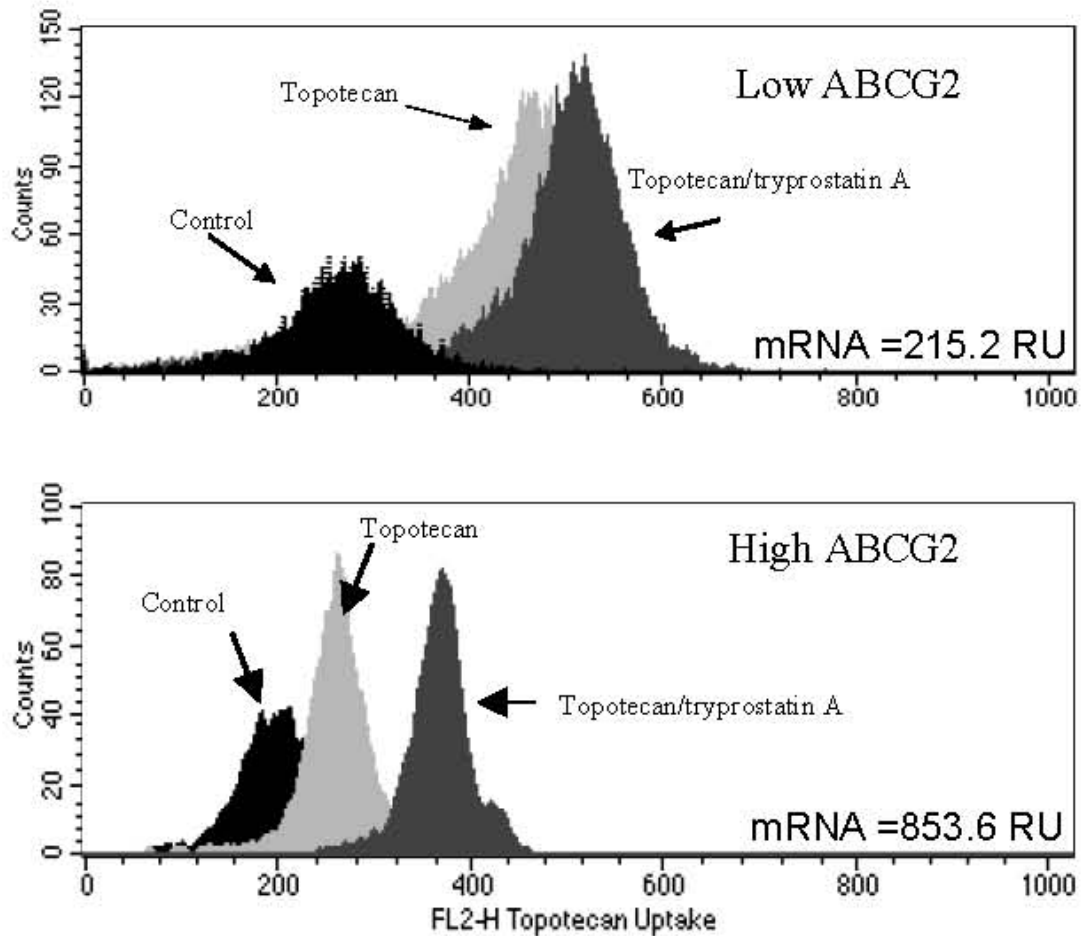


Figure 3.2. Functional assay in patient myeloma cells. Patient samples with high ABCG2 and low ABCG2 mRNA (as measured by QPCR), were assayed for ABCG2 function. The high ABCG2 expresser effluxed topotecan more efficiently than the lower expresser. Efflux was shown to be ABCG2 specific by the addition of tryprostatin A.

This analysis showed that ABCG2 protein and mRNA levels correlated well with function (Figure 3.1A, 3.1B, 3.1C, 3.1D). Similar results were found in patient bone marrow samples, where a high ABCG2 expressing sample was found to efflux topotecan more efficiently than a low expresser (Figure 3.2). In addition, doxorubicin was effluxed more efficiently by high ABCG2 expressing 8226MR than 8226 parental cells (Figure 3.3). ABCG2 efflux of doxorubicin was inhibited by the addition of the ABCG2 inhibitor tryprostatin A (Figure 3.3).

¹⁴C-Mitoxantrone uptake was also used to assay ABCG2 function, as we have previously described (Harker et al., 1995). Human myeloma cells were incubated for 2 hours with ¹⁴C-mitoxantrone with and without a large molar excess of unlabeled mitoxantrone. ¹⁴C-Mitoxantrone uptake corroborated the findings of topotecan uptake; high ABCG2 expressing 8226MR cells effluxed labeled drug more efficiently than low expressing 8226 parental cells. 8226MR cells had an equilibrium cellular radioactivity of 5,374±65.6 cpm/mg cellular protein, while parental 8226 cells had 13,187±102.9 cpm/mg cellular protein. Thus, the ABCG2 expressing 8226MR cells are able to efflux mitoxantrone more efficiently.

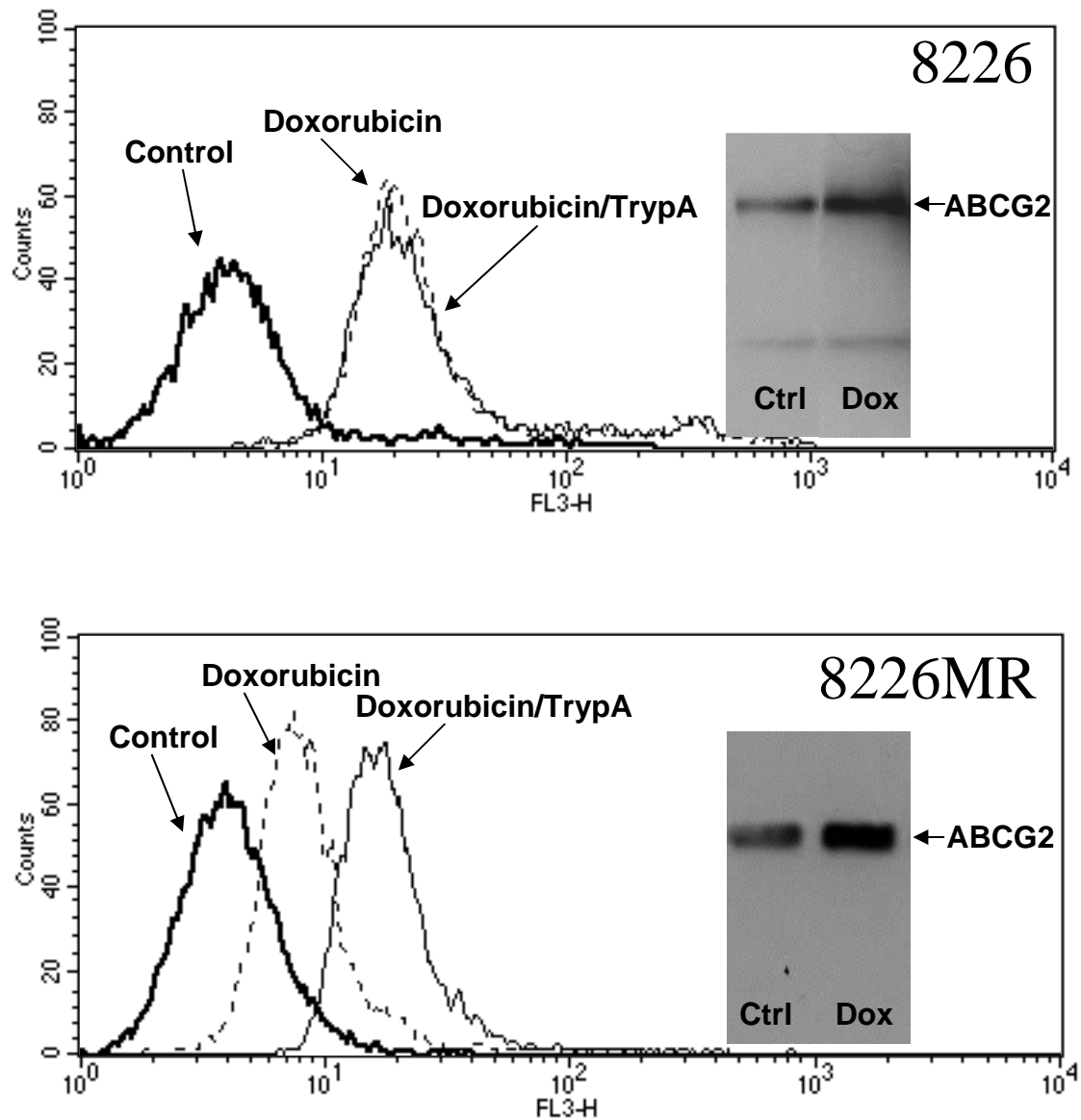


Figure 3.3. ABCG2 expression increases in response to doxorubicin exposure. ABCG2 expression was assayed by flow cytometry in 8226 and 8226MR MM cells after exposure to 1 μM doxorubicin for 20 minutes. Higher ABCG2 expressing cells were able to efflux doxorubicin more than parental 8226 cells. The ABCG2 specific inhibitor, tryprostatin A decreased efflux in the 8226MR cell line but not the 8226 parental cell line, indicating that doxorubicin efflux was mediated by ABCG2. Myeloma cells treated with low dose doxorubicin, 0.1 μM 8226MR and 1.0 μM 8226 cells exhibit an increase in protein expression as determined by Western analysis (inset of each graph). Equal amounts of protein (25 μg), was assayed. Both 8226 and 8226 MR cells demonstrated a 1.7-fold increase in ABCG2 protein after low-dose doxorubicin treatment.

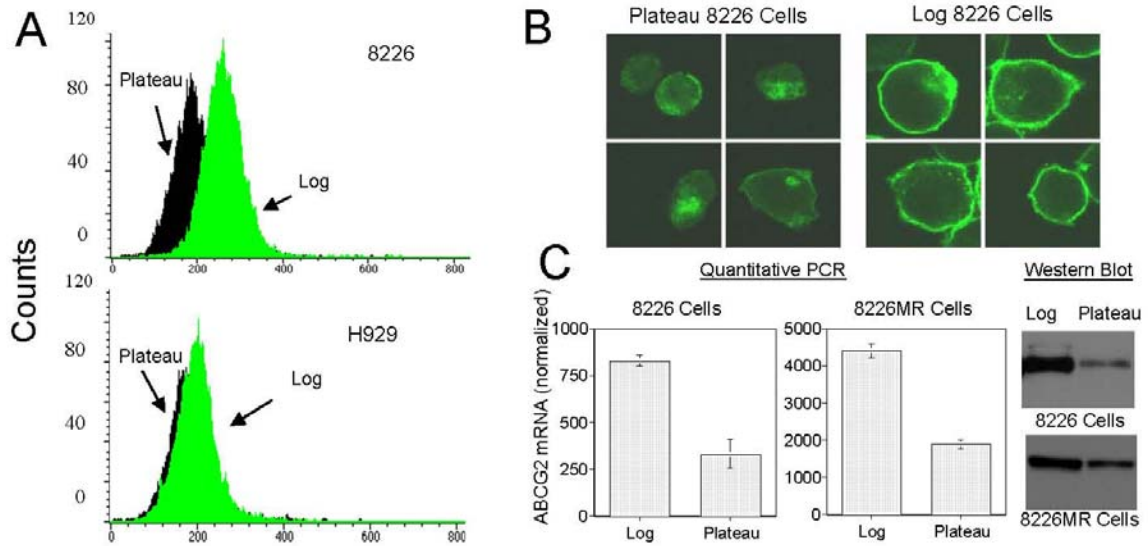


Figure 3.4. ABCG2 expression is elevated in log phase myeloma cells. (A) Flow cytometric data using an ABCG2 antibody (Chemicon) demonstrate that ABCG2 expressing 8226 cells have increased ABCG2 at log-phase density compared with log-phase H929 cells. (B-C) The FACScan data are confirmed by immunostaining for ABCG2 (B), and by QPCR and Western analyses (C). Densitometry analysis of the immunoblot shows a 4:1 ratio of log:plateau ABCG2 in 8226 cells, and a 2:1 ratio of log:plateau 8226MR ABCG2. Note, 8226 and 8226MR Western blots were exposed for different time intervals and do not reflect relative protein levels.

Effect of the microenvironment and topoisomerase inhibitors on ABCG2 expression

We also examined the expression of ABCG2 as a function of cell density (Figure 3.4), and found that log-phase (low density) 8226 and 8226MR cells express significantly more ABCG2 than plateau-phase (high density) cells, as shown by flow cytometry (Figure 3.4A), immunofluorescence microscopy (Figure 3.4B), QPCR (Figure 3.4C), and Western blot analysis (Figure 3.4C). Changes in cell density failed to induce ABCG2 protein expression in H929 cells (Figure 3.4A).

8226MR and 8226 cells were also found to increase the expression of ABCG2 in response to low dose topotecan exposure (Figure 3.5). This was shown by immunofluorescence microscopy (Figure 3.5A-B), Western blot analysis (Figure 3.5C inset), and protein expression measured as pixel intensity from immunofluorescence (Figure 3.5C). In addition, ABCG2 protein expression was measured by flow cytometry using the membrane epitope-specific antibody Bcrp1-PE (Chemicon), and showed an increase in ABCG2 protein in log-phase 8226MR and 8226 cells after exposure to low dose topotecan (1 μ M) for 20 hours (Figure 3.5D). The low ABCG2 expressing myeloma cell line H929 had no ABCG2 antibody binding (Figure 3.5D).

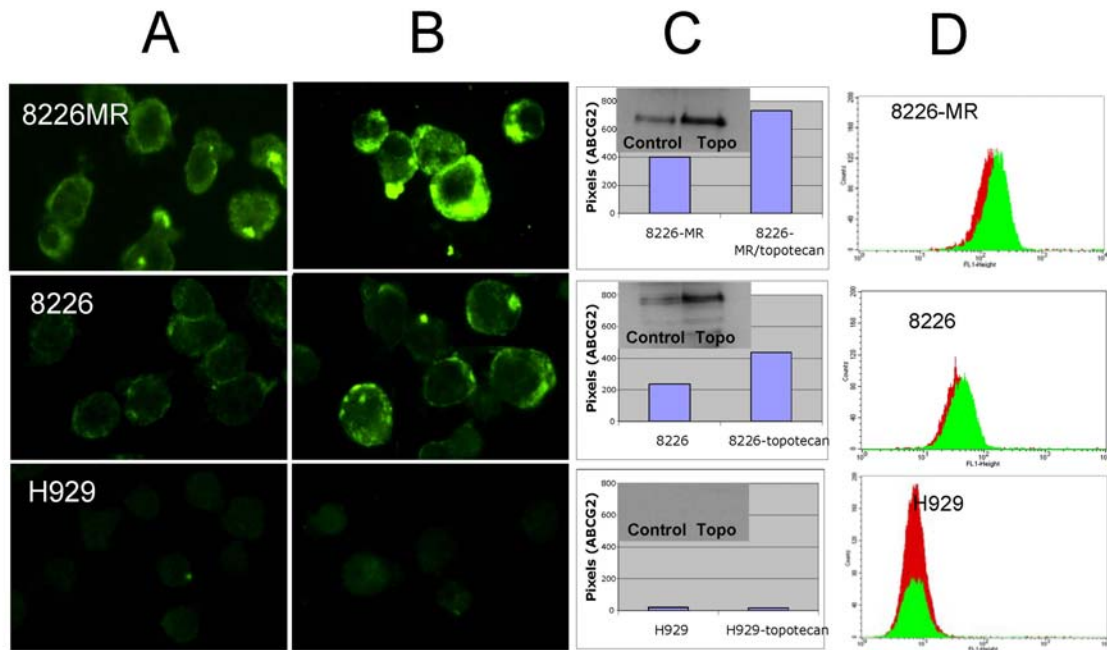


Figure 3.5. ABCG2 expression increases in response to topotecan chemotherapy. (A-B) Multiple myeloma 8226MR, 8226, and H929 cells treated with low dose topotecan (B) exhibit an increase in membrane ABCG2 over the no drug control (A), as shown by immunostaining with ABCG2 antibody (Bcrp1 FITC). (C) Protein expression of ABCG2 was quantified as pixel intensity (from immunofluorescence microscopy), and also assessed by Western analysis (inset of each graph). (D) ABCG2 expression was measured by flow cytometry and showed an increase in log-phase 8226MR and 8226 cells after exposure to low dose topotecan (1 μ M) for 24 hours. The ABCG2 non-expressing cell line (H929) shows no increase in ABCG2 antibody binding

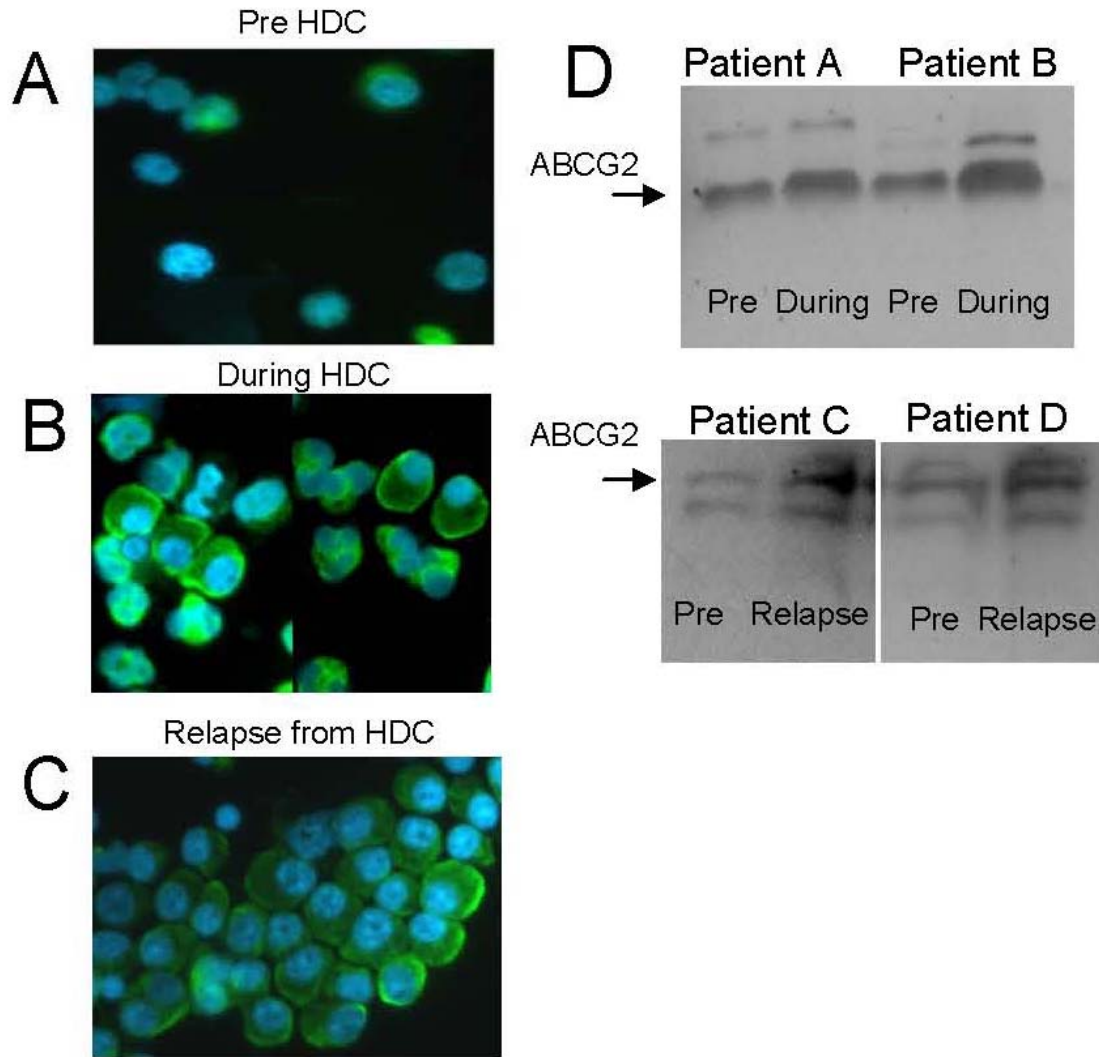


Figure 3.6. ABCG2 increases in patient plasma cells after high-dose chemotherapy and at relapse. (A-B) A patient bone marrow aspirate taken before (A) and during (B) HDC with melphalan and topotecan exhibited an increase in immunofluorescence of ABCG2 (green). (C) This same patient demonstrated an increase in ABCG2 expression by immunofluorescence at relapse as well. (D) ABCG2 protein expression pre-HDC and at relapse by Western blot in four patients on the MTV study. Laser densitometry analysis of the immunoblots shows a 1.54- and 1.94-fold increase in ABCG2 during HDC for patients A and B, respectively, and a 3.68- and 1.34-fold increase in ABCG2 at relapse for patients C and D, respectively. In all cases this is relative to the pre-HDC ABCG2 protein expression.

8226 parental and 8226MR cells treated with low dose topotecan exhibit an increase in membrane ABCG2 compared to the no drug control, as shown by immunostaining with ABCG2 antibody (MXB-21) (Figure 3.5A-B).

8226MR and 8226 parental cell cultures treated with low dose doxorubicin for 20 hours also demonstrated an increase in ABCG2 expression by Western blot analysis (insets figure 3.3). As was seen at the mRNA level (Table 3.2), patient bone marrow aspirates taken before, during, and after HDC with melphalan and topotecan showed changes in ABCG2 expression (Figure 3.6). A limited number of patient samples from the MTV trial were available for these Western and immunofluorescence analyses. The same patient sample from dose level 5 showed increased ABCG2 expression after three days of exposure *in vivo* to topotecan (Figure 3.6B), as well as at relapse (Figure 3.6C). Four different patient samples analyzed by immunoblotting (from dose levels 4 and 5 of the MTV trial) also demonstrated an increase in ABCG2 protein expression after three days of topotecan or at relapse from high-dose chemotherapy (Figure 3.6D).

ABCG2 promoter methylation

The methylation status of a previously described CpG island in the ABCG2 promoter was examined via bisulfite sequencing. Figure 3.7 shows the CpG dinucleotides that were methylated in four cell lines tested. The promoter

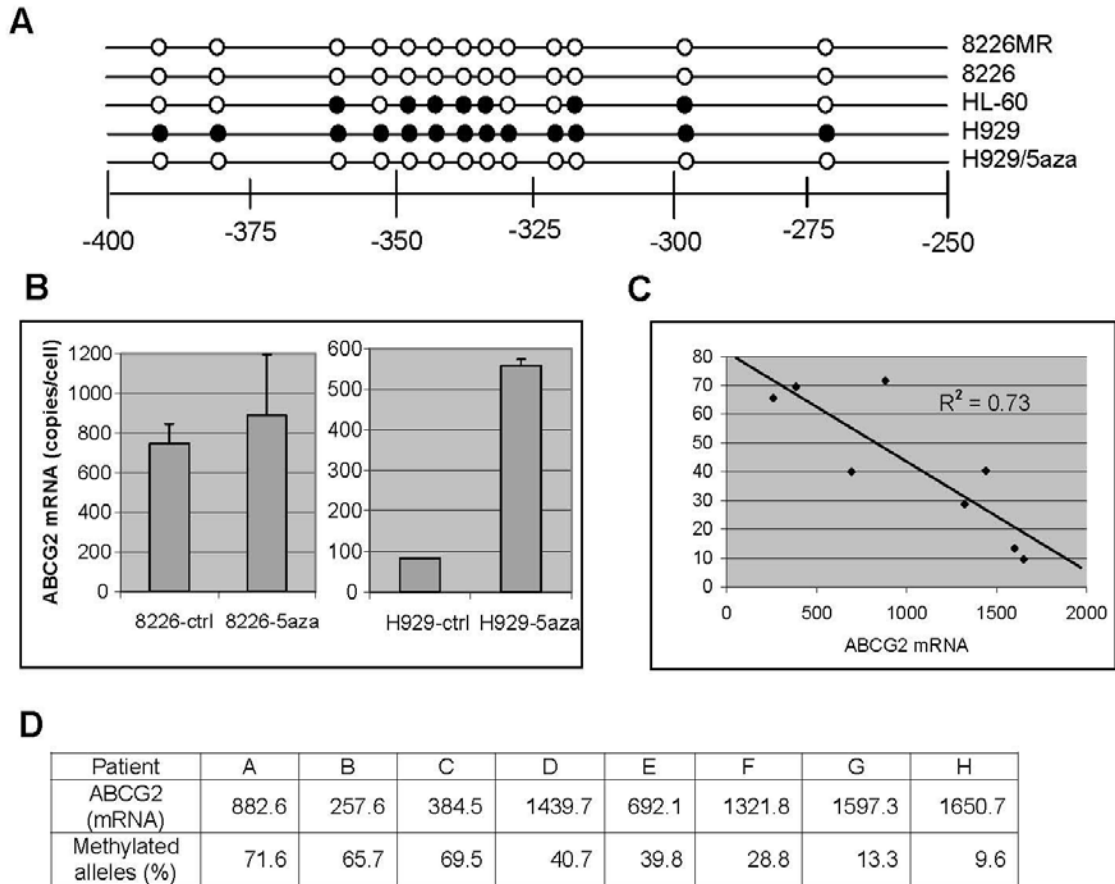


Figure 3.7. ABCG2 promoter methylation. (A) Cells were harvested at plateau-phase and the DNA extracted and assayed by bisulfite DNA sequencing analysis. The figure shows the methylation status of the putative CpG island of the ABCG2 promoter in four cell lines. Filled circles represent methylated groups and the open circles demethylated CpG. (B) H929/5aza are cells treated with 100 nM 5-aza-2'-deoxycytidine for 72 hours and the ABCG2 promoter assayed by bisulfite sequencing. 5-Aza-2'-deoxycytidine was able to augment ABCG2 transcription in low ABCG2 expressing H929 cells but had no effect in moderate expressing 8226 cells. (C, D) CD-138 selected MM patient samples were assayed for ABCG2 promoter methylation after bisulfite conversion using real-time quantitative PCR. The percentage of alleles that were methylated inversely correlated with ABCG2 mRNA expression.

region of ABCG2 over-expressing cells, 8226MR, was completely unmethylated, whereas the H929 cells, which express very little ABCG2, had 13 methylated CpG dinucleotide groups (Figure 3.7A). We found that protein, mRNA, and topotecan efflux function in live cells correlated with the methylation of CpG dinucleotides in the ABCG2 promoter (Figure 3.1). Therefore it is likely that ABCG2 expression is controlled in part by methylation of its promoter.

We also tried to increase ABCG2 expression by treating low-expressing H929 cells with 100 nM 5-aza-2'-deoxycytidine. This agent has been shown to increase gene expression by inhibiting DNA-methyltransferase I, thereby decreasing epigenetic methylation of DNA. After treatment of cells for 72 hours, we found that ABCG2 mRNA and protein increased approximately six-fold in low expressing H929 cells, whereas moderately expressing 8226 cells were unaffected (Figure 3.7B). Bisulfite sequencing of 5-aza-2'-deoxycytidine treated H929 DNA showed that the ABCG2 promoter CpG island was fully demethylated (Figure 3.7A).

Methylation-specific quantitative PCR of patient myeloma cells

The percentage of ABCG2 promoter alleles that were methylated was assayed using SYBR green based real-time quantitative PCR. These eight patient samples were all obtained pre-HDC from the MTV trial. The data are expressed in Figures 3.7C and 3.7D as the percentage of alleles methylated for

each patient sample compared to ABCG2 mRNA levels assayed by fluorescent probe based real-time quantitative PCR (as described previously in the methods). Figure 3.7C and 3.7D show that ABCG2 mRNA expression correlates inversely with the percentage of alleles methylated. High levels of methylated alleles resulted in a decrease in ABCG2 mRNA transcripts, whereas patient samples with low levels of methylated alleles had much higher amounts of ABCG2 mRNA. These data, along with cell culture methylation data, indicate that promoter methylation may contribute to the control of ABCG2 expression in human myeloma cells.

DISCUSSION

We have found that ABCG2 is present and functional in human myeloma cell lines and patient plasma cells. Using QPCR, protein assays, and functional efflux of chemotherapeutic drugs, we have found that ABCG2 is potentially involved in drug resistance to specific agents in human myeloma cells.

The principal findings of our *in vitro* experiments are that (i) human multiple myeloma cells lines have a wide range of ABCG2 expression and function, (ii) low density 8226 cells express more ABCG2 than high-density cells, (iii) human myeloma cell lines with moderate or high baseline levels of ABCG2 expression further increase this expression upon exposure to the ABCG2 substrates topotecan and doxorubicin, and (iv) methylation of the CpG island of the ABCG2 promoter correlates inversely with ABCG2 expression. *In vivo*, we

found that myeloma patients treated with topotecan had an increase in ABCG2 mRNA expression, both after three days of topotecan exposure and at the time of relapse. In addition, we found that a CpG island in the ABCG2 promoter is heavily methylated in cells that do not express ABCG2. This promoter region was completely demethylated in cells that expressed high to intermediate levels of ABCG2. Therefore, expression of ABCG2 was regulated, at least in part, by promoter methylation, both in cell lines and in patient plasma cells.

The ability to up-regulate ABCG2 in response to chemotherapy could confer a selective survival advantage to malignant plasma cells. Plasma cells are derived from hematological stem cells that have demonstrated an intrinsic ability to produce ABCG2, and therefore, myeloma cells may come by this ability naturally (Kim et al., 2002; Scharenberg et al., 2002; Zhou et al., 2001b). However, clonal selection may play a part in the further development of ABCG2 expression in myeloma. Rapidly growing log-phase myeloma cells also increased ABCG2 expression *in vitro*. Increased drug resistance conferred to rapidly growing cells could possibly produce an additive result with the unlimited replicative potential of cancer cells, one of the "hallmarks of cancer" (Hanahan and Weinberg, 2000). ABCG2 may contribute to intrinsic drug resistance in myeloma, and its effect is likely increased by exposure to chemotherapeutic drugs that are substrates for ABCG2.

In general, we observed the same results in the limited number of patient plasma cells available from the HDC MTV trial. Patient cells exposed to

topotecan *in vivo* had increased ABCG2 expression, as did plasma cells from relapse bone marrow aspirates. In addition, patient plasma cells with increased CpG island methylation of the ABCG2 promoter had decreased ABCG2 mRNA expression. In studies performed in another hematological malignancy, AML, patients demonstrated significantly increased expression of ABCG2 mRNA in the relapsed/refractory samples compared to pre-treatment (van den Heuvel-Eibrink et al., 2002). AML patients with high-levels of ABCG2 expression had significantly shorter overall survival rates (Ugla et al., 2005) and decreased ABCG2 expression predicted a complete remission of AML in adult patients (Benderra et al., 2004).

In a recent study, Raaijmakers et al (Raaijmakers et al., 2005), isolated plasma cells from normal bone marrow donors and from ten patients with myeloma prior to treatment with VAD chemotherapy using flow cytometry and an anti-CD38 antibody. They observed that ABCG2 expression was relatively high in both normal and malignant plasma cells, but that ABCG2 mediated efflux of mitoxantrone was significantly impaired in the malignant plasma cells. These results differ from ours, however, our functional assay was limited to only a high and a low expressor of ABCG2 (Figure 3.2), was not compared to drug efflux in normal plasma cells, and the plasma cells were isolated using an anti-CD138 antibody and immunomagnetic beads from patients previously treated with chemotherapy. Thus, the two studies may be comparing different populations of plasma cells.

In previous studies, ABCG2 over-expression has been observed in drug resistant cell lines (Allen et al., 1999; Candeil et al., 2004; Honjo et al., 2002; Knutsen et al., 2000; Litman et al., 2000; Miyake et al., 1999; Robey et al., 2001; Ross et al., 1999). This over-expression of ABCG2, in the majority of cases, has been attributed to heavy amplification of the gene locus (Allen et al., 1999; Knutsen et al., 2000; Miyake et al., 1999). Also, significant increases in function have been found to occur due to specific mutations, but without a concurrent increase in gene transcription or translation (Honjo et al., 2001; Honjo et al., 2002). Analysis of a putative ABCG2 promoter region presents a TATA-less promoter with several putative transcription factor binding sites. In addition, the promoter has an estrogen response element, all of which may contribute to increased gene expression levels (Bailey-Dell et al., 2001; Ee et al., 2004). It has been reported that the ABCG2 promoter contains a potential CpG island, which may regulate expression by methylation (Bailey-Dell et al., 2001). The MDR1 promoter has a similar CpG island. In a recent publication, it was found that hypermethylation of CpG dinucleotides in the MDR1 promoter region strongly contributed to differences in gene expression in related cell lines (David et al., 2004). In our study, we examined the methylation status of the ABCG2 promoter region in cell lines that differed in their respective ABCG2 expression. We found that the promoter of very low level expressing cells was almost completely methylated, whereas high and medium ABCG2 expressers were either completely or almost completely unmethylated. Analysis of the ABCG2

promoter via bisulfite sequencing showed that methylation occurred precisely at the putative CpG island as described by Bailey-Dell, et al (Bailey-Dell et al., 2001). In addition, when low ABCG2 producing H929 cells were exposed to the de-methylating agent, 5-aza-2'-deoxycytidine, the cells were induced to express ABCG2 mRNA and protein (Figure 3.7B). Methylation was also shown to be important in human myeloma patient samples. The percentage of methylated alleles inversely correlated with ABCG2 mRNA expression (Figure 3.7C and 3.7D). Therefore, our data suggest that promoter methylation contributes to gene expression of ABCG2.

In summary, our data suggest ABCG2 may be involved in the resistance of human myeloma cells, both *in vitro* and *in vivo*, to chemotherapeutic agents that are substrates of ABCG2. Doxorubicin, VP-16 and topotecan are all substrates of ABCG2. Doxorubicin is commonly used in the treatment of myeloma (vincristine + adriamycin + decadron, or VAD regimen). In this study we found that doxorubicin is actively effluxed by ABCG2 *in vitro*. Single agent topotecan has been shown to have activity in relapsed and refractory multiple myeloma patients in a SWOG trial (Kraut et al., 1998). The overall response rate in these highly pre-treated patients was 16%. We have recently combined melphalan and VP-16 phosphate with dose-escalated topotecan in a phase I/II high-dose chemotherapy trial in high risk myeloma (Harker et al., 1995; Valkov et al., 2000a). and found this to be an active and tolerable regimen. Future trials that incorporate ABCG2 transport inhibitors, such as GF120918, may increase

the efficacy of topoisomerase I inhibitors in this disease (Jonker et al., 2000; Maliepaard et al., 2001b).

DISSERTATION SUMMARY

Multiple myeloma is an incurable malignancy that kills approximately 17,000 individuals yearly in the United States. In this dissertation we have shown that myeloma is intrinsically resistant to commonly used topoisomerase inhibitors by microenvironmental factors that induce nuclear export of topo II α . In addition, during disease progression myeloma acquires additional multi-drug resistance by overexpression of the molecular transporter ABCG2. Topoisomerases are critical for cell division, especially in rapidly dividing cells such as are found in cancer. Topoisomerases are an excellent drug target to treat cancer, however, in order for topoisomerase drugs to be effective, the enzyme must be in direct contact with the DNA.

Myeloma is intrinsically resistant to topoisomerase drugs via a mechanism whereby the drug target, topo II α , is exported. We found that this mechanism is present at cell densities similar to those found in the bone marrow. High-density cells were found to be greater than 10-fold more drug resistant than low-density cells. Nuclear export could be blocked using a CRM1 inhibitor ratjadone C, CRM1 specific siRNA or a casein kinase II specific inhibitor. Blocking nuclear export was found to sensitize high-density cells to topoisomerase drugs. Sensitization to topoisomerase inhibitors was correlated with increased topoisomerase/DNA complexes and increased DNA strand breaks. This method

of sensitizing human myeloma cells suggests a new therapeutic approach to this disease.

We also examined acquired drug resistance mediated by the molecular transporter ABCG2 in multiple myeloma. We found that ABCG2 expression in myeloma cell lines increased in response to treatment with topotecan or doxorubicin. In patient studies we found that after treatment with topotecan, and at relapse, patients had an increase in ABCG2 mRNA and protein expression. Increased protein expression correlated with decreased drug uptake in functional assays. We found that expression of ABCG2 is regulated, at least in part, by promoter methylation both in cell lines and in patient plasma cells. Demethylation of the promoter increased ABCG2 mRNA and protein expression. These findings suggest that ABCG2 is expressed and functional in human myeloma cells, regulated by promoter methylation, affected by cell density, upregulated in response to chemotherapy, and may contribute to acquired drug resistance. The potential exists to utilize this effect with drug combinations which include a ABCG2 inhibitory agent.

REFERENCES

- Abbott, B.L. 2003. ABCG2 (BCRP) expression in normal and malignant hematopoietic cells. *Hematol Oncol.* 21:115-30.
- Allen, J.D., R.F. Brinkhuis, J. Wijnholds, and A.H. Schinkel. 1999. The mouse Bcrp1/Mxr/Abcp gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. *Cancer Res.* 59:4237-41.
- Allen, J.D., and A.H. Schinkel. 2002. Multidrug resistance and pharmacological protection mediated by the breast cancer resistance protein (BCRP/ABCG2). *Mol Cancer Ther.* 1:427-34.
- Ambudkar, S.V., S. Dey, C.A. Hrycyna, M. Ramachandra, I. Pastan, and M.M. Gottesman. 1999. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol.* 39:361-98.
- Anandatheerthavarada, H.K., G. Biswas, M.A. Robin, and N.G. Avadhani. 2003. Mitochondrial targeting and a novel transmembrane arrest of Alzheimer's amyloid precursor protein impairs mitochondrial function in neuronal cells. *J Cell Biol.* 161:41-54.
- Andres, D.A., I.M. Dickerson, and J.E. Dixon. 1990. Variants of the carboxyl-terminal KDEL sequence direct intracellular retention. *J Biol Chem.* 265:5952-5.
- Arnaoutov, A., Y. Azuma, K. Ribbeck, J. Joseph, Y. Boyarchuk, T. Karpova, J. McNally, and M. Dasso. 2005. Crm1 is a mitotic effector of Ran-GTP in somatic cells. *Nat Cell Biol.* 7:626-32.
- Asada, M., T. Yamada, H. Ichijo, D. Delia, K. Miyazono, K. Fukumuro, and S. Mizutani. 1999. Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. *Embo J.* 18:1223-34.
- Bailey-Dell, K.J., B. Hassel, L.A. Doyle, and D.D. Ross. 2001. Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene. *Biochim Biophys Acta.* 1520:234-41.
- Baker, E.K., R.W. Johnstone, J.R. Zalcberg, and A. El-Osta. 2005. Epigenetic changes to the MDR1 locus in response to chemotherapeutic drugs. *Oncogene.* 24:8061-75.
- Bednenko, J., G. Cingolani, and L. Gerace. 2003. Nucleocytoplasmic transport: navigating the channel. *Traffic.* 4:127-35.
- Benderra, Z., A.M. Faussat, L. Sayada, J.Y. Perrot, D. Chaoui, J.P. Marie, and O. Legrand. 2004. Breast cancer resistance protein and P-glycoprotein in 149 adult acute myeloid leukemias. *Clin Cancer Res.* 10:7896-902.

- Bertrand, R., D. Kerrigan, M. Sarang, and Y. Pommier. 1991. Cell death induced by topoisomerase inhibitors. Role of calcium in mammalian cells. *Biochem Pharmacol.* 42:77-85.
- Black, B.E., J.M. Holaska, L. Levesque, B. Ossareh-Nazari, C. Gwizdek, C. Dargemont, and B.M. Paschal. 2001. NXT1 is necessary for the terminal step of Crm1-mediated nuclear export. *J Cell Biol.* 152:141-55.
- Boehm, M., T. Yoshimoto, M.F. Crook, S. Nallamshetty, A. True, G.J. Nabel, and E.G. Nabel. 2002. A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression. *Embo J.* 21:3390-401.
- Bogerd, H.P., R.A. Fridell, R.E. Benson, J. Hua, and B.R. Cullen. 1996. Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel in vivo randomization-selection assay. *Mol Cell Biol.* 16:4207-14.
- Bonifacino, J.S., and L.M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem.* 72:395-447.
- Bourdon, J.C. 2007. p53 and its isoforms in cancer. *Br J Cancer.* 97:277-82.
- Boyer, J., E.G. McLean, S. Aroori, P. Wilson, A. McCulla, P.D. Carey, D.B. Longley, and P.G. Johnston. 2004. Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. *Clin Cancer Res.* 10:2158-67.
- Brunet, A., A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, and M.E. Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell.* 96:857-68.
- Burden, D.A., L.J. Goldsmith, and D.M. Sullivan. 1993. Cell-cycle-dependent phosphorylation and activity of Chinese-hamster ovary topoisomerase II. *Biochem J.* 293 (Pt 1):297-304.
- Candeil, L., I. Gourdiere, D. Peyron, N. Vezzio, V. Copois, F. Bibeau, B. Orsetti, G.L. Scheffer, M. Ychou, Q.A. Khan, Y. Pommier, B. Pau, P. Martineau, and M. Del Rio. 2004. ABCG2 overexpression in colon cancer cells resistant to SN38 and in irinotecan-treated metastases. *Int J Cancer.* 109:848-54.
- Chen, Q., P.C. Van der Sluis, D. Boulware, L.A. Hazlehurst, and W.S. Dalton. 2005. The FA/BRCA pathway is involved in melphalan-induced DNA interstrand cross-link repair and accounts for melphalan resistance in multiple myeloma cells. *Blood.* 106:698-705.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* 162:156-9.
- Connor, M.K., R. Kotchetkov, S. Cariou, A. Resch, R. Lupetti, R.G. Beniston, F. Melchior, L. Hengst, and J.M. Slingerland. 2003. CRM1/Ran-mediated nuclear export of p27(Kip1) involves a nuclear export signal and links p27 export and proteolysis. *Mol Biol Cell.* 14:201-13.

- Craig, E., Z.K. Zhang, K.P. Davies, and G.V. Kalpana. 2002. A masked NES in INI1/hSNF5 mediates hCRM1-dependent nuclear export: implications for tumorigenesis. *Embo J.* 21:31-42.
- Cuny, M., A. Kramar, F. Courjal, V. Johannsdottir, B. Iacopetta, H. Fontaine, J. Grenier, S. Culine, and C. Theillet. 2000. Relating genotype and phenotype in breast cancer: an analysis of the prognostic significance of amplification at eight different genes or loci and of p53 mutations. *Cancer Res.* 60:1077-83.
- Dabholkar, M., J. Vionnet, F. Bostick-Bruton, J.J. Yu, and E. Reed. 1994. Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *J Clin Invest.* 94:703-8.
- Daelemans, D., S.V. Costes, S. Lockett, and G.N. Pavlakis. 2005. Kinetic and molecular analysis of nuclear export factor CRM1 association with its cargo in vivo. *Mol Cell Biol.* 25:728-39.
- Danks, M.K., K.E. Garrett, R.C. Marion, and D.O. Whipple. 1996. Subcellular redistribution of DNA topoisomerase I in anaplastic astrocytoma cells treated with topotecan. *Cancer Res.* 56:1664-73.
- David, G.L., S. Yegnasubramanian, A. Kumar, V.L. Marchi, A.M. De Marzo, X. Lin, and W.G. Nelson. 2004. MDR1 promoter hypermethylation in MCF-7 human breast cancer cells: changes in chromatin structure induced by treatment with 5-Aza-cytidine. *Cancer Biol Ther.* 3:540-8.
- Davis, J.R., M. Kakar, and C.S. Lim. 2007. Controlling protein compartmentalization to overcome disease. *Pharm Res.* 24:17-27.
- Diestra, J.E., G.L. Scheffer, I. Catala, M. Maliepaard, J.H. Schellens, R.J. Scheper, J.R. Germa-Lluch, and M.A. Izquierdo. 2002. Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. *J Pathol.* 198:213-9.
- Doyle, L.A., and D.D. Ross. 2003. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene.* 22:7340-58.
- Doyle, L.A., W. Yang, L.V. Abruzzo, T. Krogmann, Y. Gao, A.K. Rishi, and D.D. Ross. 1998. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A.* 95:15665-70.
- Dumontet, C., and B.I. Sikic. 1999. Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol.* 17:1061-70.
- Durant, S.T., M.M. Morris, M. Illand, H.J. McKay, C. McCormick, G.L. Hirst, R.H. Borts, and R. Brown. 1999. Dependence on RAD52 and RAD1 for anticancer drug resistance mediated by inactivation of mismatch repair genes. *Curr Biol.* 9:51-4.
- Edwards, S.W., C.M. Tan, and L.E. Limbird. 2000. Localization of G-protein-coupled receptors in health and disease. *Trends Pharmacol Sci.* 21:304-8.

- Ee, P.L., S. Kamalakaran, D. Tonetti, X. He, D.D. Ross, and W.T. Beck. 2004. Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. *Cancer Res.* 64:1247-51.
- Emmons, M., D. Boulware, D.M. Sullivan, and L.A. Hazlehurst. 2006. Topoisomerase II beta levels are a determinant of melphalan-induced DNA crosslinks and sensitivity to cell death. *Biochem Pharmacol.* 72:11-8.
- Engel, R., N.I. Valkov, J.L. Gump, L. Hazlehurst, W.S. Dalton, and D.M. Sullivan. 2004. The cytoplasmic trafficking of DNA topoisomerase IIalpha correlates with etoposide resistance in human myeloma cells. *Exp Cell Res.* 295:421-31.
- Fabbro, M., and B.R. Henderson. 2003. Regulation of tumor suppressors by nuclear-cytoplasmic shuttling. *Exp Cell Res.* 282:59-69.
- Falini, B., N. Bolli, J. Shan, M.P. Martelli, A. Liso, A. Pucciarini, B. Bigerna, L. Pasqualucci, R. Mannucci, R. Rosati, P. Gorello, D. Diverio, G. Roti, E. Tiacci, G. Cazzaniga, A. Biondi, S. Schnittger, T. Haferlach, W. Hiddemann, M.F. Martelli, W. Gu, C. Mecucci, and I. Nicoletti. 2006. Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc+ AML. *Blood.* 107:4514-23.
- Feldhoff, P.W., S.E. Mirski, S.P. Cole, and D.M. Sullivan. 1994. Altered subcellular distribution of topoisomerase II alpha in a drug-resistant human small cell lung cancer cell line. *Cancer Res.* 54:756-62.
- Fink, D., S. Aebi, and S.B. Howell. 1998. The role of DNA mismatch repair in drug resistance. *Clin Cancer Res.* 4:1-6.
- Fishman, M.N., and D.M. Sullivan. 2001. Application of Resistance Reversal Agents in Hematologic Malignancies; Malignancy; Current Clinical Practice. *Hematology.* 5:343-358.
- Foster, J.S., R.I. Fernando, N. Ishida, K.I. Nakayama, and J. Wimalasena. 2003. Estrogens down-regulate p27Kip1 in breast cancer cells through Skp2 and through nuclear export mediated by the ERK pathway. *J Biol Chem.* 278:41355-66.
- Fryxell, K.B., S.B. McGee, D.K. Simoneaux, C.L. Willman, and M.M. Cornwell. 1999. Methylation analysis of the human multidrug resistance 1 gene in normal and leukemic hematopoietic cells. *Leukemia.* 13:910-7.
- Gatto, B., and E. Leo. 2003. Drugs acting on the beta isoform of human topoisomerase II (p180). *Curr Med Chem Anticancer Agents.* 3:173-85.
- Goldman, B. 2003. Multidrug resistance: can new drugs help chemotherapy score against cancer? *J Natl Cancer Inst.* 95:255-7.
- Gottesman, M.M. 2002. Mechanisms of cancer drug resistance. *Annu Rev Med.* 53:615-27.
- Gottesman, M.M., T. Fojo, and S.E. Bates. 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer.* 2:48-58.

- Gould, S.J., G.A. Keller, N. Hosken, J. Wilkinson, and S. Subramani. 1989. A conserved tripeptide sorts proteins to peroxisomes. *J Cell Biol.* 108:1657-64.
- Guo, Y.X., K. Dallmann, and J. Kwang. 2003. Identification of nucleolus localization signal of betanodavirus GGNNV protein alpha. *Virology.* 306:225-35.
- Hamamoto, T., H. Seto, and T. Beppu. 1983. Leptomycins A and B, new antifungal antibiotics. II. Structure elucidation. *J Antibiot (Tokyo).* 36:646-50.
- Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell.* 100:57-70.
- Harker, W.G., D.L. Slade, R.L. Parr, P.W. Feldhoff, D.M. Sullivan, and M.H. Holguin. 1995. Alterations in the topoisomerase II alpha gene, messenger RNA, and subcellular protein distribution as well as reduced expression of the DNA topoisomerase II beta enzyme in a mitoxantrone-resistant HL-60 human leukemia cell line. *Cancer Res.* 55:1707-16.
- Hazlehurst, L.A., R.F. Argilagos, M. Emmons, D. Boulware, C.A. Beam, D.M. Sullivan, and W.S. Dalton. 2006. Cell adhesion to fibronectin (CAM-DR) influences acquired mitoxantrone resistance in U937 cells. *Cancer Res.* 66:2338-45.
- Hazlehurst, L.A., and W.S. Dalton. 2001. Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. *Cancer Metastasis Rev.* 20:43-50.
- Hazlehurst, L.A., N.E. Foley, M.C. Gleason-Guzman, M.P. Hacker, A.E. Cress, L.W. Greenberger, M.C. De Jong, and W.S. Dalton. 1999. Multiple mechanisms confer drug resistance to mitoxantrone in the human 8226 myeloma cell line. *Cancer Res.* 59:1021-8.
- Hazlehurst, L.A., N. Valkov, L. Wisner, J.A. Storey, D. Boulware, D.M. Sullivan, and W.S. Dalton. 2001. Reduction in drug-induced DNA double-strand breaks associated with beta1 integrin-mediated adhesion correlates with drug resistance in U937 cells. *Blood.* 98:1897-903.
- Henderson, B.R., and A. Eleftheriou. 2000. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp Cell Res.* 256:213-24.
- Hodel, M.R., A.H. Corbett, and A.E. Hodel. 2001. Dissection of a nuclear localization signal. *J Biol Chem.* 276:1317-25.
- Honjo, Y., C.A. Hrycyna, Q.W. Yan, W.Y. Medina-Perez, R.W. Robey, A. van de Laar, T. Litman, M. Dean, and S.E. Bates. 2001. Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. *Cancer Res.* 61:6635-9.
- Honjo, Y., K. Morisaki, L.M. Huff, R.W. Robey, J. Hung, M. Dean, and S.E. Bates. 2002. Single-nucleotide polymorphism (SNP) analysis in the ABC half-transporter ABCG2 (MXR/BCRP/ABCP1). *Cancer Biol Ther.* 1:696-702.

- Houghton, P.J., G.S. Germain, F.C. Harwood, J.D. Schuetz, C.F. Stewart, E. Buchdunger, and P. Traxler. 2004. Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 in vitro. *Cancer Res.* 64:2333-7.
- Hwang, C.Y., I.Y. Kim, and K.S. Kwon. 2007. Cytoplasmic localization and ubiquitination of p21(Cip1) by reactive oxygen species. *Biochem Biophys Res Commun.* 358:219-25.
- Ikuta, T., H. Eguchi, T. Tachibana, Y. Yoneda, and K. Kawajiri. 1998. Nuclear localization and export signals of the human aryl hydrocarbon receptor. *J Biol Chem.* 273:2895-904.
- Ishida, N., T. Hara, T. Kamura, M. Yoshida, K. Nakayama, and K.I. Nakayama. 2002. Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export. *J Biol Chem.* 277:14355-8.
- Ishikawa, T., and F. Ali-Osman. 1993. Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance. *J Biol Chem.* 268:20116-25.
- Jonker, J.W., J.W. Smit, R.F. Brinkhuis, M. Maliepaard, J.H. Beijnen, J.H. Schellens, and A.H. Schinkel. 2000. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J Natl Cancer Inst.* 92:1651-6.
- Kalesse, M., M. Christmann, U. Bhatt, M. Quitschalle, E. Claus, A. Saeed, A. Burzlaff, C. Kasper, L.O. Haustedt, E. Hofer, T. Scheper, and W. Beil. 2001. The chemistry and biology of ratjadone. *ChemBiochem.* 2:709-14.
- Kanwal, C., H. Li, and C.S. Lim. 2002. Model system to study classical nuclear export signals. *AAPS PharmSci.* 4:E18.
- Karpa, K.D., R. Lin, N. Kabbani, and R. Levenson. 2000. The dopamine D3 receptor interacts with itself and the truncated D3 splice variant d3nf: D3-D3nf interaction causes mislocalization of D3 receptors. *Mol Pharmacol.* 58:677-83.
- Kau, T.R., F. Schroeder, S. Ramaswamy, C.L. Wojciechowski, J.J. Zhao, T.M. Roberts, J. Clardy, W.R. Sellers, and P.A. Silver. 2003. A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells. *Cancer Cell.* 4:463-76.
- Kau, T.R., and P.A. Silver. 2003. Nuclear transport as a target for cell growth. *Drug Discov Today.* 8:78-85.
- Kavallaris, M., A.S. Tait, B.J. Walsh, L. He, S.B. Horwitz, M.D. Norris, and M. Haber. 2001. Multiple microtubule alterations are associated with Vinca alkaloid resistance in human leukemia cells. *Cancer Res.* 61:5803-9.
- Kawabata, S., M. Oka, H. Soda, K. Shiozawa, K. Nakatomi, J. Tsurutani, Y. Nakamura, S. Doi, T. Kitazaki, K. Sugahara, Y. Yamada, S. Kamihira, and S. Kohno. 2003. Expression and functional analyses of breast cancer resistance protein in lung cancer. *Clin Cancer Res.* 9:3052-7.

- Keeshan, K., T.G. Cotter, and S.L. McKenna. 2003. Bcr-Abl upregulates cytosolic p21WAF-1/CIP-1 by a phosphoinositide-3-kinase (PI3K)-independent pathway. *Br J Haematol.* 123:34-44.
- Kent, C.R., J.J. Eady, G.M. Ross, and G.G. Steel. 1995. The comet moment as a measure of DNA damage in the comet assay. *Int J Radiat Biol.* 67:655-60.
- Kim, M., H. Turnquist, J. Jackson, M. Sgagias, Y. Yan, M. Gong, M. Dean, J.G. Sharp, and K. Cowan. 2002. The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. *Clin Cancer Res.* 8:22-8.
- Knutsen, T., V.K. Rao, T. Ried, L. Mickley, E. Schneider, K. Miyake, B.M. Ghadimi, H. Padilla-Nash, S. Pack, L. Greenberger, K. Cowan, M. Dean, T. Fojo, and S. Bates. 2000. Amplification of 4q21-q22 and the MXR gene in independently derived mitoxantrone-resistant cell lines. *Genes Chromosomes Cancer.* 27:110-6.
- Koster, M., S. Lykke-Andersen, Y.A. Elnakady, K. Gerth, P. Washausen, G. Hofle, F. Sasse, J. Kjems, and H. Hauser. 2003. Ratjadones inhibit nuclear export by blocking CRM1/exportin 1. *Exp Cell Res.* 286:321-31.
- Kraut, E.H., J.J. Crowley, J.L. Wade, L.R. Laufman, M. Alsina, S.A. Taylor, and S.E. Salmon. 1998. Evaluation of topotecan in resistant and relapsing multiple myeloma: a Southwest Oncology Group study. *J Clin Oncol.* 16:589-92.
- Krishna, R., and L.D. Mayer. 2000. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. *Eur J Pharm Sci.* 11:265-83.
- Kudo, N., N. Matsumori, H. Taoka, D. Fujiwara, E.P. Schreiner, B. Wolff, M. Yoshida, and S. Horinouchi. 1999. Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc Natl Acad Sci U S A.* 96:9112-7.
- Kusaba, H., M. Nakayama, T. Harada, M. Nomoto, K. Kohno, M. Kuwano, and M. Wada. 1999. Association of 5' CpG demethylation and altered chromatin structure in the promoter region with transcriptional activation of the multidrug resistance 1 gene in human cancer cells. *Eur J Biochem.* 262:924-32.
- Kutay, U., and S. Guttinger. 2005. Leucine-rich nuclear-export signals: born to be weak. *Trends Cell Biol.* 15:121-4.
- Li, X.G., P. Haluska, Jr., Y.H. Hsiang, A. Bharti, D.W. Kufe, and E.H. Rubin. 1996. Identification of topoisomerase I mutations affecting both DNA cleavage and interaction with camptothecin. *Ann N Y Acad Sci.* 803:111-27.
- Lim, R.Y., U. Aebi, and B. Fahrenkrog. 2008. Towards reconciling structure and function in the nuclear pore complex. *Histochem Cell Biol.*
- Litman, T., M. Brangi, E. Hudson, P. Fetsch, A. Abati, D.D. Ross, K. Miyake, J.H. Resau, and S.E. Bates. 2000. The multidrug-resistant phenotype

- associated with overexpression of the new ABC half-transporter, MXR (ABCG2). *J Cell Sci.* 113 (Pt 11):2011-21.
- Liu, J., X. Du, and Y. Ke. 2006. Mapping nucleolar localization sequences of 1A6/DRIM. *FEBS Lett.* 580:1405-10.
- Longley, D.B., D.P. Harkin, and P.G. Johnston. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer.* 3:330-8.
- Longley, D.B., and P.G. Johnston. 2005. Molecular mechanisms of drug resistance. *J Pathol.* 205:275-92.
- Lyu, Y.L., and J.C. Wang. 2003. Aberrant lamination in the cerebral cortex of mouse embryos lacking DNA topoisomerase IIbeta. *Proc Natl Acad Sci U S A.* 100:7123-8.
- Maliepaard, M., G.L. Scheffer, I.F. Faneyte, M.A. van Gastelen, A.C. Pijnenborg, A.H. Schinkel, M.J. van De Vijver, R.J. Scheper, and J.H. Schellens. 2001a. Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res.* 61:3458-64.
- Maliepaard, M., M.A. van Gastelen, A. Tohgo, F.H. Hausheer, R.C. van Waardenburg, L.A. de Jong, D. Pluim, J.H. Beijnen, and J.H. Schellens. 2001b. Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins in vitro using non-substrate drugs or the BCRP inhibitor GF120918. *Clin Cancer Res.* 7:935-41.
- Meissner, T., E. Krause, and U. Vinkemeier. 2004. Ratjadone and leptomycin B block CRM1-dependent nuclear export by identical mechanisms. *FEBS Lett.* 576:27-30.
- Min, Y.H., J.W. Cheong, J.Y. Kim, J.I. Eom, S.T. Lee, J.S. Hahn, Y.W. Ko, and M.H. Lee. 2004. Cytoplasmic mislocalization of p27Kip1 protein is associated with constitutive phosphorylation of Akt or protein kinase B and poor prognosis in acute myelogenous leukemia. *Cancer Res.* 64:5225-31.
- Mirski, S.E., J.C. Bielawski, and S.P. Cole. 2003. Identification of functional nuclear export sequences in human topoisomerase IIalpha and beta. *Biochem Biophys Res Commun.* 306:905-11.
- Mirski, S.E., J.H. Gerlach, H.J. Cummings, R. Zirngibl, P.A. Greer, and S.P. Cole. 1997. Bipartite nuclear localization signals in the C terminus of human topoisomerase II alpha. *Exp Cell Res.* 237:452-5.
- Miyake, K., L. Mickley, T. Litman, Z. Zhan, R. Robey, B. Cristensen, M. Brangi, L. Greenberger, M. Dean, T. Fojo, and S.E. Bates. 1999. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res.* 59:8-13.
- Mogi, M., J. Yang, J.F. Lambert, G.A. Colvin, I. Shiojima, C. Skurk, R. Summer, A. Fine, P.J. Quesenberry, and K. Walsh. 2003. Akt signaling regulates side population cell phenotype via Bcrp1 translocation. *J Biol Chem.* 278:39068-75.
- Munro, S., and H.R. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899-907.

- Nakamura, N., S. Ramaswamy, F. Vazquez, S. Signoretti, M. Loda, and W.R. Sellers. 2000. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol.* 20:8969-82.
- Nakamura, T., H. Imai, N. Tsunashima, and Y. Nakagawa. 2003. Molecular cloning and functional expression of nucleolar phospholipid hydroperoxide glutathione peroxidase in mammalian cells. *Biochem Biophys Res Commun.* 311:139-48.
- Nefedova, Y., T.H. Landowski, and W.S. Dalton. 2003. Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms. *Leukemia.* 17:1175-82.
- Neufeld, K.L., D.A. Nix, H. Bogerd, Y. Kang, M.C. Beckerle, B.R. Cullen, and R.L. White. 2000. Adenomatous polyposis coli protein contains two nuclear export signals and shuttles between the nucleus and cytoplasm. *Proc Natl Acad Sci U S A.* 97:12085-90.
- Newlands, E.S., G.J. Rustin, and M.H. Brampton. 1996. Phase I trial of elactocin. *Br J Cancer.* 74:648-9.
- Nikolaev, A.Y., M. Li, N. Puskas, J. Qin, and W. Gu. 2003. Parc: a cytoplasmic anchor for p53. *Cell.* 112:29-40.
- Nitiss, J.L. 2002. DNA topoisomerases in cancer chemotherapy: using enzymes to generate selective DNA damage. *Curr Opin Investig Drugs.* 3:1512-6.
- Noske, A., W. Weichert, S. Niesporek, A. Roske, A.C. Buckendahl, I. Koch, J. Sehoul, M. Dietel, and C. Denkert. 2008. Expression of the nuclear export protein chromosomal region maintenance/exportin 1/Xpo1 is a prognostic factor in human ovarian cancer. *Cancer.*
- O'Brate, A., and P. Giannakakou. 2003. The importance of p53 location: nuclear or cytoplasmic zip code? *Drug Resist Updat.* 6:313-22.
- Oloumi, A., S.H. MacPhail, P.J. Johnston, J.P. Banath, and P.L. Olive. 2000. Changes in subcellular distribution of topoisomerase IIalpha correlate with etoposide resistance in multicell spheroids and xenograft tumors. *Cancer Res.* 60:5747-53.
- Pichler, A., and F. Melchior. 2002. Ubiquitin-related modifier SUMO1 and nucleocytoplasmic transport. *Traffic.* 3:381-7.
- Pommier, Y., C. Redon, V.A. Rao, J.A. Seiler, O. Sordet, H. Takemura, S. Antony, L. Meng, Z. Liao, G. Kohlhagen, H. Zhang, and K.W. Kohn. 2003. Repair of and checkpoint response to topoisomerase I-mediated DNA damage. *Mutat Res.* 532:173-203.
- Poon, I.K., and D.A. Jans. 2005. Regulation of nuclear transport: central role in development and transformation? *Traffic.* 6:173-86.
- Powell, S.M., N. Zilz, Y. Beazer-Barclay, T.M. Bryan, S.R. Hamilton, S.N. Thibodeau, B. Vogelstein, and K.W. Kinzler. 1992. APC mutations occur early during colorectal tumorigenesis. *Nature.* 359:235-7.

- Raaijmakers, M.H., E.P. de Grouw, L.H. Heuver, B.A. van der Reijden, J.H. Jansen, G. Scheffer, R.J. Scheper, T.J. de Witte, and R.A. Raymakers. 2005. Impaired breast cancer resistance protein mediated drug transport in plasma cells in multiple myeloma. *Leuk Res.* 29:1455-8.
- Rabindran, S.K., D.D. Ross, L.A. Doyle, W. Yang, and L.M. Greenberger. 2000. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. *Cancer Res.* 60:47-50.
- Rasheed, Z.A., and E.H. Rubin. 2003. Mechanisms of resistance to topoisomerase I-targeting drugs. *Oncogene.* 22:7296-304.
- Rimsza, L.M., K. Campbell, W.S. Dalton, S. Salmon, G. Willcox, and T.M. Grogan. 1999. The major vault protein (MVP), a new multidrug resistance associated protein, is frequently expressed in multiple myeloma. *Leuk Lymphoma.* 34:315-24.
- Robey, R.W., W.Y. Medina-Perez, K. Nishiyama, T. Lahusen, K. Miyake, T. Litman, A.M. Senderowicz, D.D. Ross, and S.E. Bates. 2001. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. *Clin Cancer Res.* 7:145-52.
- Rodriguez-Vilarrupla, A., M. Jaumot, N. Abella, N. Canela, S. Brun, C. Diaz, J.M. Estanyol, O. Bachs, and N. Agell. 2005. Binding of calmodulin to the carboxy-terminal region of p21 induces nuclear accumulation via inhibition of protein kinase C-mediated phosphorylation of Ser153. *Mol Cell Biol.* 25:7364-74.
- Roe, M., A. Folkes, P. Ashworth, J. Brumwell, L. Chima, S. Hunjan, I. Pretswell, W. Dangerfield, H. Ryder, and P. Charlton. 1999. Reversal of P-glycoprotein mediated multidrug resistance by novel anthranilamide derivatives. *Bioorg Med Chem Lett.* 9:595-600.
- Rosin-Arbesfeld, R., A. Cliffe, T. Brabletz, and M. Bienz. 2003. Nuclear export of the APC tumour suppressor controls beta-catenin function in transcription. *Embo J.* 22:1101-13.
- Ross, D.D., W. Yang, L.V. Abruzzo, W.S. Dalton, E. Schneider, H. Lage, M. Dietel, L. Greenberger, S.P. Cole, and L.A. Doyle. 1999. Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. *J Natl Cancer Inst.* 91:429-33.
- Sakaguchi, A., and A. Kikuchi. 2004. Functional compatibility between isoform alpha and beta of type II DNA topoisomerase. *J Cell Sci.* 117:1047-54.
- Santos, A., L. Calvet, M.J. Terrier-Lacombe, A. Larsen, J. Benard, C. Pondarre, G. Aubert, J. Morizet, F. Lavelle, and G. Vassal. 2004. In vivo treatment with CPT-11 leads to differentiation of neuroblastoma xenografts and topoisomerase I alterations. *Cancer Res.* 64:3223-9.
- Sauerbrey, A., W. Sell, D. Steinbach, A. Voigt, and F. Zintl. 2002. Expression of the BCRP gene (ABCG2/MXR/ABCP) in childhood acute lymphoblastic leukaemia. *Br J Haematol.* 118:147-50.

- Scharenberg, C.W., M.A. Harkey, and B. Torok-Storb. 2002. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood*. 99:507-12.
- Schwarzenbach, H. 2002. Expression of MDR1/P-glycoprotein, the multidrug resistance protein MRP, and the lung-resistance protein LRP in multiple myeloma. *Med Oncol*. 19:87-104.
- Senda, T., A. Iizuka-Kogo, T. Onouchi, and A. Shimomura. 2007. Adenomatous polyposis coli (APC) plays multiple roles in the intestinal and colorectal epithelia. *Med Mol Morphol*. 40:68-81.
- Shin, I., J. Rotty, F.Y. Wu, and C.L. Arteaga. 2005. Phosphorylation of p27Kip1 at Thr-157 interferes with its association with importin alpha during G1 and prevents nuclear re-entry. *J Biol Chem*. 280:6055-63.
- Sordet, O., Q.A. Khan, K.W. Kohn, and Y. Pommier. 2003. Apoptosis induced by topoisomerase inhibitors. *Curr Med Chem Anticancer Agents*. 3:271-90.
- Soussi, T. 2007. p53 alterations in human cancer: more questions than answers. *Oncogene*. 26:2145-56.
- Soussi, T., and K.G. Wiman. 2007. Shaping genetic alterations in human cancer: the p53 mutation paradigm. *Cancer Cell*. 12:303-12.
- Stommel, J.M., N.D. Marchenko, G.S. Jimenez, U.M. Moll, T.J. Hope, and G.M. Wahl. 1999. A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *Embo J*. 18:1660-72.
- Sullivan, D.M., K.C. Chow, and W.E. Ross. 1989. Topoisomerase II mediated mechanisms of drug resistance. CRC Press, Boca Raton, FL. 281-292 pp.
- Sullivan, D.M., J. Partyka, and W.S. Dalton. 2001. A phase I/II study of high-dose melphalan + topotecan + VP-16 phosphate (MTV) followed by autologous stem cell rescue in multiple myeloma. *In ASCO*. Vol. 20. 14.
- Sullivan, D.M., and W.E. Ross. 1991. Resistance to inhibitors of DNA topoisomerases. Kluwer Academic Publishers, Boston, MA USA. 57-99 pp.
- Susaki, E., and K.I. Nakayama. 2007. Multiple mechanisms for p27(Kip1) translocation and degradation. *Cell Cycle*. 6:3015-20.
- Takenaka, Y., T. Fukumori, T. Yoshii, N. Oka, H. Inohara, H.R. Kim, R.S. Bresalier, and A. Raz. 2004. Nuclear export of phosphorylated galectin-3 regulates its antiapoptotic activity in response to chemotherapeutic drugs. *Mol Cell Biol*. 24:4395-406.
- Taylor, C.W., W.S. Dalton, P.R. Parrish, M.C. Gleason, W.T. Bellamy, F.H. Thompson, D.J. Roe, and J.M. Trent. 1991. Different mechanisms of decreased drug accumulation in doxorubicin and mitoxantrone resistant variants of the MCF7 human breast cancer cell line. *Br J Cancer*. 63:923-9.
- Terry, L.J., E.B. Shows, and S.R. Wentz. 2007. Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport. *Science*. 318:1412-6.

- Thomas, H., and H.M. Coley. 2003. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting p-glycoprotein. *Cancer Control*. 10:159-65.
- Turner, J.G., R. Engel, J.A. Derderian, R. Jove, and D.M. Sullivan. 2004. Human topoisomerase II alpha nuclear export is mediated by two CRM-1-dependent nuclear export signals. *J Cell Sci*. 117:3061-71.
- Uggla, B., E. Stahl, D. Wagsater, C. Paul, M.G. Karlsson, A. Sirsjo, and U. Tidefelt. 2005. BCRP mRNA expression v. clinical outcome in 40 adult AML patients. *Leuk Res*. 29:141-6.
- Valkov, N., J.L. Gump, A.N. Hernandez, and D.M. Sullivan. 2000a. Effect of sequencing high-dose melphalan, topotecan and VP-16 on the amount and localization of topoisomerases in malignant plasma cell from Multiple Myeloma. *In AACR*. Vol. 41. 212.
- Valkov, N.I., J.L. Gump, R. Engel, and D.M. Sullivan. 2000b. Cell density-dependent VP-16 sensitivity of leukaemic cells is accompanied by the translocation of topoisomerase IIalpha from the nucleus to the cytoplasm. *Br J Haematol*. 108:331-45.
- Valkov, N.I., and D.M. Sullivan. 1997. Drug resistance to DNA topoisomerase I and II inhibitors in human leukemia, lymphoma, and multiple myeloma. *Semin Hematol*. 34:48-62.
- Valkov, N.I., and D.M. Sullivan. 2003. Tumor p53 status and response to topoisomerase II inhibitors. *Drug Resist Updat*. 6:27-39.
- van den Heuvel-Eibrink, M.M., E.A. Wiemer, A. Prins, J.P. Meijerink, P.J. Vossebeld, B. van der Holt, R. Pieters, and P. Sonneveld. 2002. Increased expression of the breast cancer resistance protein (BCRP) in relapsed or refractory acute myeloid leukemia (AML). *Leukemia*. 16:833-9.
- Vogt, P.K., H. Jiang, and M. Aoki. 2005. Triple layer control: phosphorylation, acetylation and ubiquitination of FOXO proteins. *Cell Cycle*. 4:908-13.
- Wadhwa, R., T. Yaguchi, M.K. Hasan, Y. Mitsui, R.R. Reddel, and S.C. Kaul. 2002. Hsp70 family member, mot-2/mthsp70/GRP75, binds to the cytoplasmic sequestration domain of the p53 protein. *Exp Cell Res*. 274:246-53.
- Wang, J.C. 2002. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol*. 3:430-40.
- Warnecke, P.M., C. Stirzaker, J. Song, C. Grunau, J.R. Melki, and S.J. Clark. 2002. Identification and resolution of artifacts in bisulfite sequencing. *Methods*. 27:101-7.
- Weis, K. 2007. The nuclear pore complex: oily spaghetti or gummy bear? *Cell*. 130:405-7.
- Welsh, M.J., and A.E. Smith. 1993. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell*. 73:1251-4.
- Wessel, I., P.B. Jensen, J. Falck, S.E. Mirski, S.P. Cole, and M. Sehested. 1997. Loss of amino acids 1490Lys-Ser-Lys1492 in the COOH-terminal region of topoisomerase IIalpha in human small cell lung cancer cells selected for

- resistance to etoposide results in an extranuclear enzyme localization. *Cancer Res.* 57:4451-4.
- Woehlecke, H., H. Osada, A. Herrmann, and H. Lage. 2003. Reversal of breast cancer resistance protein-mediated drug resistance by tryprostatin A. *Int J Cancer.* 107:721-8.
- Xiao, H., Y. Mao, S.D. Desai, N. Zhou, C.Y. Ting, J. Hwang, and L.F. Liu. 2003. The topoisomerase II β circular clamp arrests transcription and signals a 26S proteasome pathway. *Proc Natl Acad Sci U S A.* 100:3239-44.
- Xu, J., Y. Liu, Y. Yang, S. Bates, and J.T. Zhang. 2004. Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. *J Biol Chem.* 279:19781-9.
- Xu, Y., and M.A. Villalona-Calero. 2002. Irinotecan: mechanisms of tumor resistance and novel strategies for modulating its activity. *Ann Oncol.* 13:1841-51.
- Yashiroda, Y., and M. Yoshida. 2003. Nucleo-cytoplasmic transport of proteins as a target for therapeutic drugs. *Curr Med Chem.* 10:741-8.
- Yoneda, Y., M. Hieda, E. Nagoshi, and Y. Miyamoto. 1999. Nucleocytoplasmic protein transport and recycling of Ran. *Cell Struct Funct.* 24:425-33.
- Yu, Q., S.E. Mirski, K.E. Sparks, and S.P. Cole. 1997. Two COOH-terminal truncated cytoplasmic forms of topoisomerase II α in a VP-16-selected lung cancer cell line result from partial gene deletion and alternative splicing. *Biochemistry.* 36:5868-77.
- Zeng, Q., T.T. Tran, H.X. Tan, and W. Hong. 2003. The cytoplasmic domain of Vamp4 and Vamp5 is responsible for their correct subcellular targeting: the N-terminal extension of VAMP4 contains a dominant autonomous targeting signal for the trans-Golgi network. *J Biol Chem.* 278:23046-54.
- Zhang, Y., and Y. Xiong. 2001. A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation. *Science.* 292:1910-5.
- Zhao, S., K.S. Smith, A.M. Deveau, C.M. Dieckhaus, M.A. Johnson, T.L. Macdonald, and J.M. Cook. 2002. Biological activity of the tryprostatins and their diastereomers on human carcinoma cell lines. *J Med Chem.* 45:1559-62.
- Zhou, B.P., Y. Liao, W. Xia, B. Spohn, M.H. Lee, and M.C. Hung. 2001a. Cytoplasmic localization of p21^{Cip1}/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nat Cell Biol.* 3:245-52.
- Zhou, S., J.D. Schuetz, K.D. Bunting, A.M. Colapietro, J. Sampath, J.J. Morris, I. Lagutina, G.C. Grosveld, M. Osawa, H. Nakauchi, and B.P. Sorrentino. 2001b. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med.* 7:1028-34.

About the Author

Joel Turner received a B.S. degree in Human Biology from the University of Wisconsin in 1983 and a M.S. in Molecular and Cellular Biology from the University of South Florida in 2004. He officially entered the Ph.D. program at the University of South Florida College of Arts and Sciences Biology program in the spring of 2005 and was admitted to candidacy for doctoral degree August 9, 2007.

Mr. Turner started working as a laboratory technician at the age of 17 as a college freshman and since has continued in medical research for 28 years. Mr. Turner has been a lab technician and coordinator at the H. Moffitt Cancer Center and Research Institute for eleven years. He has accumulated 40 peer-reviewed scientific publications and is a full-member of the *American Association for Cancer Research*.

In addition, he has taught undergraduate biology, anatomy and physiology during evening classes as an adjunct faculty at both Hillsborough Community College and the USF College of Public Health for the past four years.