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MECHANISM OF CELLULAR RESPONSE TO ANTICANCER DRUGS:
INVOLVEMENT OF ATM AND TOPOISOMERASE

DISSertation

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

By

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* * * * *

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2002

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ABSTRACT

As a result of evolution, cells have developed mechanisms to deal with stresses caused by internal and environmental factors, such as DNA repair and cell cycle checkpoint. This dissertation includes several aspects of these mechanisms.

The first part of the dissertation involves in the roles of ATM in histone acetylation-mediated gene activation. I present evidence that induction of p21WAF1 by histone deacetylase inhibitors (FR901228) and trichostatin A is defective in Ataxia telangiectacia (AT) cells. Caffeine and wortmannin attenuate p21WAF1 induction in normal cells, indicating that PI-3 kinase activity is essential for this process. It is also observed that ectopic expression of the wild-type ATM gene in an AT cell line restores p21WAF1 induction by the HDAC inhibitors. Besides the p21WAF1 promoter, activation of topoisomerase IIIα (topo IIIα) and SV40 promoters by the HDAC inhibitors are also decreased in the AT cell lines. Finally, despite the deficiency of induction of gene expression, the overall levels of H3 and H4 histone acetylation appear to be the same between AT and normal cells in response to HDAC inhibitor treatments. Taken together, the data in this study indicate that ATM is involved in histone acetylation-mediated gene expression regulation.
The second part of the dissertation involves investigation of drug resistance in a particular cancer, multiple myeloma (MM). Previous clinical studies have demonstrated that the topoisomerase I (topo I) inhibitor topotecan had activity in a small portion of patients (16%) with resistant or relapsed myeloma. In order to identify the mechanisms behind response and resistance to this drug, the relationship between topo I and camptothecin (CPT) cytotoxicity in six myeloma cell lines as well as the effect of camptothecin combined with a protein kinase C/CDK inhibitor UCN-01 were investigated.

Among the six cell lines I examined, three of them (NCI-H929, MC/CAR, HS-Sultan) displayed relatively high sensitivity to CPT, while the other three (8226/S, 8226/DOX40, SKO-007) were relatively resistant. Despite the fact that topo I polypeptide levels and CPT-induced DNA-topo I cleavable complex levels varied among these cell lines, cytotoxicity correlated with topo I levels as well as DNA-topo I cleavable complex levels only in those cells possessing either very high or very low complex levels. On the other hand, cell lines with intermediate levels of topo I-DNA complexes did not demonstrate a close correlation with cytotoxicity.

To identify potential approaches to overcome resistance, CPT was combined with UCN-01 to treat two CPT resistant myeloma cell lines (8226/DOX40 and SKO-007). Cytotoxicity assays showed that UCN-01 significantly enhanced CPT-induced cytotoxicity in both 8226/DOX40 and SKO-007. Flow cytometry data indicate that addition of UCN-01 to CPT specifically abrogated the S-phase checkpoint induced by CPT in these two cell lines. Furthermore, CPT-induced DNA synthesis inhibition was partially abolished in 8226/DOX40. As a result, an increase of DNA double strand breaks
(DSB) represented by γ-H2AX was observed. Our data suggest that the combination of CPT with UCN-01 has the potential to overcome the CPT resistance detected in multiple myeloma.
ACKNOWLEDGMENTS

I would like to express my appreciation to my advisor, Dr. Mark Muller, for providing me with opportunity, resources and scientific guidance as well as freedom through my years in his laboratory. I would also like to thank the members of my committee, Drs. L. Johnson, H. Park, A. Simcox and R. Tassava, for their suggestion, time and support. Thanks to all the members of the Muller lab, past and present, especially D. Lou, C. Furbee, Y. Mao, W. Staerker, C. wolfs, I. Mehl, K. Liu and Y. Wang, for their support, friendship and intelligent discussion of science. Special thanks to Dr. E. Kraut for his suggestions and support. Finally, I would like to thank my parents for their encouragement and understanding and my wife, Zhuming, for her love, help and patience.
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PUBLICATIONS


FIELD OF STUDY

Major Field: Molecular Genetics
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<th>Definition</th>
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<tr>
<td>AT</td>
<td>Ataxia-telengiectasia</td>
</tr>
<tr>
<td>ATM</td>
<td>AT mutated</td>
</tr>
<tr>
<td>BASC</td>
<td>BRCA1 associated genome surveillance complex</td>
</tr>
<tr>
<td>BLM</td>
<td>Bloom’s syndrome</td>
</tr>
<tr>
<td>BRCA</td>
<td>breast cancer associated gene</td>
</tr>
<tr>
<td>BRCT</td>
<td>BRCA1 C-terminus</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CPT</td>
<td>camptothecin</td>
</tr>
<tr>
<td>DS</td>
<td>double strand</td>
</tr>
<tr>
<td>FR</td>
<td>FR901228</td>
</tr>
<tr>
<td>γ-H2AX</td>
<td>phosphorylated histone H2AX</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>ICT</td>
<td><em>In vivo</em> Complex of Topoisomerase</td>
</tr>
<tr>
<td>IR</td>
<td>ionizing radiation</td>
</tr>
<tr>
<td>MBD2</td>
<td>methyl-CpG-binding-domain protein 2</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MeCP2</td>
<td>methyl-CpG-binding protein 2</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte enhancer factor</td>
</tr>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance protein</td>
</tr>
<tr>
<td>NcoR</td>
<td>nuclear hormone receptor corepressor</td>
</tr>
<tr>
<td>NBS</td>
<td>Nijmegen breakage syndrome</td>
</tr>
<tr>
<td>NuRD</td>
<td>nucleosome remodelling histone deacetylase</td>
</tr>
<tr>
<td>PCAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PI-3 kinase</td>
<td>phosphatidylinositol-3 kinases</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>RDS</td>
<td>radioresistant DNA synthesis</td>
</tr>
<tr>
<td>RAR</td>
<td>unliganded retinoid acid receptor</td>
</tr>
<tr>
<td>Topo</td>
<td>topoisomerase</td>
</tr>
<tr>
<td>TR</td>
<td>thyroid hormone receptor</td>
</tr>
<tr>
<td>TSA</td>
<td>trichostatin A</td>
</tr>
</tbody>
</table>
CHAPTER 1

P21WAF1 EXPRESSION IS ACTIVATED BY HISTONE DEACETYLASE INHIBITORS VIA ATM

INTRODUCTION

I. Biology of histone acetylation

A. Chromatin Structure

In eukaryotic cells, DNA is packaged with a set of basic histone proteins to form nucleosomes, the repeat unit of chromatin. A variable length of linker DNA associated with histone H1 separates contiguous nucleosomes and each nucleosome contains a histone core wrapped with 146 bp of DNA. The histone core is an octamer composed of a histone H3-H4 tetramer that interacts with two H2A-H2B dimers (Luger, 1997).

Histones are multi-domain proteins, where histone H3 and H4 have a defined apolar globular domain core and flexible N-terminal tails and H2A, H2B as well as H1 consist of conserved central globular domains and flexible N and C-terminal domains. X-ray analysis reveals that the highly charged N-terminal tails of core histones extend outward from the core of nucleosome (Wolffe et al, 1999; Cheung et al, 2000). Amino acids with
highly active side chains are present among the flexible tail domains in all the histones, such as lysine, serine, and tyrosine. Numerous studies demonstrate that these are the sites where reversible chemical modifications occur such as phosphorylation at serines and tyrosines in both N and C terminal domains of H1 as well as at N-terminal serines of H3. The modifications also involve acetylation at lysines in N-terminal domains of H2A, H2B, H3 and H4 and ubiquitination at lysines at N-terminal domains of H2A and H2B (Cheung et al, 2000; Roth et al, 2001; Zhang et al, 2001).

B. HATs and HDACs

1. Biology of HATs

Histone acetylation is the most extensively studied modification. There are two classes of enzymes involved in this process, histone acetyltransferases (HATs), which add the acetyl group to ε-amino group of lysine of core histone, and histone deacetylases (HDACs), which remove the acetyl group (Davie et al, 1998). HATs are divided into two categories: nuclear localized type A and cytoplasmic localized type B (Brownell et al, 1996). The A-type HATs acetylate nucleosomal histones in chromatin, and most likely regulate transcription. The B-type HATs acetylate newly synthesized free cytoplasmic histones destined for nuclear import, where they may be deacetylated and deposited into chromatin (Allis et al, 1985; Garcea et al, 1980; Ruiz-Carrillo et al, 1975). To date, five groups of HATs have been identified (Sterner, 2000) (summarized in Table 1.). The first group, Gcn-5-related N-acetyltransferase (GNAT), contains GCN5 (general control nonderepressible –5) and p300/CBP-binding protein-associated factor (PCAF), which are related to yeast HAT Gcn5 (Neuwald et al, 1997). This group also includes Hat1, the only
<table>
<thead>
<tr>
<th>Type A/B</th>
<th>organism</th>
<th>function</th>
<th>Histones acetylated by recombinant HAT</th>
<th>Histones acetylated by HAT complex</th>
<th>Complexes that the HAT associated with</th>
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<td>GNAT superfamily Hat 1 B</td>
<td>Yeast to humans</td>
<td>Histone deposition</td>
<td>H4</td>
<td>H4, H2A</td>
<td>Yeast HAT-B, HAT-A3 Yeast HAT-A3 ADA, SAGA; human GCN5, PACF complex Polymerase II holoenzme</td>
</tr>
<tr>
<td>Gcn5 A</td>
<td>Yeast to humans</td>
<td>Coactivator</td>
<td>H3, H4</td>
<td>H3, H2B</td>
<td></td>
</tr>
<tr>
<td>PCAF A</td>
<td>Humans, mice Yeast</td>
<td>Coactivator</td>
<td>H3, H4</td>
<td>H3, H4</td>
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<tr>
<td>Elp3 A</td>
<td>Yeast</td>
<td>Transcription elongation</td>
<td>H2A, H2B, H3, H4</td>
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<td>Yeast</td>
<td>Silencing silencing</td>
<td>Unknown</td>
<td>H3, H4</td>
<td></td>
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<td>Dosage compensation</td>
<td>H4</td>
<td>H4</td>
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<tr>
<td>MOF A</td>
<td>Fruit flies</td>
<td>Leukemogenesis, upon chromosomal translocation</td>
<td>Unknown</td>
<td>H4</td>
<td></td>
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<tr>
<td>MOZ A</td>
<td>Human</td>
<td></td>
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Table 1. Summary of HATs (continued)
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<th>HBO1</th>
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<td>A</td>
<td>Multicellular organisms</td>
<td>Global coactivator</td>
<td>H2A, H2B, H3, H4</td>
<td></td>
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<tr>
<td>P300</td>
<td>A</td>
<td>Multicellular organisms</td>
<td>Global coactivator</td>
<td>H2A, H2B, H3, H4</td>
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<tr>
<td>CBP</td>
<td>A</td>
<td>Yeast to humans</td>
<td>TBP-associated factor</td>
<td>H2A, H2B, H3, H4</td>
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<td>Yeast to humans</td>
<td>Transcriptional response to hormone signals</td>
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<td>Nuclear receptor coactivators</td>
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<td>Humans, mice</td>
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<td>H3, H4</td>
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<td>SRC1 ACTR</td>
<td>A</td>
<td>Humans, mice</td>
<td></td>
<td>H3, H4</td>
<td></td>
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type B HAT (Kleff, et al 1995; Parthun et al, 1996). The second group, MYST (named for its founding members: MOZ, Ybf2/Sas3, Sas2, and Tip60), contains Esa 1 (essential Sas family acetyltransferase), MOZ (monocytic leukemia zinc finger protein), Sas2 (something about silencing 2) (yeast homolog of the human oncogene MOZ), MORF, Tip60 (TAT-interactive protein with a mass of 60 kDa) and Hbo1 (Borrow J et al, 1996). A third group contains the closely related cyclic adenosine monophosphate response element-binding protein (CBP) and p300 (Ogryzko et al, 1996), which act as co-activators for a number of transcription factor complexes (Bannister et al, 1996). A fourth group contains the TAF250 protein, part of the basic transcription complex TFIIID that binds the TATA box (Mizzen et al, 1996). Finally, a fifth group contains the SRC-1 (Kamei et al, 1996) and ACTR that are co-activators for ligand-activated nuclear receptor (Leo et al, 2000).

HAT functions are important in many physiological process, such as DNA replication (HBO1) (Iizuka, 1999), basal transcription (TAFIID and TFIIIC ) (Mizzen et al, 1996; Kunduet al, 1999), elongation (Elp3) (Wittschieben et al, 1999), locus-specific acetylation (Schubeler et al, 2000), and transcriptional silencing (Sas2 and Sas3 and Hat1) (Ehrenhofer-Murray et al, 1997; Reifsnyder et al, 1996; Kelly et al, 2000).

Distinct functions of different HATs appear to be associated with their substrate specificity in terms of histones and sites within the histones (Brownell and Allis, 1996). For example, Hat1, as a B-type HAT (Kleff et al, 1995; Parthun et al, 1996), acetylates only H4 at lys5 or/and lys12 (Chicoine et al, 1986), consistent with the pattern of H4 diacetylation related to histone deposition (Chicoine et al, 1986; Verreault et al, 1996).
Another example is that the dosage compensation-related HAT, MOF, selectively acetylates H4 at lys16 in vitro, consistent with the in vivo pattern associated with dosage compensation (Turner et al, 1992; Bone et al, 1994). Most known HATs are able to acetylate free histones in vitro when assayed as a single polypeptide. Many, however, are unable to acetylate their probable physiological substrates, nucleosomal histones, under standard conditions in vitro. Consistently, they are found to function as multi-protein complexes in vivo (Roth et al, 2001). Among the best characterized examples are yeast Gcn5-containing HAT complexes (Grant et al, 1997, Sendra et al, 2000) and the highly related mammalian PCAF complex (Ogryzko et al, 1998; Vassilev et al, 1998). Recombinant yeast Gcn5 as a monomer acetylate free histones (H3 strongly and H4 weakly) efficiently. However, it is unable to acetylate nucleosomal histones (Brownell et al, 1996). Gcn5 exists in four distinct HAT complexes: SAGA, ADA, NuA4 and NuA3 (Sterner, 2000). Only in these complexes, is Gcn5 able to acetylate nucleosomes effectively. For example, Gcn5 in SAGA has the ability to acetylate primarily H3 and, to a less extent, H2B (Grant, 1997). The capacity to recognize nucleosomal histones is apparently conferred by other subunits in the complex and may be associated with one of Gcn5's non-activity domain (bromodomain) as deletion of this domain significantly reduces nucleosome acetylation by SAGA (Sterner et al, 1999). Consistent with its ability to acetylate nucleosomal histones, the SAGA complex and its components have been shown to be critical to certain types of transcription. In vitro, purified SAGA was able to stimulate transcription in various chromatin-template assays by ways of its combined HAT activity and interaction with acidic activators.
<table>
<thead>
<tr>
<th>SAGA complex</th>
<th>PCAF complex</th>
</tr>
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<tbody>
<tr>
<td>Gcn5</td>
<td>PCAF</td>
</tr>
<tr>
<td>Ada1</td>
<td></td>
</tr>
<tr>
<td>Ada2</td>
<td>HADa2</td>
</tr>
<tr>
<td>Ada3</td>
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</tr>
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<td>Sot3</td>
<td>HSpt3</td>
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</tr>
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<td>Tra1</td>
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<td>TAFII90</td>
<td>PAF65β</td>
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<td>TAFII61/68</td>
<td>TAF15/20</td>
</tr>
<tr>
<td>TAF60</td>
<td>PAF65α</td>
</tr>
<tr>
<td>TAF23/25</td>
<td>TAF30</td>
</tr>
<tr>
<td>TAF17/20</td>
<td>TAF31</td>
</tr>
<tr>
<td>Sin4</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Comparison of yeast HAT complex SAGA and human HAT complex

PCAF
(Ikeda et al, 1999; Utley et al, 1998; Wallberg et al, 1999). The in vivo significance of SAGA has been demonstrated by examination of mutants of its components, which have verified that the complex has an important role in transcriptional activation at a subset of genes, such as GAL1 (Dudley et al, 1999), TRP3 and HIS3 (Belotserkovskaya et al, 2000). The complex similar to SAGA in mammalian system is PCAF complex. Table 2 lists the components of SAGA and PCAF complex. As observed in yeast SAGA, the other factors in the PCAF complex has an effect on the core HAT, PCAF, in terms of its activity. Although recombinant PCAF can acetylate nucleosomal histones, primarily H3, the PCAF complex acetylates H3 much more strongly (Ogryzko, 1998). One interesting component of HAT complexes is Tra1 of SAGA (Grant et al, 1998; Saleh et al, 1998). Tra1 shares homology with PAF400 in the PCAF complex (Vassilev et al, 1998) as well as TRRAP in the TIP60 complex of mammalian cells (Ikura et al, 2000). TRRAP is a cofactor for c-myc and E2F and is important for both transcriptional activation and transforming properties of these oncogenes (McMahon et al, 2000). Sequence analysis reveals that TRRAP, RAF400 and Tra1 are homologous to the ATM superfamily of phosphatidylinositol-3 kinases (PI-3 kinase); however, these factors lack particular amino acids associated with protein kinase activity.

2. Biology of HDACs

To date, 17 human HDAC isoforms have been identified (Gray et al, 2001; Bertos et al, 2001; Fischle et al, 2001; Zhou et al, 2001; Fischer et al, 2001). They can be divided into three classes according to sequence homology, intracellular localization, and association with proteins that form the DNA binding complex (Gray et al, 2001).
<table>
<thead>
<tr>
<th>Class</th>
<th>S. cerevisiae homolog</th>
<th>Expression pattern in tissues</th>
<th>function</th>
<th>Sensitivity to inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>RPD3</td>
<td>Ubiquitous</td>
<td>Transcription repression</td>
<td>Yes</td>
</tr>
<tr>
<td>II</td>
<td>HDA1p</td>
<td>Tissue-specific</td>
<td>Differentiation</td>
<td>Yes</td>
</tr>
<tr>
<td>III</td>
<td>Sir2</td>
<td>SIRT1-7</td>
<td>Gene silencing</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 3. Summary of human HDACs
HDAC1, HDAC2, HDAC3 and HDAC8 belong to class I based on their similarity to the yeast gene Rpd3 (Bertos et al., 2001; Fischle et al., 2001) while HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 belong to class II. HDAC1, 2 and 3 are ubiquitously expressed in many types of tissues (Yang et al., 1997; Dangond et al., 1998). HDAC4 mRNA displayed differential expression in different tissues with the highest in skeletal muscle, thymus and small intestine (Fischle et al., 2001). HDAC5 expression overlaps with that of HDAC4 and was detected in brain, heart, skeletal muscle and placenta, whereas the highest expression of HDAC6 was found in heart, liver, kidney and pancreas (Grozinger et al., 1999).

A third HDAC class contains 7 human genes that share homology with yeast silent information regulator gene (Sir2) (Afshar et al., 1999; Frye et al., 1999). They are structurally unrelated to class I and class II HDACs. Different from the class I and II HDACs, the class III are insensitive to known HDAC inhibitors (Gray et al., 2001; Bertos et al., 2001; Min et al., 2001).

Like HATs, HDACs work in multisubunit complexes in vivo, where they mediate transcriptional regulation, hormonal signaling, cell cycle, differentiation and DNA repair (reviewed by Gray et al., 2001). Among the best understood are two major histone deacetylase complexes involving class I HDAC, namely, HDAC 1 and HDAC 2: Sin3-HDAC complex (Thrash et al., 1984; Kasten et al., 1997) and NuRD (nucleosome remodelling histone deacetylase) complex (Ng et al., 2000). Sin3 complex was identified from yeast at first. It has been clearly demonstrated that Ume6, a yeast transcriptional repressor, can recruit Sin3 complex to chromatin leading to localized deacetylation of
nucleosomal histones in vivo and transcriptional repression (Kadosh et al, 1998; Rundlett et al, 1998). In mammalian cells, Sin3 complex is found to interact with various proteins to mediate transcriptional repression, including unliganded retinoid acid receptor (RAR), thyroid hormone receptor (TR) via the corepressor protein SMRT (silencing mediator of retinoid and thyroid hormone receptor) and NcoR (nuclear hormone reector coreprssor), and methyl-CpG-bing protein 2 (MeCP2) (Xu et al, 1999). On the other hand, NuRD complex interacts in vitro with a methyl-CpG-bind protein MBD2 (methyl-CpG-binding-domain protein 2), raising the possibility that MBD2 might recruit the NuRD complex to methylated DNA (Zhang et al, 1999). The functions of class II may be involved in cellular proliferation/differentiation. One example is that HDAC4 and HDAC5 associate with MEF2 (myocyte enhancer factor) family of transcription facotrs to regulate muscle-specific genes and both smooth and skeleton muscle differentiation (Busby et al, 2000; McKinsey et al, 2001; Dressel et al, 2001).

B. Histone acetylation and transcription

Nucleosomes are thought to act by repressing transcriptional initiation (Grunstein, 1990). For example, global nucleosome depletion in vivo (due to down-regulation of H4 synthesis) resulted in activation of TATA-containing promoters (Owen-Hughes et al, 1994). Histone acetylation is a well-established mechanism for reversing nucleosome repression (Logie et al, 1999; Edmondson et al, 1996). As early as 1964, Allfrey et al showed that acetylated core histones inhibited RNA synthesis in vitro to a lesser extent than unacetylated histones (Allfrey et al, 1977). Furthermore, chromatin fractions where genes are actively transcribed are associated with highly acetylated core histones, while
silent genes are localized to hypoacetylated chromatin (Grunstein, 1997; Kouzarides et al, 1999; Kuo et al, 1998). It is also known that lysine residues within the core histone tails are required for inducible gene expression in yeast (Durrin et al, 1991). Given the fact that histone acetylation precedes transcription and that nontranscribed regions of the genome can be acetylated, acetylation cannot be a consequence of transcription (Roth et al, 2001). Finally, and as noted above, HAT and HDAC activities have been identified to associate with activation and repression, respectively, in many transcription-related complexes. Based on these results, it is well accepted that histone acetylation regulates gene transcription.

Despite the advances that relate acetylation to transcription, the detailed biochemical mechanism is poorly defined. Currently, the most accepted model is that acetylation neutralizes the positive charges localized at the core histone tails and, in turn, releases the tails from DNA. As a result, the loose chromatin structure generates a more open DNA conformation that improves transcription factors access to DNA thereby leads to activate transcription (Marks et al, 2000). Since hyperacetylated nucleosomes exhibit only minor structural differences in vitro, acetylation alone probably does not impact directly nucleosome structure (Luger et al, 1997). Instead, it may affect organization of nucleosomes into high-order structures or the interactions between nucleosomes and the other factors, such as the ATP-dependent chromatin remodeling complex (Luger et al, 1997).

Besides direct modulation of chromatin structure, it is also proposed that histone acetylation may also serve as a recognition code for transacting factors (Roth, 2001). The
<table>
<thead>
<tr>
<th>Effective concentration</th>
<th>example</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Millimolar</td>
<td>Butyric acid</td>
<td>In clinical trials</td>
</tr>
<tr>
<td>Nanomolar</td>
<td>TSA</td>
<td>Highly specific, reversible HDAC binding</td>
</tr>
<tr>
<td>Nanomolar</td>
<td>SAHA oxamflatin</td>
<td></td>
</tr>
<tr>
<td>Nanomolar</td>
<td>Trapoxin A</td>
<td>Covalent binding to HDAC</td>
</tr>
<tr>
<td>Nanomolar</td>
<td>FR901228</td>
<td></td>
</tr>
<tr>
<td>Micromolar</td>
<td>MS-27-275</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Summary of HDAC inhibitors
fact that bromodomains present in several HATs interact selectively with acetylated histones supports this model. Additionally, the yeast transcription repressor Tup1p has a lower affinity for acetylated histones (Edmondson et al, 1996), suggesting acetylation favors formation of activation protein complexes. Nevertheless, more detailed studies will be required before the mechanism is completely understood.

C. HDAC inhibitors

Five classes of HDAC inhibitors have been reported (see table 4) (Marks et al, 2000). An example of the first class is the short-chain fatty acid butyrate (Newmark et al, 1994b). Butyrates are the only HDAC inhibitors that have been approved for cancer treatment. As high as millimolar concentrations of butyrate are needed to effectively inhibit HDAC activity; therefore, the cellular effects coming from butyrate treatment may not be all specifically related to HDAC inhibition. The second class contains hydroxamic acid such as trichostatin A (TSA) (Tsuji et al, 1976; Yoshida et al, 1990), suberoylanilide hydroxamic acid (SAHA) (Richon et al, 1998) and oxamflatin (Kim et al, 1999). TSA is a reversible potent HDAC inhibitor and active at nanomolar concentrations (Yoshida et al, 1995). Specificity of TSA is supported by the fact that a HDAC is mutated in TSA-resistant cell lines (Yoshida et al, 1990). Besides TSA, a series of hydroxamic acid-based HDAC inhibitors have been synthesized that inhibit HDACs at micromolar concentrations and their structure-activity relationship has been extensively studied (Braunstein et al, 1991). The third class includes cyclic tetrapeptide with a 2-amino-8-oxo-9, 10-epoxy-decanoyl (AOE) moiety, such as trapoxin A (Kijima et al, 1993). Trapoxin A irreversibly binds to HDACs and inhibits their activities at nanomolar concentrations. The fourth class includes cyclic peptides without the AOE moiety,
such as FR901228 (FR), also known as depsipeptide. FR901228, isolated from *Chromobacterium violaceum* (Ueda et al, 1994), inhibits HDAC activity at nanomolar concentrations (Nakajima et al, 1998). The final class includes the benzamides, such as MS-27-275 (Saito et al, 1999), which has activity at the micromolar.

HDAC inhibitors display anti-proliferation activity in culture and some have been shown to suppress tumor growth in animal models. For example, FR increased the life span of mice bearing ascitic tumors, such as P388 and L1210 leukemia cells as well as B16 melanoma cells and inhibited the growth of murine solid tumors and human solid tumors implanted into normal and nude mice (Ueda et al, 1994). The antitumor activity appears to result from HDAC inhibitor-mediated cell cycle arrest, differentiation and apoptosis caused by changes in the expression of regulators linked to these processes with increased histone acetylation. For example, both TSA and FR caused arrest of the cell cycle at both G1 and G2 phases (Nakajima et al, 1998; Yoshida et al, 1988). The basis for G1 arrest is most likely due to the dismantling of transcriptional repressor effects of the HDACs, on key cell-cycle regulating gene such as p21WAF1 (Archer et al, 1998), while the reason for G2 arrest remains to be elucidated. Increased expression of p21WAF1, together with phosphorylation of bcl-2 and activation of p33 MBP (myelin basic protein), was also responsible for FR-mediated apoptosis in breast cancer cell lines MCF-7 and MDA-MB231 (Rajgolikar et al, 1998). Butyrate-induced apoptosis appears to be mediated by caspase (Medina et al, 1997) and involved in different cell death signaling regarding to status of Bcl-2 and bax in different cancer cell lines (Hague et al, 1997). In addition to cell-cycle arrest and apoptosis, induction of differentiation is
another hallmark of HDAC inhibitors (Newmark et al, 1994). TSA treatment of human carcinoma cell lines T24 and Hela led to dramatic morphologic alterations and the reappearance of actin stress fibers. Consistently gelsolin, an actin regulatory protein, was induced by 7-fold (T24) and 12-fold (Hela) after treatment with TSA (Hoshikawa et al, 1994).

Even though HDAC inhibitors affect many physiological processes, it has been shown that only a small percentage (2%) of mammalian genes were influenced based on a gene array data when two HDAC inhibitors, TSA and tryoxin (TPX), were used (Van Lint, 1996). These genes, not surprisingly, include a number of regulators of cell cycle and apoptosis such as, c-myc (Janson et al, 1997), plasminogen activator (Reeder et al, 1993; Dong-Le Bourhis et al, 1998), gelsolin (Hoshikawa et al, 1994) and p21WAF1 (Nakano et al, 1997; Richon et al, 2000; Saito et al, 1999; Kim et al, 1999; Drlica et al, 1992; DiGiuseppe et al, 1999). However, the mechanisms by which genes are selectively induced or repressed by changes in the acetylation status of chromatin remains to be well understood.

**D. P21WAF1 and HDAC inhibitors**

P21WAF1 was first identified as a cyclin-dependent kinase (cdk) inhibitor whose expression is activated by an increase of p53 in response to DNA damage (el-Deiry et al, 1993; Gu et al, 1993; Dulic et al, 1994). In this pathway, suppression of cdk activity results in the accumulation of hypophosphorylated Rb and this, in turn, leads to assembly of the Rb-E2F complex (Dulic et al, 1994; Harper et al, 1993; Chellappan et al, 1991; Kaelin et al, 1991). Since release of E2F is essential for expression of many genes
DNA damages trigger posttranslational accumulation of p53, which binds the p21\textsuperscript{WAF1} promoter and activates the expression of p21\textsuperscript{WAF1} protein. The elevated p21\textsuperscript{WAF1} protein associates with cyclin/ckd complex and inhibits its kinase activity, which, in turn, blocks phosphorylation of the Rb protein and results in dissociation of E2F. Only unbound E2F activates genes whose products are required for passage from G1 to S phase of the cell cycle at the transcriptional level, thus cells are arrested at G1 checkpoint.
DNA damage

↓

P53 accumulation

↓

P21^{WAF1} expression

↓

cyclin/CDK

Rb/E2F ➔ Rb-p + E2F

G1 phase ➔ S phase

Figure 1
responsible for G1 to S transition, formation of Rb-E2F complex arrests cells at G1 checkpoint (summarized in Figure 1) (Nevins et al, 1998; Dyson et al, 1998).

Expression of p21WAF can be up-regulated in a p53-independent manner by various stimuli, including transforming growth factor-β (TGF-β) (Hu et al, 1999), progesteron (Gareth et al, 1998) and nerve growth factor (Billon et al, 1999). p21WAF is also induced by almost all HDAC inhibitors tested (Newmark et al, 1994; Carducci et al, 1997; Nakajima et al, 1998; Xiao et al, 1999) and this induction is found to be p53-independent. It occurs at the transcriptional level and is regulated by the chromatin remodeling resulting from the accumulation of acetylated histones H3 and H4 in p21WAF promoter region (Huang et al, 2000). Moreover, recent studies have demonstrated that HDAC inhibitors activate p21WAF induction via Sp1 sites through Sp1 family transcription factors, Sp1 and/or Sp3, similar to TGF-β or nerve growth factor (Huang et al, 2000; Saito et al, 1999). However, there have been no reports addressing the possible link between HDAC-mediated transcriptional activation of the p21WAF gene expression and the function of ATM.

II. Biology of ATM

Ataxia telangiectasia (AT) is an autosomal recessive disorder. AT patients exhibit cerebellar ataxia, dilated blood vessels in the eyes and skin (oculocutaneous telangiectasia), immunodeficiency, hypersensitivity to ionizing radiation (IR) and elevated risk of certain cancers (Swift et al, 1991; Taylor et al, 1994).
A. ATM gene

The gene that is responsible for AT, designated ATM (Ataxia telangiectasia-mutated), was identified by positional cloning and localized to chromosome 11q22-23 (Savitsky et al, 1995; Gilad et al, 1996). It encodes a 3056-amino acid protein with a calculated molecular mass of 350 kDa. ATM shares homology with a gene family in which all the members have a 300-amino-acid motif at their C termini that resemble the catalytic domain of PI-3 kinases. Members of this gene family are usually involved in the detection of DNA damage and control of cell cycle progression (Hunter et al, 1995; Jackson et al, 1996; Meyn et al, 1995). Examples include Saccharomyces cerevisiae proteins TEL1 and MEC1 (Greenwell et al, 1995; Morrow et al, 1995), Schizosaccharomyces pombe RAD3 (Bently et al, 1996), Drosophila MEI-41 (Hari et al, 1995), ATM-related protein kinase (ATR) (Chicoine et al, 1996) and human DNA-PK (Hartley et al, 1995). All the evidence indicates that the ATM gene product is a protein kinase rather than a lipid kinase (Hunter et al, 1995; Lavin et al, 1995).

B. The cell biology of AT

The cells derived from AT patients display several cellular defects including chromosome instability, defective cell cycle checkpoints (Beamish et al, 2001; Paules et al, 1995) and radio-resistant DNA synthesis (Houldsworth et al, 1980) in response to IR. Later studies indicated that the loss of cell cycle checkpoints were due to failure of the corresponding signal transduction pathways (reviewed by Khanna et al, 2001). By using AT cells the signal transduction networks with regard...
Figure 2. ATM-mediated signaling through the G1 checkpoint.

DNA double strand breaks (dsbs) cause accumulation of p53 in G1 phase via activation of ATM. ATM regulates this process by an indirect pathway involving the Chk2-mediated phosphorylation of Ser 20 on p53 and by direct phosphorylation of Ser 15 on p53 and Ser 395 on MDM2. In addition, the DNA damage signal is also passed through the ATR pathway by Chk1-mediated phosphorylation of Ser20 on p53. The consequence of p53 phosphorylation is disassembly of p53-MDM2 dimer, which, in turn, leads to G1 arrest and/or apoptosis due to the accumulation of p53.
DNA double strand breaks

ATR
Chk1
Chk2

G1 arrest
Apoptosis

Figure 2
to cell cycle checkpoints and DNA repair have been dissected. In the past two years, tremendous advances have been achieved in identifying the substrates of ATM protein and furthermore understanding the cellular mechanisms of ATM and its substrates to regulate the cell cycle progression in response to the cellular and environmental genotoxic stresses (Abraham et al, 2001).

1. Involvement of ATM in the G1 checkpoint pathway

ATM-mediated G1 checkpoints involve p53 both directly and indirectly (Giaccia et al, 1998) (see Figure 2). p53 is rapidly induced by many different genotoxic insults, a response that is mediated primarily through an increase in its stability (Ko et al, 1996). In the absence of DNA damage, p53 interacts with MDM2, a protein that targets p53 for ubiquitination, thereby leading to nuclear export and proteosomal degradation (Freedman et al, 1999; Juven-Gershon et al, 1999). With regard to the G1 checkpoint, the downstream target of p53 is the cyclin-dependent kinase (cdk) inhibitor, p21\textsuperscript{WAF1}. p53 activates expression of p21\textsuperscript{WAF1}, which suppresses cyclin E- and cyclin A-associated cdk2 activities, thereby preventing G1-to-S phase progression (as discussed above). Besides p21\textsuperscript{WAF1}, elevated p53 also activates many other genes that induce apoptosis (Yu et al, 1999).

In response to IR, p53 is phosphorylated at Serine15 by ATM (Banin et al, 1998; Canman et al, 1998). Although Ser15 phosphorylation is not sufficient to dissociate p53 from MDM2 (Dumaz et al, 1999), it facilitates the subsequent phosphorylation of p53 at Ser20 by casein kinase I (Dumaz et al, 1999). The modifications at these two sites significantly enhance the dissociation between p53
Figure 3. S-phase checkpoint pathway involving ATM.

Activation of ATM initiated by ionizing radiation leads to phosphorylation of Chk2, which promotes the proteosome-mediated degradation of Cdc25A. The failure to dephosphorylate cyclin/cdk2, which results from the lack of Cdc25A activity, results in inhibition of DNA synthesis. ATM also regulates homologous recombination-mediated DNA repair via NBS1 (Nijmegen breakage syndrome) and 53BP1 (p53 binding protein 1).
Homologous recombination / DNA repair

Inhibition of DNA synthesis

Figure. 3
and MDM2. Ser15 phosphorylation also stimulates transactivation of p53 by enhancing its binding to the co-activator p300 (Dumaz et al, 1999). Additionally, ATM stabilizes p53 indirectly (Ahn et al, 2000), where ATM activates an intermediate protein kinase Chk2 by phosphorylating its N-terminal Thr residue, and Chk2, in turn, phosphorylates the serine residue Ser20 in p53 (Chehab et al, 2000; Hirao et al, 2000; Shieh et al, 2000). The second indirect mechanism is phosphorylation of MDM2 at Ser395 by the ATM protein. This modification may decrease the shuttling activity of MDM2, which is essential to export p53 to the cytoplasm for degradation (Maya et al, 2001). The data suggest that these collective modifications dissociate p53 and MDM2, thereby favoring the p53 accumulation in response to IR-induced DNA damage.

2. Involvement of ATM in the S-phase checkpoint pathway

In normal cells, exposure of IR provokes a rapid but reversible decrease in DNA synthesis. In contrast, AT cells display radioresistant DNA synthesis (RDS) (Painter et al, 1980). Studies suggested that the S phase checkpoint involves the ATM-Chk2 pathway (Falck et al, 2001). Specifically, these studies reveal that IR exposure activates ATM during S-phase, and that activated ATM subsequently phosphorylates Chk2 as happens in G1; however, the downstream target in S phase is cdc25A (Falck et al, 2001), a protein tyrosine phosphatase that activates cyclin A-cdk2 complex as the cell progresses from G1 to S phase (Donaldson et al, 1999). Chk2 phosphorylates cdc25A at Ser123 causing cdc25 to be degraded by ubiquitin-dependent pathways.
Inactivation of cyclinA-cdk2 terminates initiation of replication origins during S phase (Figure 3) (Takisawa et al., 2000). ATM protein also regulates the S phase checkpoint in a second pathway, NBS1. NBS1 is the gene mutated in the human chromosomal instability disorder, NBS (Shiloh, 1997; Carney, 1999; Petrini, 1999). NBS (Nijmegen breakage syndrome) patients exhibit considerable but not complete overlapping clinical features with AT patients, one of which is the RDS phenotype. The data showed that ATM directly phosphorylates NBS1 at up to three serine residues (Gatei, 2000; Lim, 2000); however, the exact roles of these modifications remain unclear.

3. Involvement of ATM in the G2 checkpoint pathway

When exposed to IR during G1 or S phase, AT cells become arrested prior to initiation of mitosis. However, when AT cells are irradiated in G2 phase, they fail to arrest and proceed on into mitosis (Beamish et al., 2001; Scott et al., 1994). These results suggest that the ATM protein plays a role in the G2 checkpoint only when the DNA damage occurs in the G2 phase. Recent studies indicated that the ATM-Chk2 pathway might be the responsible for this phenomenon (Brown et al., 1999). Unlike the downstream target of the ATM-Chk2 pathway in S phase checkpoint, activation of Chk2 in the G2 checkpoint phosphorylates the mitosis-promoting phosphatase, Cdc25C. This modification creates a binding site for 14-3-3 protein, and, in the 14-3-3 bound form, Cdc25C is either catalytically inhibited or sequestered in the cytoplasm (or both). In either case, the 14-3-3 bound form of Cdc25C fails to
Figure 4. ATM-mediated G2 checkpoint signaling pathway.

DNA damage during the G2 phase activates Chk2 via ATM, which, in turn, phosphorylates Cdc25C. Phosphorylation of Cdc25C leads to assembly of a complex of Cdc25 and 14-3-3 protein, which blocks dephosphorylation and activation of the mitotic cyclin B/cdk2 kinase activity. As a result, damaged cells are arrested in G2 checkpoint.
Figure 4
dephosphorylate and activate the mitotic cyclin B-Cdc2 kinase, thereby, blocking the irradiated cells from entering mitosis (Figure 4) (Peng et al, 1997).

III. The relationship between ATM and histone acetylation

When cells are exposed to IR, the ATM protein phosphorylates HDAC1 (Kim et al, 1999) thereby establishing a direct link between ATM and histone acetylation. In addition, ATR, an ATM family member that is structurally and functionally related to PI-3 kinases, is found to associate with HDAC (Schmidt et al, 1999). ATM has also been reported to be a part of a BRCA1-centered supercomplex BASC (BRCA1 associated genome surveillance complex) (Wang et al, 2000). The other components of this complex include MSH1 (mismatch-repair1), MSH2, and MSH6, the Bloom’s syndrome helicase (BLM), and the Mre11-Rad50-NBS1 complex (Wang et al, 2000). The latter complex has been shown to play important roles in the recombination repair of DNA double strand breaks (Shiloh et al, 1997). Interestingly, the chromatin-remodeling complex is also a part of this supercomplex (Biggs et al, 2000). Moreover, studies indicated that p300 associates with BRCA1 (Pao et al, 2000) and modulates the BRCA1 inhibition of estrogen receptor activity (Fan et al, 2002). Taken together, these studies suggested that ATM may associate with HATs or HDACs via BRCA1 to regulate gene transcription; however, there is no direct evidence indicating that ATM influences histone acetylation-mediated transcription activation.

In this study, p21WAF1 is used as a model system to investigate whether ATM is involved in histone acetylation-mediated gene activation. The results show that
activation is defective in AT cells and PI-3 kinase activity appears to be essential as wortmannin and caffeine attenuate HDAC inhibitor-induced p21\textsuperscript{WAF1} activation in ATM wild type cells. Ectopic expression of a wild type ATM gene in the AT cells restores p21\textsuperscript{WAF1} induction by the same inhibitors. Nevertheless, no dramatic difference in global acetylation of histone H3 and H4 was detected between AT and normal cells after treatment of the HDAC inhibitors.

**MATERIALS AND METHODS**

**Cell Cultures**

Cells were grown in a humidified incubator at 37°C and 5% CO\textsubscript{2}. Cell lines used in this study were summarized in Table 4. GM 9607, GM5849, GM8505, GM0637 and GM0639 were purchased from Coriell Cell Repository (Camden, NJ). WI-38 cells were from American Type Culture Collection (ATCC). They were grown in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum (Life Technologies, Inc. Rockville, MA), 2x concentrations of essential amino acid and 50 μg/ml of gentamicin. AT221JE pEBS7 and AT221JE pFBS-YZ5 cells were generous gifts from Dr. Michael B. Kastan at St. Jude Children’s Research Hospital. They were originally constructed in Dr. Yosef Shiloh's lab (Tel Aviv University) by transfecting an immortalized fibroblast line AT221JE with the mammalian expression vector pEBS7 and pEBS7 plus full-length ATM reading frame, respectively (Ziv, 1997). AT221JE pEBS7 and AT221JE pFBS-YZ5 cells were cultured in the same medium as described above plus 100 ug/ml hygromycin B. All cells were in exponential growth phase at the time of harvest.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI-38</td>
<td>wild type human fibroblast</td>
</tr>
<tr>
<td>GM 00637</td>
<td>SV 40-transformed human fibroblast</td>
</tr>
<tr>
<td>GM00639</td>
<td>SV 40-transformed human (galactosemia) fibroblast</td>
</tr>
<tr>
<td>GM05849</td>
<td>SV 40-transformed human (Ataxia-Telangiectasia) fibroblast</td>
</tr>
<tr>
<td>GM9607</td>
<td>SV 40-transformed human (Ataxia-Telangiectasia) fibroblast</td>
</tr>
<tr>
<td>GM 8505</td>
<td>SV 40-transformed human (Bloom's syndrome) fibroblast</td>
</tr>
<tr>
<td>AT22IJE pEBS7</td>
<td>SV 40-transformed human (Ataxia-Telangiectasia) fibroblast, transfected with a vector pEBS7</td>
</tr>
<tr>
<td>AT22IJpFBS-YZ5</td>
<td>SV 40-transformed human (Ataxia-Telangiectasia) fibroblast, transfected with an ATM cDNA</td>
</tr>
</tbody>
</table>

Table 4. Cell lines used in this study
Drugs, enzymes and antibodies

Trichostatin A (TSA) was purchased from Sigma (St. Louis, MO) and dissolved in DMSO at 100 mg/ml. FR 901228 (FR) (Fujisawa Pharmaceutic Co., Ltd., Osaka, Japan) was dissolved in DMSO and stored as 5 mM aliquots at -20°C.

Mouse monoclonal p21\textsuperscript{WAFl} antibody was from PharMingen (Los Angeles, CA). Actin antibody was purchased from Sigma (St. Louis, MO). Rabbit anti-acetylated histone H3 and H4 antibodies were from Upstate Biotechnology (Upstate, NY). Mouse monoclonal anti-Rb antibody was purchased from Calbiochem (Los Angeles, CA). The plasmid carrying p21\textsuperscript{WAFl} cDNA, pCEP-WAFl, was a kind gift from Dr. Bert Vogelstein (John Hopkins Oncology Center). Restriction enzymes and DNA ladder marker were from Life Technologies, Inc. (Rockville, MA).

Isolation of the p21\textsuperscript{WAFl} and the topoisomerase III\textalpha{} promoters

To amplify the p21\textsuperscript{WAFl} promoter, two primers were synthesized according to Richon et al (Richon VM, 2000b). The sequence of upstream and downstream primers were: 5'- GGT GTC TAG GTG CTC CAG GT-3', 5'-CCG GCT CCA CAA GGA ACT GA-3', respectively. The 30 cycle PCR reaction was carried out as follows: 50°C for 45 seconds, 72°C for 30 seconds and 94°C for 1 minute. The resulting fragment was subcloned into pCRH-TOPO vector (Invitrogen, Carlsbad, CA) to yield pCRII-p21p and orientations were determined by sequencing. The 659-bp Kpn I-Xhol fragment of p21\textsuperscript{wafl} was cut out of pCRII-p21p and cloned into pGL3 vector (Promega, Madison, WI) and designated p21-luc.
To amplify the topo IIIα promoter, two primers were synthesized according to the published topo IIIα promoter sequence (Kim, 1998): upstream 5'-ATA GGT ACC CAA AAC GGC CTC ACG AAG CCA CTC ACG AAG CCA C -3', downstream 5'-TCA CTC GAG TCT TCG GCC CGT CGC AGC CAC CGG A-3'. The resulting fragment spanned from +305 to -1, 262 covering YY1, USF and four Sp1 sites. Genomic DNA was extracted from GM0637 and used as the template. A 30 cycle PCR reaction was carried out as follows: 94°C for 1 minute, 72°C for 1 minute and 60°C for 1 minute. The 1.5-kb PCR fragment was digested with Kpn I, Xho I and subcloned into Bluescript II SK-phagemid to create the human topo IIIα promoter clone pBS-topoIIIp. After sequencing, pBS-topoIIIp was digested with KpnI and XhoI and the 1.5-kb fragment of topo IIIα promoter was subcloned into pGL3 to yield pTopoIII-luc.

Transfections and Luciferase assays.

For the transient assays, cells (1 x 10^5) were transfected with p21-luc or pTopoIII-luc using a lipofectamine, LF2000, (Life Technologies, Inc., Rockville, MA) in 24-well plates. After 24 hours, the transfected cells were subcultured into new 24-well plates. Another 24 hours later, cells were treated with FR901228 or TSA or 0.1% DMSO in fresh medium. After an additional 24 hours, the cells were lysed and measured for luciferase activity by using Luciferase Assay Systems according to the manufacturer's recommendation (Promega, Madison, WI). In brief, 1x Cell Culture Lysis Reagent (200 µl /each well) was added into 24-well plates and lysis was
carried out for 10 minutes at room temperature. The lysates were then transferred to microcentrifuge tubes and centrifuged at 12,000 g for one minute (4°C). Luciferase activity was measured on Luminometer (LUMAT LB9507, BERTHOLD GmbH & Co. KG, Bad Wildbad, Germany) by mixing 20 µl of cell lysate supernatant with 100 µl of Luciferase Assay Reagent. The luciferase activity assays were normalized to the total protein concentration of lysates. Fold increase was calculated by dividing the luciferase activity with FR with the luciferase activity without FR. A mean of triplicates was represented and error bars indicated standard deviation. Three independent experiments were performed and typical one was shown in the dissertation.

For stable transfections, cells (1 x 10^5) were co-transfected with pTopoIII-luc or p21-luc plus pcDNA3.1 (Invitrogen, Carlsbad, CA) using LF2000. Twenty-four hours after transfection, cells were trypsinized and split 1:10 into two 100 mm petri dishes to give the appropriate numbers of colonies. After another 24 hours, 800 µg G418/ml was added into the media and two weeks later, G418 resistant colonies were either pooled or picked individually, then transferred and amplified in the new plates.

**Extraction of Histones**

Histones were extracted according to published methods (Yoshida, 1990). Briefly, cells were pelleted, washed with PBS, and then suspended in buffer A (100 mM NaCl, 50 mM KCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM PMSF, 10% glycerol, 0.2% NP-40, 0.1% Triton X-100) to release nuclei. The nuclei
were deposited by centrifugation (1,000 g for 5 minutes, 4°C), the cytosol fraction removed and the nuclei resuspended in 100 μl ddH₂O. Concentrated H₂SO₄ (1 μl) was then added and the nuclear extract incubated on ice for 1 hour to dissolve the acid-soluble nuclear proteins. The acid-insoluble fraction was removed by centrifugation at 12,000 rpm for 10 minutes at 4°C. The acid-soluble proteins were precipitated with 1 ml of acetone at 4°C overnight and, precipitates recovered by centrifugation (12,000 g for 10 minutes at 4°C). The pellet was suspended in a solubilizing buffer (0.1% SDS, 100 mM Tris-HCl pH 6.8, 1 mM PMSF, 1 μg/ml aprotinin, leupeptin and pepstatin) and any precipitable materials removed by centrifugation (12,000 g for 10 minutes). Protein concentrations were determined using a Bio-Rad DC protein assay kit according to the manufacturer instructions (Bio-Rad, Hercules, CA). In brief, diluted or undiluted cellular extracts (100 μl) were added into 0.5 ml of reagent A, followed by addition of 4.0 ml reagent B. After 15 minutes at room temperature, absorbences were read at 750 nm.

**Western Blot Analysis**

To obtain the whole cell extracts, cells were pelleted, washed with PBS and then suspended in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25%, Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, leupeptin, pepstatin each, 1 mM Na₃VO₄, 1 mM NaF) and placed on ice for 10 minutes. Debris was removed by centrifugation (12,000 g, 10 minutes at 4°C) and protein concentrations in the supernatant determined using a Bio-Rad DC protein assay kit described as above. Proteins were electrophoresed at 200 V on SDS-polyacrylamide gels and
transferred to Hybond ECL membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Membranes were blocked for 2 hrs in TBST (0.2 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.1% Tween, 5% non fat dry milk), probed overnight with primary antibody in TBST followed by a 2 hour incubation in the secondary antibody (peroxidase-conjugated goat anti-mouse or rabbit IgG) in TBST. Signals were visualized and quantified using BM Chemiluminescence’s Western Blotting Kit and Lumi-Imager (Roche Diagnostics, Indianapolis, IN).

RNA extraction

Total RNA was extracted by using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. In brief, cells were washed with PBS once and lysed by addition of Buffer RLT from the kit (350 μl for 10^6 cells). After adding 1 volume of 70% ethanol, the lysate was loaded onto a RNeasy column and centrifuged (15 second: 8,000 g). The column-bound RNA was washed twice with Buffer RPE and eluted with 30 μl of water.

Preparation of the radioactive p21waf1 probe for Northern blot

The p21waf1 cDNA fragment was isolated by digesting the plasmid carrying p21waf1 cDNA, pCEP-WAF1, with Hind III and EcoRI. After gel purifying, the fragment was boiled for 10 minutes to denature DNA. Labeling was performed using Random Primed DNA Labeling Kit (Boehringer Mannheim GmbH, Germany). In brief, the following were added into a microcentrifuge tube: 50 ng denatured p21waf1 cDNA fragment, 3 μl dATP, dTTP and dGTP mixture, 2 μl reaction mixture, 5 μl α-[32P]-dCTP, 1 μl Klenow enzyme and water to make up the final volume to 20 μl. After a 37°C
incubation for 1 hour, the reaction was stopped by heating for 10 minutes at 65°C. Following addition of 80 µl of STE (150 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA), the diluted mixture was loaded into a ProbeQuant G-50 Micro Column (Amersham, Piscataway, NJ), which was centrifuged for 1 minute at 735 x g to separate the radioactive probe from unincorporated α-[32P]-dCTP. The purified DNA probe was collected in the bottom of the tube. Prior to hybridization, the probe was denatured by heating at 100°C for 10 minutes.

**Northern blot analysis**

Northern blotting was performed as described by Sambrook et al (Sambrook, 1989). Briefly, total RNA was denatured in formaldehyde and formamide buffer by heating at 65°C for 10 minutes and then chilled on ice. A total of 5 µg RNA was loaded onto a 1.2 % formaldehyde agarose gel. RNA was transferred from the agarose gel to Hybond-N+ nucleic acid transfer membranes (Amersham, Piscataway, NJ) using capillary action. In brief, the agarose gel was placed on a piece of Whatman 3MM paper supported by a piece of glass on a container filled with 20 x SSC (3.0 M NaCl, 0.3 M sodium acetate). Two ends of 3 MM paper were immersed into 20 x SSC. On top of the gel were the membrane, Whatman 3 MM paper, stacked paper towels and a 500g object. After overnight transfer, the membrane was peeled from the gel, washed with 6 x SSC and baked at 80°C for 2 hours. Prehybridization was carried out in a hybridization bottle by incubating the membrane in ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA) for 2 hours at 65°C. At the end of prehybridization, the denatured radioactive probe (5 x 10⁷ cpm) was directly added into the bottle to give a final radioactivity at 10⁶ cpm/ml.
and the membrane was hybridized at 65°C overnight. The membrane was washed with 1 x SSC, 0.1% SDS once at room temperature, followed by three washes at 65°C with 0.2 x SSC, 0.1% SDS before exposed to X-ray film for 2 days at −70°C.

**Flow cytometry analysis.**

Flow cytometric analysis of PI-stained cells was performed as described (Bonifacino JS, 1998). Briefly, cells were harvested and fixed in 70% ethanol, washed with PBS, and suspended in propidium iodide (PI) solution containing 0.2 mg/ml RNase A and 20 µg/ml PI for at least 30 minutes at room temperature. DNA content of at least 15,000 cells was determined by a FACScan flow cytometry. Typical data are shown.

**RESULTS**

1. **Induction of p21WAF1 by HDAC inhibitors is defective in AT cells**

HDAC inhibitors induce p21WAF1 in a p53-independent manner in normal cells (Richon, 2000). To test whether FR might induce p21WAF1 expression in AT cells, SV40-transformed AT cells (GM5849) were treated with FR at different concentrations and for different intervals. As a control, a matched SV40-transformed wild type cell line (GM 0637) was also treated in the same way. Figure 5 reveals that in the wild type cell line GM0637, p21WAF1 was induced 23 fold by FR at 5 nM and saturated at 50 nM (47-fold increase). In contrast, in the AT cells (GM5849), p21WAF1 was increased less than 5 fold even when the cells were treated with FR at concentrations as high as 500 nM. Furthermore, the wild type cells started expressing p21WAF1 12 hours after addition of 50 nM FR and reached saturated levels at 24
Figure 5. Dose response of \textsuperscript{p21}WAFI protein induction by FR.

Wild type (GM0637) or AT (GM5849) cells were cultured with the indicated concentrations of FR for 24 hours. Cells were extracted and 100 µg of protein was loaded onto 15% SDS-polyacrylamide gel. \textsuperscript{p21}WAFI protein was detected by using the mouse monoclonal anti-p21\textsuperscript{WAFI} antibody (SX118) at 1:500. A parallel blot was probed with an anti-actin antibody to demonstrate that all lanes were loaded with similar amount of protein. Panel A: Western blot results show the positions of \textsuperscript{p21}WAFI and actin on the right. The amount of FR is indicated above each lane. Panel B: Western blot signals in A were quantified as described in “Materials and Methods” and are shown. The value for the control (no FR) sample was adjusted to a value of 1 to determine fold increase after treatment of FR.
Figure 5
Figure 6. Time course of p21\textsuperscript{WAF1} induction by FR in AT cells.

Wild type (GM0637) and AT (GM5849) cells were cultured with FR (50 nM) for the indicated times. Cells were harvested and a total of 100 μg extract was loaded onto 15% SDS-polyacrylamide gel. Panel A, Western blot performed to detect p21\textsuperscript{WAF1} protein and actin as described in Figure 5. Panel B, quantitation of Western blot data.
Figure. 6

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Figure 7. Structures of FR and TSA.
Figure 7
hours; however, \( p21^{WAFI} \) was hardly detected at 24 hours in AT cells (Figure 6). These data indicate that FR cannot effectively induce \( p21^{WAFI} \) expression in AT cells. Based on these results, we performed subsequent experiments using 50 nM FR for 24 hours as a standard treatment regimen (except as indicated otherwise). Despite its completely different structure, trichostatin A (TSA) is also a specific HDAC inhibitor (Figure 7) (Yoshida, 1990). The data in Figure 8 show that TSA failed to induce \( p21^{WAFI} \) in AT cells. Thus, TSA and FR both display the same influence over \( p21^{WAFI} \) expression in AT cells. The commonality between these two structurally diverse agents is their inhibitory activity on HDACs.

To assess whether \( p21^{WAFI} \) induction deficiency only occurred in the AT cell line (GM5849) and to exclude the possibility that secondary mutations might be responsible (due to an unstable genome caused by loss of ATM), a different SV40-transformed AT cell line (GM9607) was evaluated. Although \( p21^{WAFI} \) induction in GM9607 was not quite as deficient (relative to GM5849) (Figure 6 and 7), \( p21^{WAFI} \) induction was nonetheless significantly reduced compared to the wild type cells (Figure 9). These results argue against the notion that impaired \( p21^{WAFI} \) expression is due exclusively to the ATM-unrelated secondary mutations. Finally to confirm that our results were specific to AT cells, SV40-transformed Bloom’s syndrome (BLM) cells (GM8505) and glactosemia cells (GM0639) were treated with FR. Figure 10 shows that both lines of cells expressed similar levels of \( p21^{WAFI} \) to the wild type cells in response to FR, indicating \( p21^{WAFI} \) induction deficiency is AT-specific.
Figure 8. P21<sup>WAF1</sup> induction by TSA in AT cells.

Wild type GM0637 and AT mutant GM5849 cells were incubated with 0.5 μg/ml of TSA for 24 hours. Cells were harvested and Western blotting carried out as described in Figure 5.
Figure 8

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Figure 9. p21\textsuperscript{WAF1} induction by FR and TSA in another AT cell line. GM9607 and GM 0637 cells were treated with FR (50 nM) for 24 hours. Cells were lysed and protein extracts were prepared. Western blotting as detailed in the “Materials and Methods”. Panel A, Western blot performed as described in Figure 5. Panel B, quantitation of Western blot data. Lanes labeled Ctl are negative drug controls.
Figure 9

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Figure 10. p21\textsuperscript{WAF1} induction by FR in different mutant cell lines.

GM0637 (wild type), GM0639 (galactosemia), GM8505 (Bloom's syndrome), GM5849 (AT) and WI-38 cells were cultured with FR (50 nM) for 24 hours. Cells were processed for Western blotting as described in Figure 5.
Figure 10

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Figure 11. Induction of p21<sub>WAF1</sub> mRNA by FR.

WT (GM0637) and AT (GM5849) cells were treated with indicated concentrations of FR for 24 hours. Then total RNA was extracted and 5 µg loaded into 1.2 % agarose gel. Northern blot was performed as described in the “Materials and Methods” using a [32P]-random-labeled human cDNA (p21<sub>WAF1</sub>) as probe. Panel A, actual Northern blot. Panel B, quantified signals from Panel A. The value for the control (no FR) sample was adjusted to a value of 1 to determine fold increase after treatment of FR.
Figure 11
In addition, normal (i.e., not SV-40 transformed) fibroblast cell line WI-38 exhibited a high level induction in response to FR, indicating SV-40 transformation alone cannot explain these results (Figure 10B).

2. HDAC inhibitor-mediated induction deficiency occurs at the promoter level and is not confined to p21\textsuperscript{WAF1}

Previous studies have demonstrated that HDAC inhibitors induce p21\textsuperscript{WAF1} expression by activating its promoter (Richon, 1998). To test whether the HDAC inhibitor-mediated induction deficiency was transcriptional or post-transcriptional, the mRNA level of p21\textsuperscript{WAF1} in response to FR was examined. As shown in Figure 11, the p21\textsuperscript{WAF1} mRNA levels were significantly elevated when treated with FR at as low as 5nM and saturated at 500 nM in the wild type cells. On the contrary, only a slight increase of p21\textsuperscript{WAF1} mRNA was seen in the AT cells even when the FR concentration reached as high as 500 nM. Given the similar pattern between the p21\textsuperscript{WAF1} peptide (Figure 5) and mRNA (Figure 11) in response to FR suggests that the deficiency likely occurs at the transcriptional level.

To further evaluate this, activation of the p21\textsuperscript{WAF1} promoter was investigated. A 650-bp fragment of the p21\textsuperscript{WAF1} promoter was PCR amplified, cloned and validated by sequencing (Figure 12). To examine whether HDAC inhibitor-mediated induction deficiency was transcriptional or post-transcriptional, the p21\textsuperscript{WAF1} promoter was fused with the luciferase gene and the construct was transfected into both AT and wild type cell lines. Figure 13 shows that in wild type GM0637 cells the episomal p21\textsuperscript{WAF1} promoter was activated from 38 fold (5 nM FR) to as much as 80...
Figure 12. PCR Amplification of the p21\textsuperscript{WAF1} promoter

Genomic DNA was extracted from wild type cells (GM0637) as a template to conduct PCR reactions to amplify the 650-bp p21\textsuperscript{WAF1} promoter. PCR conditions are described in “Material and Methods. PCR reaction products were loaded onto 1.0% agarose gel.
Figure 12
Figure 13. Activation of p21WAF1 promoter by FR in transient transfections

Plasmid DNA (p21-luc) was transfected into the AT cell line GM5849 and the wild type cell line GM0637. Approximately 48 hours after the transfection, cells were cultured with 0.1% DMSO (-FR) or indicated concentrations of FR (+FR) for 24 hours. Cells were lysed and luciferase activity was measured. Mean fold increase from triplicate samples was determined by normalizing FR-treated luciferase activity to DMSO-treated luciferase activity.
Figure 13
Figure 14. Activation of the p21<sub>WAF1</sub> promoter by FR in stable transfection

DNAs (p21-luc plus pcDNA3.1) were co-transfected into AT cell line GM5849 and wild type cell line GM0637. The transfectants from both cell lines were selected based on their resistance to G418. More than 100 clones from each cell line were pooled. The pooled clones were cultured with 0.1% DMSO (-FR) or indicated concentrations of FR (+FR) for 24 hours. Cells were processed and luciferase was assayed as described in Figure 13.
Figure 14
fold (500 nM FR). In contrast, there was an across the board 20 fold induction of the promoter activity in AT cells that did not respond to increases in FR concentration. A similar profile was observed with the integrated p21^{WAF1} promoter except that differences between wild type and AT cells were far more dramatic (Figure 14). In AT cells, the integrated p21^{WAF1} promoter was activated only about 5 fold in response to FR (all concentrations). On the other hand, in wild type cells, the integrated and episomal p21^{WAF1} promoter displayed a similar level of activation (80 fold by 50 nM of FR). The integrated p21^{WAF1} promoter appeared to be saturated at 50 nM of FR. Thus the results from stable assays appear to be more consistent with Western blot data shown in Figure 5. Taken together, these data indicate that the p21^{WAF1} induction deficiency is due to poor p21^{WAF1} promoter activation.

HDAC inhibitors affect expression of about 2% of all genes (Marks, 2000). Thus it was of interest to see whether loss of ATM affected other genes besides p21^{WAF1}. To test this possibility, the topoisomerase IIIα (topo IIIα) promoter was also examined. This promoter was PCR cloned from wild type cells (Figure 15). Figure 16 shows that this promoter was activated by FR up to 5 fold in the wild type cells, but not at all in AT cells. A constitutive promoter from SV40 also yielded similar pattern. In addition, after stable transfection of topo IIIα –luciferase gene into the AT and wild type cell lines, the integrated promoter was activated to a much higher degree wild type relative to AT clones in response to FR (Figure 17). It is also worth noting that in wild type cells, the topo IIIα promoter was activated to different extents in the various stably transfected clones.
Figure 15. Amplification of the topoisomerase IIIα promoter

Genomic DNA was extracted from GM0637 cells as a template to conduct PCR reactions to amplify a 1.5-kb topoisomerase IIIα promoter. PCR conditions are described in “Material and Methods”. PCR reaction products were loaded onto 1.0% agarose gel.
**Figure 15**

[Image of a gel electrophoresis with markers and labeled bands.]

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**Figure 16. Activation of topo IIIα promoter by FR in transient transfections**

The constructs containing either full-length 1.5-kb or truncated 600-bp topo IIIα promoter fused to the luciferase gene were transfected into AT cells (GM5849) or normal cells (GM0637). As a control, pGL3 (SV-40 promoter-luciferase gene) was introduced into the cells in the same way. Cells were processed and luciferase was measured as described in Figure 13. Panel A: Activation of topo IIIα promoter represented; Panel B: Graphic map of the constructs.
Figure 16
Figure 17. FR activation of topo IIα promoter in stable transfections

A construct containing full-length 1.5-kb topoIIα promoter fused to luciferase was transfected into both GM0637 and GM5849 cells. The stable transfectants were selected based on their resistance against G418. After screening for their luciferase activities, six of clones from each cell line were treated with or without FR. The fold increases were calculated by normalizing FR-treated luciferase activity to DMSO-treated controls.
Figure 17

Wild type

Cell line

Fold increase (+/- FR)

AT

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Figure 18. Ectopic expression of wild type ATM gene restores p21\textsuperscript{WAF1} induction by FR.

AT cells (AT22IJE) bearing the empty vector (pEBS7) and the recombinant ATM gene (pFBS-YZ5), as well as wild type cells (GM0637) were treated with or without 50 nM FR for 24 hours. Cells were analyzed for p21\textsuperscript{WAF1} and actin protein levels by Western blotting as described in Figure 5.
Figure 18

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3. **Ectopic expression of ATM restores p21^{WAF1} induction by FR901228 in AT cells.**

Genomic instability is a hallmark of ATM deficiency in AT cells largely due to random secondary mutations (Shiloh, 2001). If HDAC inhibitor-mediated p21^{WAF1} induction deficiency in AT cells is due to this type of mutation, it should be irreversible. That is, ectopic expression of a wild type ATM in AT cells should not restore the defective induction. To test this, we obtained two AT fibroblast lines, AT22IJ E pEBS7, derived from an immortalized fibroblast line AT22IJ E transfected with the “empty” mammalian expression vector pEBS7, and AT22IJ E pEBS7-YZ5, derived from the same cell lines but transfected with an ATM cDNA. Both lines were treated with FR. Figure 18 shows that AT cells with the vector displayed only barely detectable amounts of p21^{WAF1} in response to FR induction. In contrast, p21^{WAF1} expression in the ATM-complemented cells was essentially identical to wild type cell line GM0637. These data suggest that expression of the wild type ATM gene restores p21^{WAF1} induction deficiency in AT cells. The lack of FR induction appears to be a consequence of the direct loss of ATM activity rather than downstream secondary mutations.

4. **PI-3 kinase activity of ATM is required for induction of p21^{WAF1} by HDAC inhibitors**

PI-3 kinase activity appears to be closely connected to many cellular defects in AT cells and expression of an ATM gene lacking PI-3 kinase domain confers AT phenotypes on wild type cells (Morgan, 1997). To test the role of PI-3 kinase
Figure 19. Effects of caffeine and wortmannin on $p21^{WAF1}$ induction by FR

Wild type GM0637 cells were incubated with or without 50 nM FR together with either 2 mM caffeine or 10 uM wortmannin as indicated for 24 hours. Cell extracts were analyzed by Western blotting as described in Figure 5. Panel A, Western blotting. Panel B, quantitation of Western blot data. The value for FR-only (no caffeine or wortmannin) sample was adjusted as 100% to determine the percentage of $p21^{WAF1}$ protein induction after treatment of caffeine or wortmannin.

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Figure 19

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Figure 20. Effect of caffeine on the p21\textsuperscript{WAF1} induction by TSA

Wild type GM0637 cells were treated with or without TSA (0.2 ug/ml) together with 2mM caffeine as indicated for 24 hours. Cell extracts were analyzed by Western blotting as described in Fig. 5. Panel A, Western blot results. Panel B, quantitation of Western blot data.
Figure 20

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Figure 21. Effect of caffeine on activation of p21\textsuperscript{WAF1} promoter by FR

The pooled clones from wild type line (GM0637) carrying integrated p21-luc were treated with indicated concentrations of FR combined with caffeine (2 mM) as indicated. Luciferase activity was measured and fold increase calculated as described in Figure 13.
Figure 21

Fold increase

FR conc. (nM)

no caffeine

caffeine
activity on ATM-mediated p21WAFI expression, two PI-3 kinase inhibitors (caffeine and wortmannin) were used together with FR or TSA. As shown in Figure 19, wortmannin and caffeine reduced FR-induced p21WAFI expression by 33% and 60%, respectively. A similar result was obtained with TSA as shown in Figure 20. Moreover, caffeine inhibits FR-mediated p21WAFI promoter activation. As shown in Figure 21, when the pooled wild type clones carrying integrated the p21WAFI promoter were treated with FR, FR alone induced the p21WAFI promoter activity by 60-80 fold, depending on concentration. Addition of caffeine reduced this increase to 30 fold at 5 nM. These results suggest that caffeine and wortmannin repress the FR-mediated p21WAFI induction at the transcriptional level. Taken together, these results suggest that PI-3 kinase activity is essential for HDAC inhibitor-mediated p21WAFI induction.

6. HDAC inhibitors fail to induce Rb dephosphorylation and G1 arrest in AT cells

Dephosphorylation of Rb is one downstream consequence of elevated p21WAFI in the G1 checkpoint pathway (DelSal et al, 1996). To test role of a defective p21WAFI, we examined the Rb phosphorylation status and cell cycle progression in AT cells in response to FR/TSA. Figure 22 shows that elevated p21WAFI was correlated with a decreased phosphorylated Rb in the wild type cells (GM637); however, consistent with the defective p21WAFI induction, the levels of hyperphosphorylated Rb remained nearly unchanged in FR treated AT cells lines (GM5849 and GM9607).
Figure 22. Effects of FR on Rb phosphorylation

The phosphorylation status of Rb was determined by electrophoretic mobility on 8% SDS-polyacrylamide gel. Rb was detected in AT (GM5849, GM9607) and wild type (GM0637) whole cell lysates (100 µg) by immunoblot using an antibody that recognizes both phosphorylated (pRb) and unphosphorylated (Rb) forms of Rb, following 24 hours of treatment with FR (50 nM) (+) or DMSO (-) as indicated. Actin was used as a loading control.
Figure 22
Figure 23. Effect of FR on cell cycle distribution

Fluorescence cell sorting and cell cycle analysis were used to generate cell cycle profiles for three cell lines. GM5849 (AT), GM8505 (Bim) and GM0637 (WT) cells were treated with or without FR 50 (nM) for 24 hours, fixed and stained with propidium iodide. After sorting data were plotted as DNA content versus cell number. $G_1$, S, $G_2/M$ and sub-$G_1$ phases are shown.
Figure 23
Figure 24. Effect of TSA on cell cycle distribution

GM0637 (WT) and GM5849 (AT) cells were treated with TSA (0.1 ug/ml) for 24 hours. Cells were fixed, stained, and cell cycle profiles determined as described in Figure 23.
Figure 24

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Moreover, GM5849 in particular displayed significantly higher levels of Rb than wild type cells, consistent with the previous studies (Sugita et al, 1992).

FR is also known to induce G1 and G2 arrest (Nakajima, 1998). G1 arrest is the result of elevated p21\textsuperscript{WAF1} while the mechanism of G2 arrest remains unclear (Sandor et al, 2000). Flow cytometry of cell cycle progression revealed that the AT cell line GM5849 had an attenuated arrest at the G1 checkpoint while the G2 checkpoint appeared to be intact (Figure 23). As a control, the Bloom's syndrome cell line (GM8508) exhibited a normal G1 arrest, consistent with its normal p21\textsuperscript{WAF1} induction. Interestingly, this cell line failed to arrest at the G2 checkpoint when treated with FR. In addition, a higher percentage of apoptotic bodies represented by sub-G1 population were observed in AT cells, indicating a higher sensitivity to FR. Similar results were seen when TSA was applied, further suggesting that the effects are indeed due to inhibition of HDACs (Figure 24).

7. Analysis of H3 and H4 histone acetylation

To determine if the defective promoter activation was due to alterations in global histone acetylation, histones were isolated from the cells after exposure to FR for 0, 2, 4 and 8 hours. Western blot data show that before incubation with FR (0 hour), the levels of acetylated H3 and H4 were low in both the normal and AT cells (Figure 25). After adding FR, the acetylated H3 and H4 started to accumulate in the both cell lines. There was a relatively minor difference in H4 acetylation pattern in AT cells compared to the wild type controls (see acetylated H4 in AT blot, Figure 25); however, other differences were not obvious between the two cell lines.
Figure 25. Effect of FR on histone acetylation

GM5849 (AT) and GM0637 (WT) cells were treated with 50 nM FR for the indicated times (in hours). Histones were acid extracted as described in “Materials and Methods” and a total of 5 µg histone protein was loaded onto 20% SDS-polyacrylamide gel. Acetylation was detected by using anti-acetylated H3 and H4 antibodies. A parallel gel was stained with Coomassie blue to demonstrate that all lanes were loaded with the similar amount of histone protein (labeled as core histones). Positions of acetylated H3 and H4 are indicated on the right of the blot.
Figure 25

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DISCUSSION

Previous studies revealed that both FR and TSA induce the expression of p21\textsuperscript{WAFI} in normal cells (Sowa, 1997; Biggs, 1996; Rajgolikar, 1998). In this study, p21\textsuperscript{WAFI} induction by HDAC specific inhibitors is shown to be defective in the AT cell. Since the defects were observed in three independent AT cell lines and not in the other non-AT mutant lines such as Bloom’s syndrome and galactosemia, it is unlikely that this particular defect is caused by random mutations unrelated to loss of ATM function. One of the AT lines (GM9607) displayed a less severe defect relative to the other two. From this, I conclude that different ATM mutations may have distinct effects.

Like the other HDAC inhibitors, FR and TSA affect many biological processes, such as differentiation and apoptosis of transformed cells in culture, and inhibition of tumor growth in animals (Sugita, 1992b; Yoshida, 1990; Yoshida, 1987). The global effects of FR and TSA may reflect the multiple roles of HDACs. It is also possible that FR or TSA affect other physiological targets and processes besides HDACs; however, given their complete unrelated structures, any common effects caused by both FR and TSA are likely due to an HDAC target. Therefore, I conclude that the defective induction by FR or TSA is directly associated with the expected targets for these drugs, HDACs.

The fact that ectopic expression of the ATM gene restores defective p21\textsuperscript{WAFI} induction strongly supports the notion that ATM is directly associated with HDAC
inhibitor-mediated p21 induction. The data also suggest that repression of FR/TSA induced p21WAF expression by caffeine or wortmannin is related to ATM instead of ATR although ATR has been reported to associate with HDAC2 (Schmidt, 1999). Taken together, I conclude that the ATM protein with PI-3 kinase activity is essential for histone acetylation-dependent p21WAF expression. While both caffeine and wortmannin behaved similarly, caffeine was a more efficient inhibitor of the FR/TSA-mediated p21WAF induction, for reasons that are not clear (note that we cannot rule out stability differences between caffeine and wortmannin over the 24 hour incubation in vitro).

Although FR and TSA inhibit most HDACs, they only affect expression of about 2% of mammalian genes (Van Lint, 1996). It was therefore of interest to determine the generality of the response with other promoters besides p21WAF. To address this, we tested two other promoters, SV40 and topoisomerase IIIα. The topo IIIα promoter is TATA-less, sharing similarity to a number of housekeeping genes (Kim et al, 1998). The SV40 promoter is constitutive "promiscuous" with a prototypic TATA box and has shown to be activated by FR and TSA (Nakajima et al, 1998a). In spite of these differences, both appear to be induced by the HDAC inhibitors in the wild type cells in this study. Activation was diminished in AT cells, however. From these data I conclude that the defect in acetylation-dependent gene expression in AT cells is not limited to p21WAF alone.
In stably transfected wild type cells containing integrated topo IIIα promoter-luciferase DNA, it was noted that the different clones exhibited different degrees of activation by FR. From this observation, it appears that positional effects are relevant to the FR activation phenomenon reported here. In contrast to wild type cells, stably transfected AT cells exhibited very low levels of FR activation. These results imply that the loss of ATM function may have a global effect on histone acetylation-mediated gene transcription.

Based on these observations, decreases in HDAC inhibitor-mediated induction in AT cells might be explained in two ways. One is that the ATM protein directly modulates HAT and HDAC activities, such that overall acetylation is less robust in the absence of a fully functional ATM gene product. Therefore, reducing HDAC activity (by addition of FR or TSA) does not culminate in excess acetylation since HAT activity is less active. The other possibility is that the loss of ATM function does not directly influence histone acetylation but influences the events downstream of histone acetylation presumably in chromatin. That is, an AT cell will have normal levels of acetylation in response to FR or TSA, but somehow the acetylation fails to result in transcription activation. Assessment of FR-induced histone acetylation (Figure 25) appears to support the second possibility. There were no substantive differences observed in histone H3 acetylation between the normal cells and AT cells after FR treatment. Although H4 displays a delay in histone acetylation in response to FR, the final acetylation levels were no different between the normal cells and AT cells.
The ATM protein is known to localize in chromatin and the nuclear matrix. Since ionizing radiation does not change its localization or amount and since chromatin structure in AT cells appears to be abnormal (Beamish et al, 2001), it is possible that the ATM function is required to maintain an appropriate chromatin structure as a prerequisite for histone acetylation-dependent gene regulation. Struhl (Struhl, 1998) has proposed models in which histone acetylation might selectively affect expression of one gene over another. One of his models is that histone acetylations are generally targeted to promoter elements and the selection is due to inherent differences in the promoters. For example, acetylation-sensitive promoters are associated with more tightly packed and/or positioned nucleosomes, and acetylation changes the nucleosome organization to facilitate accessibility of the Pol II machinery to the promoters. In contrast, acetylation-insensitive promoters are located in less tightly packed and/or positioned nucleosome region and the organization state of nucleosomes does not affect the accessibility of the Pol II machinery. This model may apply to regulation of p21 or topo IIIα. That is, these promoters are acetylation-sensitive in the presence of normal ATM protein and loss of ATM turns these promoters into an acetylation insensitive mode.

Since we have not examined the change of local histone acetylation (at the gene level) in response to FR or TSA, we cannot exclude the possibilities that the loss of ATM results in disassembly of certain HAT complexes on the promoters we tested, which, in turn, fail to acetylate the histones on those promoters. In fact, a recent
study (Wang et al, 2000) indicated that ATM is a part of a super complex, BASC (BRCA1-associated genome surveillance complex). Besides ATM, this BRCA1-centered complex also contains other DNA damage repair proteins, such as BLM, MSH2, MSH6 and NBS1. Furthermore, BRCA1 was reported to interact with the components of a histone deacetylase complex, which consists of RbAp46, RbAp48 (retinoblastoma-binding protein), HDAC1 and HDAC2 (Yarden et al, 1999), indicating that BRCA1 interacts with NuRD complex. In addition, p300/CBP, a HAT, also associated with BRCA1 at the same domain as HDACs (Neish et al, 1998; Pao et al, 2000). Combined these data with the one from this study, I propose a possible model as follows: ATM controls p21WAFI transcription by modulating BRCA1 activity (probably by phosphorylation), which, in turn, regulates the association between BRCA1 and HDACs or p300/CBP. The fact that overexpression of BRCA1 in the BRCA1 wild type cells transactivates transcription of p21WAFI (Somasundaram et al, 1997) further supports this model. Another report that ATM phosphorylates BRCA1 at multiple sites (Cortez et al, 1999) also favors the model. Although such phosphorylations were only investigated under conditions of IR, the careful examination of the results from non-radiation reveals that BRCA1 from AT cells was less phosphorylated than the one from wild type cells (Cortez et al, 1999; Yazdi et al, 2002). This incates that ATM may phosphorylate BRCA1 even without IR treatment and this modification may be essential for association between BRCA1 and HDACs.
A large body of evidence supports the idea that acetylation of histones activates transcription; however, exactly how histone acetylation leads to transcription remains unclear. In this study, I show that the ATM gene product plays a role in this process. Further understanding of this phenomenon will not only provide an insight into the mechanism of histone acetylation and transcription, but also open a new angle to understand multiple functions of ATM.
Figure 26. A proposed model to explain the relationship between ATM and histone acetylation-mediated gene (p21^{WAF1}) activation.

ATM controls p21^{WAF1} transcription by modulating BRCA1 activity (probably by phosphorylation), which, in turn, regulates the association between BRCA1 and HDACs or p300/CBP at BRCT (BRCA1 C-terminus) domain.
BRCA1

ATM

BRCT

P300/CBP
(HAT)

Histone
deacetylase
complex

transcription activation

transcription repression

Figure 26

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CHAPTER 2

CIRCUMVENTION OF CAMPTOTHECIN RESISTANCE BY UCN-01 IN MULTIPLE MYELOMA CELLS

INTRODUCTION

DNA topoisomerases are enzymes that alter the topological state of DNA by generating transient breaks in DNA backbone. Topoiomerases are divided into two classes based primarily on their modes of cleaving DNA. Type I topoisomerases catalyze DNA topology change by making a transient single strand break in DNA, pass another strand through the nick (Champoux et al, 1978; Liu et al, 1989). Type II topoisomerases act by transiently nicking both strands of DNA, passing another double-stranded DNA segment through the gap (Wang, 1996). Among type I topoisomerases, two subfamilies, type IA and type IB, have been characterized (Champoux, 2001). The two subfamilies share no homology and form different DNA adducts. Type IA subfamily members form a covalent phosphotyrosine linkage to the 5’end of DNA cleavage during catalysis while subfamily IB members attach to 3’ end. In eukaryotic cells, topoisomerase II (topo II) is a type II enzyme while topoisomerase I (topo I) and III (topo III) belong to type IB and type IA, respectively (Berger et al, 1998).
Topoisomerases are important, often functionally essential to cells. They participate in nearly all aspects of DNA metabolism including replication, transcription, recombination and DNA repair (Liu et al, 1989; Wang, 1996; Nitiss, 1998).

Topoisomerase II is also involved in chromosome condensation and segregation of newly replicated chromosomes. Recent studies also indicated that topoisomerases are associated with maintaining genome stability (Hu et al, 2001; Chakraverty et al, 2001; Wallis et al, 1989).

1. Topoisomerase I

Human topo I is a 100kD monomeric protein (765 amino acids) encoded by a single copy gene. The topo I gene is spread over at least 85 kb of genomic DNA on the long arm of chromosome 20 (20q12-13.2) (Liu et al, 1981; D'Arpa et al, 1988; Juan, 1988; Kunze et al, 1990). The topo I protein can be divided into four domains: the N-terminal domain that contains the nuclear translocation signal, the core domain, the linker region and the C-terminal domain in which the active site tyrosine (tyr723) is localized. The N-terminal domain is responsible for the nuclear localization of topo I but dispensable for relaxation activity (Stewart et al, 1996; Pommier et al, 1998). This domain is also involved in protein-protein interactions. The core region and C-terminal domains are fairly conserved, protease resistant and essential for activity. Many mutations causing drug resistance occur in these two domains.

Yeast topo I is non-essential, as demonstrated in mutants of S. cerevisiae (Thrash et al, 1984) and Saccharomyces pombe (Uemura, 1984) which has less than 1% of normal topo I activity, but grow normally despite the fact that deletion of the Topo I gene results
in an increased rate of deletion in the rDNA (Christman et al, 1988). However, the nuclear *Drosophila* and mouse topo I are not dispensable for embryonic growth and development since topo I-null embryos cannot develop (Fernandez-Beros et al, 1992; Bjornst et al, 1989; Madden et al, 1992). On the other hand, overexpression of eukaryotic topo I in E. coli is lethal (Fernandez-Beros et al, 1992; Bjornst et al, 1989), and the extent of topo I overexpression is also limited in mammalian cells (Madden et al, 1992). One of the major functions for topo I is to unwind the supercoiled DNA resulting from replication. With SV40, bi-directional replication is stimulated in the presence of topo I (Ishimi et al, 1991) and the treatment of SV40 infected cells with the topo I poison camptothecin (CPT) inhibits viral replication fork movement (Avemann et al, 1988).

Topo I is also involved in transcription. It is found to be associated with the TATA binding protein (TBP) and to be involved in selective activation of transcription initiation (Merino et al, 1993; Kretzschmar et al, 1993). In addition, Topo I from vaccinia virus causes lambda prophage excision by recombination and efficiently resolves Holiday junctions (Shuman et al, 1989). Moreover, topo I is structurally closely related to site-specific recombinases; thus it probably participates in recombination. Topo I may be also involved in DNA repair. For example, Topo I has been shown to recognize and nick base mismatch at the Topo I cleavage site (Yeh et al, 1994). Evidence also indicates that Topo I is important in UV-related DNA repair (Mao et al, 2000).

The topo I promoter region exhibits sequence features found in a number of housekeeping genes, including high G+C content, high frequency of CpG islands, and absence of a TATA and CCAAT box (Kunze et al, 1990). The topo I
promoter also contains binding motifs for several transcriptional factors such as Sp1, the cAMP-responsive element, NF-kB and members of basic helix-loop-helix/leucine-zipper proteins (Kunze et al, 1990; Heiland et al, 1993; Hwong et al, 1989). Topo I is usually present throughout the cell cycle (Heck et al, 1988); however, its transcriptional level is also regulated under certain physiological conditions. For instance, topo I mRNA increases by 20-fold in regenerating rat liver (Duguet et al, 1983) and 10-fold in human skin fibroblasts upon exposure to phorbol 12-myristate 13-acetate (PMA) (Hwong et al, 1989). In addition, topo I also increases during cell proliferation (Sobczak et al, 1989) and in response to adenovirus infection (Romig et al, 1990).

Besides transcriptional regulation, topo I activity is modified at the post-translational levels as well, such as phosphorylation and poly(adenosine-diphosphoribosylation) [poly(ADP-ribosylation)]. In vitro, tyrosine phosphorylation was shown to inactivate calf thymus Topo I while phosphorylation at serine residues by protein kinase C enhances topo I activity (Samuels, 1989). The fact that topoI co-purifies with kinase C and is activated by the kinase suggests that protein kinase C is a physiological regulator of topo I (Pommier et al, 1990; Kasid et al, 1989; Ferro et al, 1984). Poly(ADP-ribosylation) of topo I decreases its catalytic activity in vitro (Park et al, 1991). Consistently, inhibitors of poly(ADP-ribosylation) increase the levels of DNA breaks and cytotoxicity in response to CPT (Mattern et al, 1987). Cell lines deficient in poly(ADP-ribosylation) are CPT-hypersensitive (Chatterjee et al, 989).

Although topo I exhibits catalytic activity as a monomer, it is reported to interact with other proteins. Such interactions likely influence the enzyme activity. One of

2. The catalytic mechanism of Topo I

The catalytic reaction of topo I can be divided into four steps (Chen, 1994): (1) binding of topo I to DNA: eukaryotic topo I preferentially binds double strand DNA (dsDNA) and has greater affinity for supercoiled DNA over relaxed DNA. (2) DNA cleavage of one strand with covalent attachment of topo I to the one of termini of nicked DNA. The tyrosine hydroxyl group of eukaryotic topo I linkes to the 3’ phosphate of DNA, releasing the 5’-OH group to generate a single strand break. (3) Single strand passage. Two possible models are proposed for the passage. The first one is that during cleavage, the enzyme covalently binds to one end of DNA and leaves the non-covalently bound DNA end to rotate through the break before being recaptured by the enzyme. The second one is that the enzyme serves as a bridge in a DNA strand by holding both ends of the cleaved strand via either covalent or non-covalent interactions. Then the cleaved strand rotates around the intact strand, thereby changing the linking number. (4) Ligation of the cleaved DNA. In this step, topo I is released and cleaved DNA is religated (Wang, 1996).
The DNA cleavage and religation reaction of topo I is governed by an equilibrium process. Under normal conditions, the equilibrium lies toward religation favoring the sealed product (Chen, 1994). In other words, the intermediate DNA-topo I cleavable complex has a short half-life and thereby, is hard to detect. A group of chemicals, such as camptothecin, block the ligation reaction and, thereby, favor formation of the DNA-topo I complex.

3. Camptothecin (CPT) and its derivatives

Camptothecin was identified as an antitumor alkloid from the tree *camptotheca acuminata* three decades ago (Wall et al, 1966). Topo I was found to be its target (summarized by Pommier et al, 1998). Although CPT inhibits topo I catalytic activity, further studies indicated that it is not an inhibitor by traditional definitions. Instead of inhibiting topo I activity, CPT was found to be able to stabilize the enzyme reaction intermediate—DNA-topo I covalently bound complex (DNA-topo I cleavable complex) (Hsiang et al, 1983; Covey et al, 1989). The detailed mechanism of this stabilization is not completely understood. The present evidence suggested that CPT may bind to both topo I and DNA (Champoux et al, 2000). The notion that CPT is a topo I poison instead of inhibitor is supported by the following observations: (1) the DNA-topo I cleavable complex can be easily detected in presence of CPT and its derivatives (Nitiss et al, 1988); (2) the elevated topo I level confers on cells a higher sensitivity to CPT (Bjomsti et al, 1989; Madden et al, 1992). The extreme example is that the yeast cells in which topo I is deleted are resistant to CPT (Nitiss et al, 1988) and expression of wild-type topo I gene restores CPT sensitivity (Bjomsti et al, 1989; Madden et al, 1992).
A variety of CPT derivatives were developed by modifying CPT to increase its antitumor activity and decrease its side effects, such as 9-aminocamptothecin, topotecan and CPT-11 (Pommier et al, 1998; Tanizawa et al, 1994; Tanizawa et al, 1995). CPT-11 is a weak topo I poison, however, its metabolite, SN-38 has potent activity.

4. Generation of Cytotoxicity by CPT

Although CPT is found to be toxic to proliferating cells, covalent topo I-DNA complexes stabilized by camptothecin during G1 and G2 phases of the cell cycle are much less toxic than those stabilized during S phase (Horwitz, 1973). This suggests that the topo I-DNA complexes per se are not the cytotoxic lesions and therefore, not sufficient to insure cell death. Processes downstream of DNA-topo I complex formation play key roles as well.

a. Processing of cleavable complexes

Although some evidence indicated that CPT is toxic to nonproliferating neuronal cells at relatively high concentrations (10uM) (Morris et al, 1996), in most cases DNA synthesis is essential for final cytotoxicity. DNA synthesis inhibitors such as aphidicolin or hydroxyurea have been shown to abolish cytotoxicity of CPT in mammalian cells (Hsiang et al, 1989; Snapka et al, 1986; Shin et al, 1990). Addition of camptothecin to cells containing replicating SV 40 results in double-strand (ds) breaks in both the leading and lagging strands of the viral genome, indicating that replication machinery converts the topo I-DNA complexes into DNA ds breaks. This is further supported by the finding that small amounts of DNA ds breaks have been detected in newly replicated genomic DNA of camptothecin-treated cells (Ryan, 1991). These results suggested DNA
ds breaks converted from DNA-Topo I cleavable complexes are likely the toxic lesions leading to cell death. The fact that deletion of rad53 (a gene required for ds break repair) confers the yeast cells hypersensitivity to CPT further adds the evidence to support this view (Eng et al, 1988; Nitiss et al, 1988). Collectively, these studies support the model that replication machinery at the advancing replication fork collides with DNA-topo I complex, causes releasing of the cleaved strand, and leads to ds DNA breaks and cell death.

b. Consequences of DNA damage caused by CPT

CPT has been shown to induce G1, S and G2 arrest (Tobey et al, 1972; Zucker et al, 1991). The currently accepted theory is that cell cycle arrests give cells time to repair DNA damage, and, thereby, avoid proceeding throughout cell cycle with damaged DNA. Waldman et al. reported that colon cancer cells with disrupted p53 displayed hypersensitivity to CPT (Waldman et al, 1996), consistent with the notion that G1 arrest gives the cells an opportunity to repair DNA damage. On the other hand, Slichenmyer et al. demonstrated that RKO colon cancer cells with or without functional p53 showed no difference in colony formation with response to CPT (Slichenmyer, 1993). These results suggest that the presence p53 and G1 arrest may not necessarily be the sole factors accounting for the final result with CPT.

DNA repair pathways play significant roles determining the final fate of CPT-treated cells. Disruption of DNA repair pathway-related genes increases CPT sensitivity. For instance, Ataxia telangiectasia (AT) cells, in which ATM gene is mutated, display hypersensitivity to the cytotoxic effect of CPT (Davies et al, 1989). Loss of rad53
described as above also renders the corresponding yeast cells hypersensitivity to CPT (Nitiss and Wang, 1988; Eng, 1988). Furthermore, the cells surviving CPT-treatment have increased frequency of chromosomal aberrations and mutations (Andersson, 1992). More detailed studies have revealed that mutations induced by CPT treatment often involve large deletion and insertions occurring in the vicinity of preferred topoisomerase cleavage sites (Berger, 1991; Hashimoto, 1995). These results implied probable mechanisms to repair DNA breaks resulting from CPT treatment.

CPT and its derivatives have been demonstrated to kill tumor cells by inducing apoptosis (Mesner, 1997). Like apoptosis induced by the other DNA damaging agents, a series of biochemical changes occur after treatment with CPT (Mesner, 1997; Martins, 1997). These events include: (a) expression of certain genes such as the transcription factor Jun (Rubin, 1991) and the tumor necrosis factor-α homology Fas ligand (Friesen, 1996; Kasibhatla, 1998); (b) release of cytochrome c from the intermembrane space of mitochondria into the cytosol (Yang, 1997); (c) activation of caspase-3 and the other effector proteases (Datta, 1996; Ibrado, 1996; Martins, 1997; Faleiro, 1997); (d) selective cleavage of a variety of intracellular proteins (Villa, 1997; Porter, 1997); (e) cleavage of DNA (Walker, 1991); (f) loss of membrane integrity. After exposure to DNA damage agents, whether cells decide to repair damaged DNA or execute apoptosis depends on many factors, such as the extent of DNA damage, the capacity for DNA repair, and the threshold of apoptosis. In cancer chemotherapy, these are important factors that affect drug resistance of cancer cells.
5. CPT resistance in tumor cells

Drug resistance is one of the biggest obstacles in the cancer treatment. Many cellular events can affect the final cytotoxicity of antitumor drugs and provide cancer cells with resistance. The events involved in topo drugs can be classified into three categories: pre-target, drug-target interaction and post-target (Larsen et al, 1998).

The pre-target events include the factors involved in availability of drugs, such as drug accumulation, drug metabolism and intracellular distribution. Many drug resistant cell lines overexpress membrane-localized transporters, which belong to a superfamily of ATP-dependent transporters. They recognize structurally unrelated antitumor agents, and reduce the drug accumulation or alter intracellular drug distribution. The examples of these transporters are Pgp (P-glycoprotein) (Gottesman et al, 1988) and MRP (multidrug resistance protein) (Cole et al, 1992). Overexpression of Pgp renders cells resistant to a broad-spectrum of antitumor drugs but not CPT or its derivatives (Jonsson et al, 1997). In contrast, studies carried out with more than 200 fresh tumor samples displayed a clear correlation between MRP expression and topotecan resistance (Jonsson et al, 1997). An ATP-binding protein was also identified in yeast that renders the cells resistance to CPT (Reid et al, 1997). Another energy-dependent pump has been isolated from mitoxantrone-resistant cells and found to render cells highly resistance to CPT (Yang et al, 1995). CPT resistance thereby can be a result of overexpression of the transporters.

Cells can also modify topo drugs to change the way the drugs interact with enzyme and/or DNA. For example, CPT-11 is a prodrug that has to be converted into SN-38 to have activity by cellular carboxylesterases. In a CPT-11 resistant cell line, SN-38 was
observed to decrease although the total CPT-11 level does not change when compared to the parental sensitive cell line (Kanzawa et al., 1990), suggesting the cells can acquire the CPT resistance by modifying the drugs.

Drug resistance resulting from drug-target interaction includes the accessibility of the DNA substrate, the abundance and activity of topo as well as the mutations in the topo protein affecting the DNA-topo cleavable complex formation. Highly compact chromatin will block the interaction between DNA and topo I, therefore, making DNA unavailable to topo I damage (Dufer et al., 1995). Since CPT is a topo I poison, a higher topo I protein and activity level will lead to formation of more cleavable complexes. There are extensive reports that the levels of topo I expression vary widely between and within tumor types, suggesting that down-regulation of topo I expression may account for CPT resistance in some cancer cells (Larsen et al., 1998; Beidler et al., 1995; Danks et al., 1996). As described above in this introduction, hyper- and hypophosphorylation as well as poly(ADP-ribosylation) regulate the topo I activity, thereby, the cancer cells can also acquire resistance by down-regulating the topo I activity as well.

A high percentage of CPT resistance is associated with topo I mutations which may or may not affect the topo I activity (Larsen et al., 1998). Almost all these mutations occur in the topo I core region and C-terminal domain, for example, mutations at amino acid residues 361, 362, 363, 364 in the core region, and mutations at 722, 721, 717 in the C-terminal domain. Unlike the broad-spectrum resistance resulting from MRP, the resistance caused by topo I mutations is specifically against CPT. For example,
substitution of Arg and Ala next to the active site tyrosine results in CPT resistance but has no effect on the sensitivity of another drug netropsin (Knab et al., 1995).

Since DNA-topo I complex *per se* is not cytotoxic, the events downstream of the cleavable complex obviously play significant roles to determine the final cytotoxicity. These events include DNA replication, cell cycle progression, DNA repair and recombination as well as regulation of apoptosis. Generation of ds DNA breaks has been proven to be the result of collision between the cleavable complex and replication forks. Therefore, cells that display strong S-phase checkpoint probably are more resistant (Goldwasser et al., 1996). Although loss of the G1 checkpoint does not necessarily result in decreased viability, G2 arrest is clearly associated with increased viability following the drug treatment. Naturally resistant cells often have capacity to arrest cells at G2 checkpoint for prolonged period of time (Goldwasser et al., 1996; Dubrez et al., 1995; Bailly et al., 1997), suggesting that cell cycle checkpoints probably contribute to drug resistance as well.

Different cell types have very different tolerance toward drug-induced lesions. This is partially determined by levels of apoptosis-related proteins. Bcl-2 family protein (Bcl-2, Bcl-XL, Bag1, Mcl-1, Al) appear to confer on cells a protective effect to antineoplastic agents although the roles of Bcl-2 in drug resistance are not consistent. Transfection of Bcl-2 into murine B-cell lymphoma cells resulted in both inhibition of apoptosis and elevated clonogenic survival after etoposide treatment, a topo II drug (Kamesaki et al., 1993). In contrast, overexpression of Bcl-2 into Hela cells inhibited etoposide-induced apoptosis but had no effect on clonogenic survival (Kuhl et al., 1997). On the other hand,
Bcl-\(X_L\) seems more clearly to involve in drug resistance. In a P388 murine leukemia subline, a broad spectrum of drug resistance is associated with a decrease of Bcl-2 and overexpression of Bcl-\(X_L\). In addition, transfection of human Bcl-\(X_L\) into parental P388 cells conferred drug resistance as well. Similar results were observed in myeloma cells (Schwarze et al, 1995).

6. DNA-topo I cleavable complex as a parameter to predict the effect of prognosis

In cancer chemotherapy, the frequency for patients to respond to any given anticancer drugs never approaches 100%. In other words, a certain portion of a cancer patient population receives no benefits from the treatment. In the mean time, those patients have to suffer the side effects. To resolve such a problem, one attractive strategy is to establish certain parameters to identify those prospective patients who will respond to the particular drugs and transfer the rest of the patients to the other programs.

In the chemotherapies involving CPT and its derivitives, using topo I protein level as a predictor of response has been proposed since topo I was found to vary widely among different cancers. However, the disadvantages of such a parameter are obvious. Apparently, topo I levels do not necessarily represent DNA damage. As discussed above, drug accumulation, distribution, metabolism can change the interaction between topo I and drugs so that topo I, even at a high level, may not be accessible to the drugs. Topo I protein itself can also be modified to decrease its activity or mutated to lose its binding to CPT. In such cases, low levels of DNA damage will be achieved. Thus, topo I levels alone are ideal predictors of drug sensitivity and DNA damage in presence of CPT (Goldwasser et al, 1996).
Compared to topo I, DNA-topo I complex appears to be a more attractive parameter to predict DNA damage and cytotoxicity as it represents the net results of all the pre-target and drug-target interaction events. For example, if a drug is not available to topo I resulting from drug metabolism, a low level of DNA-cleavable complex will be detected even in the cells with a high level of topo I. As a result, the low levels of cleavable complex will be consistent with the low cytotoxicity. Similarly, low levels of cytotoxicity caused by topo I mutations, low topo I activity, changed chromosome structure or subcellular localization will also be reflected by the levels of cleavable complex. However, since the cleavable complex per se is not cytotoxic, the cleavable complex alone may not always be closely connected with final cytotoxicity outcome noting that, in theory, cytotoxicity is the net result of DNA-topo I complex formation and the other downstream events, such as the capacity of cells to convert the complex into DNA damage, DNA repair and regulation of apoptosis. Only when the other events are similar, can the complex level become the major determinant.

7. Biology of myeloma

Multiple myeloma (MM) is an incurable B-cell malignancy that has slow-growing monoclonal immunoglobulin-produced plasma cells in the bone marrow (Hallek, 1998). MM accounts for 1% of all cancers and slightly more than 10% of all hematologic cancers. Currently, the major chemotherapy for MM includes: (1) steroid (prednisone, dexamethasone) based on presence of glucocorticoid receptors in MM and the fact that steroids induce apoptosis in MM; (2) alkylating agents (melphalan), which cause DNA damage; (3) combination of one of the above agents with other antineoplastic agents,
such as the topo II drug adriamycin. A series of new therapies are also under investigation, including proteasome inhibitor PS341, tyrosine kinase inhibitors (qinazolines and pyridopyrimidines), anti-angiogenic agents (Thalidomide) and histone deacetylase inhibitors (FR and SAHA). However, the median survival of patients treated with conventional chemotherapy is only 3 to 4 years. Even with the most advanced strategies, stringently defined complete remission is only 20-40% of patients. Most patients subsequently relapse (Anderson et al, 2001).

Multiple drug resistance mechanisms have been identified in MM (Sonneveld et al, 1997). Resistance to steroid therapies is found to be associated with down-regulation or loss of glucocorticoid receptor expression (Gomi et al, 1990). In addition, overexpression of the drug efflux pumps such as Pgp was detected from patients with refractory MM and drug resistant cell lines (Dalton et al, 1986; Dalton et al, 1989; Epstein et al, 1989). Consistent with the notion that many antineoplastic agents kill tumor cells by inducing apoptosis, antiapoptotic proteins such as Bcl- Xₗ and Bcl-2 were reported to be higher in chemoresistant MM cell lines and refractory MM patient cells (Tu et al, 1998). Lastly, resistant MM cells were identified to metabolize drugs more efficiently than drug-sensitive cells. One of the reasons may be due to increased expression of glutathione (GSH) and GSH-related enzymes, which may be responsible for resistance to alkylation agents (Sonneveld et al, 1997).

A CPT derivative, topotecan, has been tested on patients with refractory in a clinical trial by Kraut et al (Kraut, 1998). Although the responding rate was relatively low (17%), some individuals exhibited complete remission. Therefore, it is essential to find out
useful parameters to identify the responding patients and certain ways to effectively circumvent CPT resistance in MM.

One strategy is to combine CPT with other anticancer agents, such as UCN-01. UCN-01, originally identified as a protein kinase C inhibitor, potentiates the cytotoxicity of a variety of anticancer agents, including as mitomycin C, cisplatin, and ionizing radiation (Wang Q, 1996; Bunch, 1996). It is believed that UCN-01 sensitizes cells to DNA damage by abrogating the G₂ and/or S phase checkpoints. The reason UCN-01 abrogates the G₂ checkpoint is mainly due to inhibition of Chk1 and subsequent effect of the Chk1-Cdc25C signaling. Shao et al. also reported that UCN-01 abrogates CPT-induced S-phase checkpoint and enhances CPT cytotoxicity only in human colon carcinoma p53 mutant HT29 cells (Shao et al, 1997; Jones et al, 2000).

In this study, we investigated CPT cytotoxicity and its relationship to CPT-induced DNA-topo I cleavable complexes in six MM cell lines. Our data reveal that DNA-topo I cleavable complexes may serve as a parameter to predict the consequences of CPT cytotoxicity when the DNA-topo I complex levels are extremely high or low in myeloma. Furthermore, UCN-01 can enhance CPT cytotoxicity in CPT-resistant myeloma cell lines. The mechanism may be related to the specific abrogation of S-phase checkpoint and DNA synthesis inhibition as well as the subsequent formation of DNA ds breaks.
MATERIALS AND METHODS

Drugs, Chemicals, and antibodies

UCN-01 was provided by the Drug Synthesis Branch, Division of Cancer Treatment, NCI. Aliquots were frozen at 10 mM in DMSO, and diluted immediately before each experiment. Camptothecin was purchased from Sigma Chemical Co. (St. Louis, MO), and dissolved in DMSO at 10 mM. Aliquots were stored at -20°C until immediately before the experiments.

Anti-phosphorylated histone H2AX (γ-H2AX) antibody was purchased from Trevigen (Gaithersburg, MD). Anti-topoisomerase I was a gift from Topogen, Inc. (Columbus, OH). [14C] Thymidine (57 mCi/mmol), and [methyl-3H] thymidine (20 Ci/mmol) were purchased from ICN biomedicals, Inc. (Costa Mesa, CA).

Cell culture.

Myeloma cell lines NCI-H929, MC\CAR, HS-Sultan, SKO-007 were purchased from American Type Culture Collection (ATCC) (Rockville, MD) and cultured under the following conditions: NCI-H929 in RPMI medium 1640 with 0.05 mM 2-mercaptoethanol and 10 % of fetal bovine serum (FBS); Mc\CAR in Iscove's modified Dulbecco's medium with 20% FBS; HS-Sultan in RPMI medium 1640 with 10 % FBS; SKO-007 in RPMI medium 1640 with 15% of FBS and 1 mM sodium pyruvate. 8226/S and 8226/DOX40 were kindly provided by Dr. William S. Dalton and grown at 37°C in...
the presence of 5% CO₂ in DMEM supplemented with 5% fetal bovine serum and 50 μg/ml gentamicin (Life Technologies, Inc., Rockville, MD).

**Cytotoxicity assays.**

MTT assay was performed to examine the cytotoxicity using a kit from Promega (Madison, WI). Briefly, 1-2 x 10⁴ cells in 0.1 ml of complete medium were dispensed in 96-well microtiter plates. Drugs were added to the appropriate concentrations and the cells were cultured for 48-72 hours. At the end of drug incubation, 15 μl of MTT was added to each well and the plates were incubated at 37°C for 4 hours followed by an overnight culture at 37°C after 100 μl of solubilization/stop solution was added. The absorbance 550 nm was measured the next day with a microplate ELISA reader. Triplicate wells were run for each group. Survival percentage was determined by calculating \( \frac{A_{550 \text{nm (experimental group)}}}{A_{550 \text{nm (control group)}}} \times 100 \).

**Histone extraction**

Histones were extracted by using a modified procedure from Yoshida (Yoshida, 1990). Briefly, cells were pelleted, washed with PBS, and then suspended in buffer A (100 mM NaCl, 50 mM KCl, 20 mM Tris-HCl pH7.5, 0.1 mM EDTA, 1 mM PMSF, 10% glycerol, 0.2% NP-40, 0.1% Triton X-100) to release nuclei. The nuclei were deposited by centrifugation (1000g for 5 minutes, 4°C), the cytosol fraction removed and nuclei resuspended in 100 μl ddH₂O. Concentrated H₂SO₄ (1 μl) was then added and the extract incubated on ice for 1 hour to dissolve the acid-soluble nuclear proteins. The acid-insoluble fraction was removed by centrifugation (14,000 g 10 minutes). The acid-soluble proteins were precipitated with 1 ml of
acetone (4°C overnight). The next day, precipitates were recovered by centrifugation at 12,000 g for 10 minutes at 4°C. The pellet was suspended in solubilizing buffer (0.1% SDS, 100 mM Tris-HCl pH6.8, 1 mM PMSF, 1 μg/ml apotinin, leupeptin, pepstatin each). The insoluble fraction was removed by centrifugation at 12,000 g for 10 minutes. The protein concentration of supernatant was determined using a Bio-Rad DC protein assay kit according to the manufacturer instructions (Bio-Rad, Hercules, CA)

**Western blot analysis.**

To obtain the whole cell extracts, cells were pelleted, washed with PBS and then suspended in RIPA buffer (50 mM Tris-HCl pH7.4, 1% NP-40, 0.25%, Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 μg/ml apotinin, leupeptin, pepstatin each, 1 mM Na3VO4, 1 mM NaF) on ice for 10 minutes. Debris was removed by centrifugation (12,000 g, 10 minutes at 4°C) and protein concentrations in the supernatant determined using a Bio-Rad DC protein assay kit described as above. Proteins were electrophoresed at 200 V on SDS-polyacrylamide gels and transferred to Hybond ECL membranes (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Membranes were blocked for 2 hrs in TBST (0.2 mM Tris-HCl (pH7.5), 150 mM NaCl, 0.1% Tween, 5% non fat dry milk), probed overnight with primary antibodies in TBST followed by a 2 hour incubation in the secondary antibody (peroxidase-conjugated goat anti-mouse or rabbit IgG) in TBST. The signals were visualized by using BM Chemiluminescence’s Western Blotting Kit according to the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN).
In vivo complex of topo I (ICT) bioassay.

ICT bioassay was performed as described by Subramanian (Subramanian et al, 1995; Subramanian et al, 2001). In brief, exponentially growing cells were incubated at 37°C with 2 μM of CPT for 30 minutes. At the end of incubation, sarkosyl was added to a final concentration of 1% to lyse the cells. Lysates were stored at -20°C or processed directly. Then the lysates were overlaid onto a four-layer CsCl density gradient containing followed by 24 hour ultracentrifugation in a Beckman SW41 rotor at 31,000 rpm at 20°C. Fractions were collected from the bottom of the gradients. The fractions containing DNA were identified and combined. DNA concentrations were determined by fluorometry. Titrations of DNA were applied to Hybond C nitrocellulose membrane (Amersham, Arlington, IL) using a slot blot vacuum manifold. The nitrocellulose membranes were blocked with 5% nonfat dried milk in TBST for 2 hours followed by an overnight incubation of anti-topo I antibody (1:1,000). After three TBST washes, the membranes were blotted with [125I]–labeled Protein A (ICN, Costa Mesa, CA) in TBST for 2 hours. The membranes were washed three times again and visualized/quantified with PhosphorImagin e. The DNA-topo I complex signals were normalized to the amount of loaded DNA.

Flow cytometry analysis.

Flow cytometric analysis of PI-stained cells was performed as described (Bonifacino JS, 1998). Briefly, cells were harvested and fixed in 70% ethanol, washed
with PBS, and suspended in propidium iodide (PI) solution containing 0.2 mg/ml RNase A and 20 μg/ml PI for at least 30 minutes at room temperature. DNA content of at least 15,000 cells was determined by a FACScan flow cytometry.

**DNA synthesis assay.**

Exponentially growing cells were prelabeled with 0.001 μCi/ml of [14C] thymidine for 16 hours at 37°C, and incubated with 100 nM of CPT with or without various concentrations of UCN-01 plus [methyl-3H] thymidine (final concentration of 1 μCi/ml) followed by another incubation for 7 hours. [3H] incorporation was stopped by washing cell twice with PBS. The cells were harvested and lysed in 2 ml of 0.1 mM NaOH. Then lysates were neutralized with 0.1 ml of 1 M HCl before 2 ml of 10% trichloroacetic acid (TCA) was added to precipitate DNA. The precipitates were collected on glass fiber filters and washed with 5% TCA and ethanol. The dried filters were subjected to dual label liquid scintillation counting and [3H] values were normalized by [14C] radioactivity.

**RESULTS**

1. **Myeloma cell lines displayed various CPT sensitivities.**

To assess the sensitivity of myeloma cells to CPT, six myeloma cell lines, SKO-007, NCI-H929, 8226/S, 8226/DOX40, MC\CAR and HS-Sultan, were exposed to various concentrations of CPT. Figure 27 shows that NCI-H929 exhibited the highest susceptibility and SKO-007 displayed the lowest one. Furthermore, the six tested cell lines could be roughly divided into two groups, three of them, SKO-007, 8226/S and...
Figure 27. CPT cytotoxicity on the multiple myeloma cell lines.

Six myeloma cell lines were treated with various concentrations of CPT for 72 hrs. Viability was determined using MTT kit from Promega as described in Materials and Methods. Each concentration point was performed in triplicate and data were presented as means. The values from the samples without CPT were regarded as 100%.
Figure 27

Survival percentage (%) vs. CPT conc. (µM)

- SKD-007
- NO-H929
- HSSultan
- 8226/Dox40
- 8226/S
- Mec/CAR

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8226/DOX, were more resistant to CPT than the other three, NCI-H929, HS-Sultan and McCAR. These data indicate that there are differences in CPT sensitivity among the myeloma cell lines.

2. Topoisomerase I expression and CPT-induced DNA-topo I cleavable complex represent the final outcome of cell killing in some MM cell lines.

Since topoisomerase I (topo I) is the target of CPT, we examined topo I levels in each cell line to determine if the topo I level was responsible for the difference in cytotoxicity. Figure 28 reveals that the topo I levels among the six cell lines were different. NCI-H929 had a much higher level of topo I compared to SKO-007, which was consistent with the CPT sensitivities of these two cell lines shown in Figure 27. The other four cell lines, however, did not show such consistency. In other words, despite the significant difference in CPT sensitivity, these four cell lines displayed only slight variations in topo I protein level.

CPT-induced DNA damage is not only determined by the level of topo I, but also by factors affecting the access of CPT to its target topo I, such as CPT metabolism, efflux, topo I localization, enzyme activity, and mutations affecting CPT binding. Therefore, it is reasonable to speculate that topo I level alone is not sufficient to determine the outcome of CPT cytotoxicity. On the other hand, since the level of CPT-induced DNA-topo I complex is the net result of all events of pre-target and CPT-topo I interaction, we considered the DNA-topo I complex level as a better measurement of DNA damage than topo I level; thus, it should have a better chance to serve as a parameter to predict the clinical effect of CPT and its derivatives. The in vivo complex of topoisomerase
Figure 28. Western blot analysis of topo I expression in six MM cell lines.

A total of 50 μg cell crude extract from each cell line was loaded onto 8% SDS-polyacrylamide gel. Topo I protein was detected with an anti-topo I antibody at 1:1,000 dilution.
Figure 28

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Cells treated with topo I poisons to stabilized topo I-DNA cleavable complexes are lysed with an ionic detergent, sarkosyl, which dissociates noncovalently bound topo I and DNA while trapping covalent complexes. The complexes are separated from free topo I by loading the lysates on cesium chloride gradients. Fractions are collected and DNA DNA profiles determined by fluorometry. The DNA-bound topo I is detected by immunoslot blots using an anti-topo I antibody. Only in the presence of a poison, such as CPT, is topo I detected in the DNA peak indicating the formation of topo I-DNA cleavable complex.
Figure. 29
Figure 30. Typical results of ICT bioassay from MM cell line SKO-007 and NCI-H929.

Cells (10^7) (SKO-007 and NCI-H929) were treated without (control) or with CPT (2 μM) for 30 minutes, lysed with 1% of sarkosyl and loaded onto a CsCl gradient. Fractions containing DNA from the gradients were pooled and DNA concentration was determined by Fluorometry. DNA fractions were loaded onto a nitrocellulose filter in a titration manner. DNA-topo I complex was detected by immunoslot blot with an anti-topo I antibody at 1:1,000 dilution.
Figure 30
Figure 31. ICT bioassay of CPT-induced DNA-topo I cleavable complexes in the MM cell lines.

Exponentially growing cells from all six lines were treated with 2 μM of CPT for 30 minutes. Cells were lysed with sarkosyl, and subjected to the ICT bioassay using an anti-topo I antibody described as in Figure 30. Signals from the slot blots were quantified using phosphorimaging and normalized to the amount of DNA. The signals from NCI-H929 were regarded as 100%.
Figure 31

MM cell lines

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(ICT) (Subramanian, 1995) technique was developed to measure CPT-induced DNA-topo I complex levels. The graphic presentation of ICT bioassay is showed in Figure 29. The DNA-topo I complex levels of all six MM cell lines were examined after treated with the same concentration of CPT (2 μM). Figure 30 shows the typical ICT bioassay results of NCI-H929 and SKO-007. The slot blot signals were quantified and presented in Figure 31. Consistent with their CPT sensitivity and topo I levels, NCI-H929 had the highest DNA-topo I complex level while SKO-007 had the lowest one. Furthermore, the DNA-topo I complex levels of 8226/S and 8226/DOX40 matched their sensitivities to CPT better than topo I levels. However, even though McCAR and HS-Sultan had lower DNA-topo I complex levels than 8226/S and 8226/DOX40, McCAR and HS-Sultan were more sensitive to CPT than 8226/S and 8226/DOX40. These data indicate that CPT-induced DNA-topo I complexes may represent the degree of DNA damage, but it is not sufficient to reflect the final outcome of cell killing among these four myeloma cell lines. Instead, the factors downstream of cleavable complex formation probably play more significant roles.

3. UCN-01 enhanced CPT-induced cytotoxicity in the CPT resistant myeloma cell lines

To overcome CPT resistance due to either the low topo I level or post-target mechanisms, different drugs were combined with CPT to treat the CPT resistant cell lines, SKO-007 and 8226/DOX40. Figure 32 and 33 show that CPT combined with 50 nM of UCN-01 greatly enhanced cytotoxicity in both SKO-007 and 8226/DOX40 compared with CPT alone, respectively. The pattern of enhancement, however, was
Figure 32. UCN-01 enhanced CPT-mediated cytotoxicity in 8226/DOX cells. 8226/DOX cells were treated with various concentrations of CPT as indicated with or without 50 nM UCN-01 for 72 hours. Viability was determined by MTT method described in Figure 27.
Figure 32

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Figure 33. UCN-01 enhances CPT-mediated cytotoxicity in SKO-007.

SKO-007 cells were treated with various concentrations of CPT as indicated with or without 50 nM UCN-01 for 72 hours. Viability was determined by MTT method described Figure 27.
Figure 33

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Figure 34. Effects of UCN-01 alone on SKO-007 and 8226/40.

SKO-007 and 8226/DOX40 were treated with different concentrations of UCN-01 as indicated for 72 hours. Viability was determined using MTT method as described in Figure 26.
Figure 34

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Figure 35. UCN-01 abrogated CPT-induced S-phase checkpoint in SKO-007 and 8226/DOX40

Cells (SKO-007 and 8226/DOX40) were treated with CPT and UCN-01 as indicated for 24 hrs. Then cells were harvested, fixed with 70% ethanol, stained with propidium iodide and assessed by flow cytometry. Percentage of cells in G1, S and G2/M phase was presented.
Figure 35

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Figure 36. UCN-01 abolished CPT-induced DNA synthesis inhibition in 8226/DOX40 cells.

8226/DOX cells were pre-labeled with \([^{14}\text{C}]\) thymidine, followed by the indicated drugs and \([^{3}\text{H}]\) thymidine (final concentration 1 µCi/ml). After 7 hours of incubation, cells were harvested and the ratio of \([^{3}\text{H}] : [^{14}\text{C}]\) was obtained by a liquid scintillation counting as described in “Materials and methods”.

1. DMSO, 2. 100 nM CPT, 3. 50 nM UCN-01, 4. 100 nM UCN-01, 5. 200 nM UCN-01, 6. 100 nM CPT + 50 nM UCN-01, 7. 100 nM CPT + 100 nM UCN-01, 8. 100 nM CPT + 200 nM UCN-01.
Figure 36

H-3 incorporation (%)

0 20 40 60 80 100 120 140
1 2 3 4 5 6 7 8
different between the two cell lines. UCN-01 (50 nM) alone indeed appeared to be toxic to SKO-007, but not to 8226/DOX40, indicating that SKO-007 is more sensitive to UCN-01 than 8226/DOX40, which is consistent with the results from the further experiment shown as Figure 34.

4. UCN-01 specifically abrogated the CPT-induced S-phase checkpoint as well as CPT-induced DNA synthesis inhibition.

To understand the mechanisms of UCN-01 enhancement, the effects of UCN-01 on the cell cycle distribution of CPT-treated SKO-007 and 8226/DOX 40 cells were investigated by using flow cytometry. Figure 35 shows that 100 nM of CPT induced mainly S-phase in both cell lines and fairly G2 arrest in 8226/Dox 40. However, UCN-01 only abrogated S-phase arrest, different from the previous report that UCN-01 abrogated both the S and G2 checkpoints (Shao RG, 1997). Especially in 8226/Dox40 cell line, abrogation of the S-phase checkpoint caused the cells to accumulate at G2 phase. In other words, UCN-01 was unable to abrogate the G2 checkpoint, at least in 8226/Dox40. This is different from the previous studies that UCN-01 inhibited Chk1 and abrogated the G2 arrest (14-16).

To further dissect the mechanism of UCN-01 effect, we investigated whether UCN-01 abolished DNA synthesis inhibition caused by CPT. Figure 36 shows that 0.1 μM CPT inhibited DNA synthesis by approximately 80% relative to the control, and such inhibition was partially reversed by the addition of UCN-01. Furthermore reverse levels were enhanced with the increase of UCN-01 concentration while UCN-01 alone does not
significantly affected DNA synthesis, suggesting UCN-01 releases the CPT-caused DNA synthesis inhibition in a concentration-dependent manner.

5. Release of CPT-induced DNA synthesis inhibition by UCN-01 resulted in an increase of DNA ds breaks in 8226/DOX40.

To understand the consequences abolishing CPT-induced DNA synthesis inhibition, we investigated the formation of DNA double strand breaks. It has been reported (Rogakou, 1998; Rappold, 2001) that a histone, H2AX, is quickly phosphorylated (γ-H2AX) following the production of DNA ds breaks; thereby, it can be used to represent the creation of DNA double strand breaks. By using the anti-γ-H2AX antibody, we examined the production of DNA double strand breaks after treating 8226/DOX40 with CPT alone or combined with UCN-01. Figure 37 shows that with an extended incubation period of UCN-01, the γ-H2AX levels elevated, indicating an increase of DNA double strand breaks.

To further test if the production of DNA ds breaks resulted from the failure of DNA synthesis inhibition, a DNA synthesis inhibitor, aphidicolin, was used. Figure 38 shows that aphidicolin blocked the production of the DNA ds breaks induced by both CPT alone or CPT plus UCN-01, suggesting that UCN-01 indeed abolished the DNA synthesis inhibition and as result, led to the production of DNA ds breaks.
Figure 37. UCN-01 enhanced CPT-induced DNA double strand breaks formation in 8226/DOX40.

Exponentially growing 8226/DOX 40 cells were treated with 100 nM CPT for 4 hours following the addition of 0.1% DMSO or 200 nM UCN-01. Cells were harvested at different time points as indicated. Histones were isolated by acid extraction and subjected to western blot using an anti-γ-H2AX antibody. A parallel gel was stained with Coomassie blue to demonstrate that all lanes were loaded with the same amount of histone protein (labeled as core histone). Position of -γ-H2AX is indicated on the right of the blot.
UCN-01 (200 nM) - - - 1 2 3 hrs
CPT (100 nM) - 5 6 7 4 4 4 hrs

Coommasie Cores histone

γ-H2AX

Figure 37

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Figure 38. DNA synthesis-dependent formation of DNA double strand breaks caused by combination of UCN-01 and CPT.

Exponentially growing 8226/DOX 40 were treated with CPT (1 μM), aphidicolin (10 μM), and UCN-01 (50 nM), as indicated for 2 hours. Cells were harvested and histones were isolated by acid extraction. Western blots were performed using an anti-γ-H2AX antibody as described in Figure 37.
Figure 38
DISCUSSION

Previous clinical trials demonstrated that the MM patients possessed high CPT resistance. In this study, two strategies are considered to circumvent CPT resistance. One is to find parameters to predict the chemotherapeutic outcomes of CPT and its derivatives in order to identify the responsive patients, so that the patients can be sorted into different chemotherapeutic programs. Thus, all the patients will receive appropriate treatments in the early stage and avoid unnecessary side effects. The second strategy is to find ways to directly overcome resistance.

Currently, the most acceptable theory for CPT cytotoxicity is that CPT stabilizes the intermediate of the topo I enzyme reaction (DNA-topo I cleavable complex) (Hsiang et al, 1985) and collision between the complexes and DNA replication machinery results in the production of DNA double strand breaks (Holm et al, 1989; D’Arpa et al, 1990). Given three categories of the events that affect the final cytotoxicity (Larsen, 1998), we considered the DNA-topo I cleavable complex level as a better parameter to predict the outcome of CPT cytotoxicity compared to the topo I protein level. We believe this based on 1) The DNA-topo I complex excludes the effect of pre-target factors. Ishikawa et al (Ishikawa et al, 1993) showed that 76% of myeloma cases had significant expression of GST-pi protein. In another study 72% of the evaluated samples were positive for p-170 glycoprotein and 82% for GST-pi (Petrini et al, 1995), suggesting pre-target factors may play a significant role in chemotherapy. Therefore, it is possible that a high level of topo I generate neither a high level of DNA-topo I complex nor a higher level of DNA damage;
2) the cleavable complex excludes the factors affecting drug-target interaction. Topo I mutants that lack CPT binding have been identified and proved to be one of the most important mechanisms of CPT resistance. In these cases, consistent with the CPT resistance, no DNA-topo I complex will be detected. Taken together, DNA-topo I complex levels will reveal a closer connection to cytotoxicity than topo I protein levels alone. On the other hand, there are limitations in using the DNA-topo I complex level as a prediagnosis parameter. For example, it cannot predict the factors concerning post-target events, such as processing of DNA-topo I complexes, repair of DNA damage as well as regulation of apoptosis that determines the tolerance of cells to DNA damage.

In this study, we identified a range of CPT sensitivities in six MM cell lines. Three of them (SKO-007, 8226/S and 8226/40) are apparently more resistant to CPT than the other three (HS-Sultan, Mc\CAR and NCI-H929) (Figure 26). After we investigated the cleavable complex levels upon addition of the same concentration of CPT, we found that only at the extreme ends, NCI-H929 with the highest cleavable complex level and SKO-007 with the lowest one, the cleavable complex levels display close relation with cytotoxicity. Furthermore, since 8226/DOX40 was obtained by culturing its parental cell line 8226/S in the presence of doxorubicin, it is reasonable to assume that 8226/S and 8226/DOX40 share similar genetic backgrounds. Under this circumstance it appears that cytotoxicity is a function of the cleavable complex levels. However, when comparing 8226/S and 8226/DOX40 with Mc\CAR and HS-Sultan, which presumably have different genetic backgrounds, the connection between cytotoxicity and cleavable complex levels cannot be clearly established. These data suggest that only when cleavable complex level
is extremely high or low or other factors downstream of the cleavable complex are quite similar, the cleavable complex level can be a key predictor to determine cytotoxicity outcome of CPT. Otherwise the post-target events may prevail in the final outcome of cell killing in the MM cells we tested. Thereby, DNA-topo I cleavable complex may be essential but not sufficient to predict the final cell killing outcome.

As for the factors attributing to cleavable complex formation, topo I seems to play a role in most of the cells we tested, such as NCI-H929, MCAR, HS-Sultan and SKO-007. However, it is not true for all the cell lines. 8226/S and 8226/DOX40 have similar amount or even less topo I than HS-Sultan and MC\CAR, but they both have more cleavable complexes than the latter ones. These results indicate that in 8226/S and 8226/40 cells CPT may either have greater access to topo I in these two cell lines is higher than in HS-Sultan and MC\CAR. In addition, 8226/DOX has higher levels of topo I but lower levels of cleavable complex than 8226/S. Since 8226/DOX40 has high levels of p-glycoprotein, it indicates that p-glycoprotein may pumps out CPT although not as efficiently as it pump out topo II drugs.

Drug resistance can be bypassed by finding approaches to predict the effect of CPT so that the patients can be grouped based upon unexpected responses. However, when the predicting parameters are not available, it becomes important to discover the alternatives to enhance the sensitivity of the resistant tumor cells. Since SKO-007 has very low levels of cleavable DNA-topo I complex and a high level of CPT resistance, it is highly likely that CPT resistance in SKO-007 is only due to the lack of enough DNA damage resulting from the low levels of cleavable complex. On the other hand, the mechanism of CPT
resistance in 8226/DOX 40 is more likely due to post-target events because: 1) this cell line has an intermediate amount of cleavable complex. 2) 8226/DOX40 was reported to express a relatively high level of Bcl-XL (Tu, 1998), which may confer 8226/40 with a higher threshold to apoptosis.

To date, the combination of more than one drug is a common practice to overcome drug resistance. In this study, we investigated the combinations of CPT with a series of drugs (data not shown) in an attempt to overcome CPT resistance in SKO-007 and 8226/DOX40. Our results indicate that UCN-01 has great potential to overcome drug resistance when combined with CPT in both SKO-007 and 8226/DOX40. Interestingly, the mechanism of UCN-01 is apparently different between the two cell lines. As shown in Figure 32, SKO-007 is even sensitive to UCN-01 alone. The combination of UCN-01 and CPT may reflect additive cytotoxicity. This data further demonstrate that CPT resistance in SKO-007 is due to inadequate DNA-topo I complex formation rather than the events downstream of the cleavable complex. In contrast to SKO-007, 8226/DOX40 is resistant to either UCN-01 or CPT alone. However the combination of the two drugs significantly enhances cytotoxicity.

UCN-01 was reported to abrogate the G2 checkpoint by inhibiting Chk1 (Busby et al, 2000; Graves et al, 2000). This was considered to be the mechanism by which UCN-01 enhances the cytotoxicity of several treatments, such as mitomycin C, cisplatin and radiation. Recently Feijoo et al. (Feijoo et al, 2001) also reported that UCN-01 abrogates the hydroxyurea-induced intra-S phase checkpoint by inhibiting Chk1. In the present study, our results indicate that in 8226/DOX4, UCN-01 specifically abrogates S phase
checkpoint but not G2 checkpoint. Nevertheless, UCN-01 enhances CPT-induced cytotoxicity. These data suggest that even though the G2 checkpoint is important, abrogation of S-phase checkpoint is the key factor enhancing CPT-induced cytotoxicity. We think that the CPT-induced ds breaks can activate the S-phase checkpoint and cause replication to slow or stall, therefore, preventing the further collisions between cleavable complexes and DNA replication machinery. As a result, it allows cells to keep ds breaks to a minimum level. UCN-01 inactivates the signal transduction pathways that arrest cells at S-phase and stop the DNA synthesis, thereby leading to the production of more ds breaks.

It is reported that 8226/DOX40 cells have multiple resistance mechanisms. One is that 8226/DOX40 cells have overexpressed p-glycoprotein that effluxes drugs like topo inhibitors. This mechanism keeps the drug concentration at a very low level and thereby, protects the cells from the cytotoxicity of those inhibitors. Another is that 8226/DOX40 cells have a higher level of Bcl-X_L leading to an elevated threshold for apoptosis. As a result, they are resistant to different anti-tumor drugs. Nonetheless, a non-toxic concentration of UCN-01 can potentiate the cells to a similar extent as the sensitive cell lines. Thereby, UCN-01 has a promising potential in CPT derivative therapy. The possible application of the combination of a topo I inhibitor and UCN-01 is being considered for evaluation in MM patients.
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