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Abrogating the p53 Response:

Implications For Tumor Promotion and Drug Resistance

by

Tarek A. Abbas

A dissertation submitted to Graduate Faculty in Biology in Partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2004

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Abstract

Abrogating the p53 Response:

Implications For Tumor Promotion and Drug Resistance

by

Tarek A. Abbas

Advisor: Professor Jill Bargonetti, Ph.D.

The p53 tumor suppressor protein plays a central role in cell cycle regulation and tumor suppression by inducing growth arrest and/or apoptosis. While studies investigating the various aspects of p53 regulation are plentiful, little is known about the regulation of p53 during the course of tumor development. In this study, we investigate signaling to and from the tumor suppressor p53 protein in the context of mitogenic signaling, tumor promotion and the cytotoxic response to the chemotherapeutic agent mitomycin C (MC). We show that the well-defined tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) prevents DNA damage-induced p53 up-regulation by a mechanism involving the down-regulation of protein kinase C delta (PKCδ). We provide evidence that TPA treatment of cells, or inhibition of PKCδ inhibits the basal transcription from the *p53* gene, maintains low p53 mRNA levels in cells with damaged DNA, and blocks p53-dependent apoptosis. Our data provides a mechanistic understanding of tumor promoting activity of the phorbol ester TPA, and suggests that transcriptional regulation of *p53* may be a critical determinant of its tumor suppresser functions.

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Many chemotherapeutic regimes aim at activating the p53 apoptotic pathway. Mitomycin C (MC), a natural antibiotic and a DNA-alkylating and cross-linking agent, has a cytotoxic chemotherapeutic activity and is known to activate p53. We provide data showing that the various DNA-adducts formed upon treating cells with MC are differentially recognized by the DNA damage sensor p53. We show that there exists a clear correlation between the ability of the various MC-DNA adducts to induce p53 accumulation and their cytotoxic activities. In particular, we show that the DNA-mono-adducts formed upon treating cells with the major intracellular MC metabolite, 2,7-diaminomitosine; 2, 7-DAM, are incapable of signaling through p53, and are therefore non-cytotoxic. Our data suggests that the DNA cross-linking activity of MC is required for its ability to signal to p53 and is responsible for its apparent cytotoxicity. Finally, we show that the MC analog and DNA cross-linking agent 10-decarbamoyl-MC (DMC), similar to MC is capable of signaling through p53 and is cytotoxic, but unlike MC is also capable of inducing p53-independent apoptosis.

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Acknowledgments, and Dedication

To

My wife, Margarita, and my two daughters, Nadia, and Dalia,

whose sacrifice and love has made it possible to complete this work

And to

those who taught, inspired, assisted, and guided me through out my study

And to

My Mentor, Jill Bargonetti,

Who instilled in me a love for science and an enthusiasm towards problems solving

And to

My Parents,

Who were invaluable source of strength and inspiration during the most difficult hours,

and whose prayers were always with me

And to

all the support from friends, colleges, extended family, and the grace of God that has

seen me through very difficult times

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Chapter 1

Introduction

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The p53 tumor suppressor protein

The p53 tumor suppressor protein plays a central role in mediating stress and DNA damage-induced growth arrest and/ or apoptosis. The loss of control over genomic stability is central in the development of cancer, and p53 by virtue of its ability to delicately respond to the various environmental insults and launch the appropriate protective responses, is a key element in maintaining genomic stability (Agarwal *et al.*, 1998). Thus functional inactivation of p53 by mutation and deletion, protein degradation or viral oncogene binding renders mammalian cells susceptible to oncogenic stimuli and environmental insults that promote growth deregulation and malignant transformation. In fact, the tumor suppressor *p53* is the most frequently inactivated gene in human malignancy analyzed to date (Nigro *et al.*, 1989).

The p53 protein is a phosphoprotein that functions as a transcription factor (Raycroft *et al.*, 1990; Farmer *et al.*, 1992) by binding to p53 response elements with a consensus binding sites (5'-PuPuPuC(A/T)(T/A)GPyPyPy-3') identified in many genes (el-Deiry *et al.*, 1992). The p53 protein is functionally divided into four domains: an N-terminal trans-activation domain, a DNA-binding domain, a tetramerization domain and a carboxyl-terminal regulatory domain (Figure 1.1). Genes under the transcriptional regulation of p53 include (but are not limited to), *p21/Waf1* (el-Deiry *et al.*, 1995), *mdm2* (Barak *et al.*, 1993), growth arrest and DNA damage (*GADD45*) (Kastan *et al., 1992*), *Bax1* (Miyashita and Reed, 1995), and *Cyclin G* gene (Okamoto and Beach, 1994). The p53 target gene *p21/Waf1* encodes a 21 kD protein that is of a particular interest due to its

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function as a cyclin-dependent kinase inhibitor that blocks progression through the G1/S phase of the cell cycle by inhibiting the phosphorylation of the retinoblastoma Rb protein.

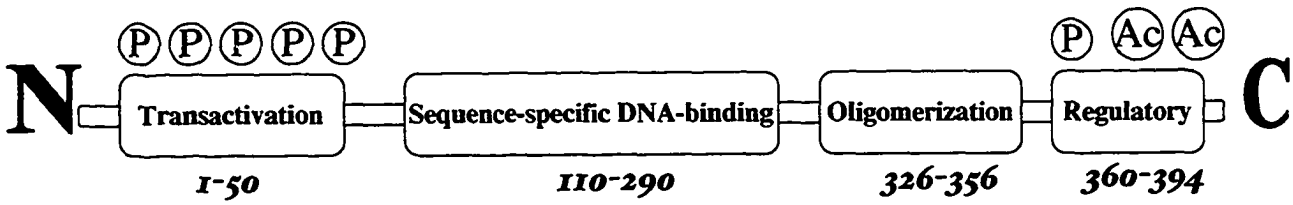

Figure 1.1 Schematic representation of the p53 protein domain structure.

Post-translational regulation of the p53 tumor suppressor protein

Because of the ability of p53 to control cell cycle checkpoints, it is not surprising that multiple mechanisms have evolved to regulate its activity and it became apparent that regulating the p53 function is highly complex and involves mechanisms regulating its stability, nuclear translocation, activation and turnover (Liang and Clarke, 2001; Bargonetti, 2002). The basal level of p53 protein in normal cells is hardly detectable (with a short half-life about 5-20 min), but rapidly increases in response to different types of cellular stresses such as DNA damage (Fiscella *et al.*, 1993; Burma *et al.*, 1999; Kapoor *et al.*, 2000), hypoxia (Alarcon *et al.*, 1999), or nucleotide depletion (Linke *et al.*, 1996; Chernova *et al.*, 1998). The mechanism by which the p53 protein is stabilized in response to these stimuli is not clear, but posttranslational modification is thought to play a pivotal role. Mdm2, the product of the proto-oncogene *mdm2* (*hdm2* in human), and a target for p53 trans-activation, binds to the N-terminus of p53, thereby reducing the p53

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trans-activating function, and functioning as ubiquitin ligase (Honda and Yasuda, 2000), targets p53 for ubiquitination and subsequent proteolytic degradation (Freedman and Levine, 1998; Roth *et al.*, 1998), thereby negatively regulating its own inducer (Figure 1.2). The significance of this auto-regulatory loop between p53 and Mdm2 is best manifested by the finding that targeted disruption of the *p53 gene* was able to rescue the early embryonic lethality caused by the homozygous deletion of *mdm2* (de Oca Luna *et al.*, 1995). In addition to targeting p53 for degradation, Mdm2 also forms a complex with p53, near the amino-terminal portion of both proteins, thereby blocking the interaction of p53 with the transcriptional machinery and resulting in abrogation of the ability of p53 to trans-activate its specific downstream target genes (Oliner *et al.*, 1993; Chin *et al.*, 1997). Moreover, the complex between p53 and Mdm2 can function directly as a potent transcriptional repressor (Thut *et al.*, 1997).

In response to stress or DNA damage, the p53 amino terminus is phosphorylated at multiple serine and threonine residues (Bargonetti, 2002). Phosphorylation of p53 at the amino terminus disrupts its interaction with Mdm2 protein, thereby leading to p53 stabilization and accumulation (Shieh *et al.*, 1999; Chehab *et al.*, 2000; Hirao *et al.*, 2000; Sakaguchi *et al.*, 2000; Shieh *et al.*, 2000). In response to DNA damage for example, serine 15 in the p53 N-terminus is phosphorylated by the product of the *ATM* gene mutated in patients with the genetic disorder ataxia-telangiectasia (A-T). Other members of the phosphatidylinositol-3-kinase (PI3k) super-family have been shown to phosphorylate ser-15 *in vitro* (Banin *et al.*, 1998; Canman *et al.*, 1998; Lakin and Jackson, 1999). Phosphorylation of ser-15 similar to phosphorylation at thr-18 and ser-20

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in the stress response, reduces binding of Mdm2, stimulates p53-dependent trans-activation, and increases its interactions with factors involved in general transcription (Lu and Levine, 1995; Thut *et al.*, 1995; Avantaggiati *et al.*, 1997; Gu and Roeder, 1997; Lill *et al.*, 1997; Lambert *et al.*, 1998; Dumaz and Meek, 1999).

Deregulated or aberrant growth signals can also stabilize and activate p53. In many cases, including c-myc, ras, E2F1, and adenovirus E1A, stabilization of p53 is accomplished via activation of the tumor suppressor protein ARF (Bates *et al.*, 1998; de Stanchina *et al.*, 1998; Palmero *et al.*, 1998; Zindy *et al.*, 1998). ARF, the product of an alternative transcript of the *INK4a* tumor suppressor locus (Sharpless and DePinho, 1999; Sherr and Weber, 2000), binds to the Mdm2 protein, and sequesters Mdm2 in the nucleolus, thereby neutralizing the inhibitory effect of Mdm2 on p53. In addition, the binding of ARF to Mdm2 directly inhibits the E3 ubiquitin ligase activity of Mdm2 (Honda and Yasuda, 1999; Tao and Levine, 1999; Weber *et al.*, 1999; Lohrum *et al.*, 2000). Stabilization of p53 via the ARF pathway is thought to work as a default safe mechanism to protect against the deleterious consequences of inappropriate oncogenic or mitogenic activation (Sherr, 1998). Oncogenic Ras for example, suppresses epithelial cell transformation by activating the ARF-p53 pathway (Lin and Lowe, 2001). The ARF tumor suppressor functions however, may extend beyond its ability to stabilize p53 (Carnero *et al.*, 2000; Esteller *et al.*, 2000).

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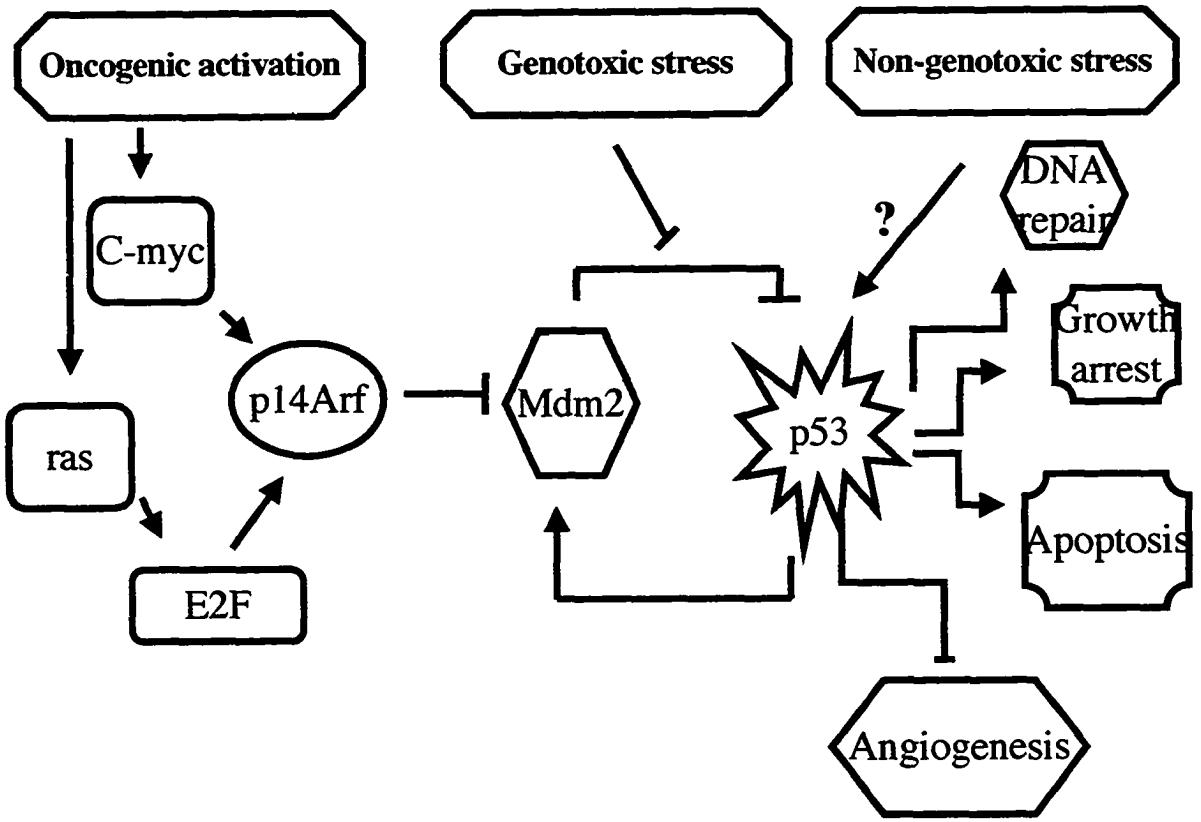

Figure 1.2 Regulation of the p53 tumor suppressor protein

The p53 protein is a sensor of multiple stresses. Activation of the p53 tumor suppressor pathway in response to oncogenic activation such as c-Myc, Ras, and E2F involves activation of the tumor suppresser p14Arf, and the inhibition of the inhibitory effect of Mdm2 on p53. The activation of the p53 pathway in response to genotoxic stress involves posttranslational modifications of p53 and/or Mdm2 in such a way that Mdm2 can no longer interacts with p53, thereby stabilizing the p53, and ultimately the activation of downstream target genes involved in DNA repair, growth arrest, and apoptosis among others processes. Other non-oncogenic non-genotoxic stresses such as hypoxia, nucleotide depletion are also capable of signaling via the p53 pathway, albeit the mechanism by which p53 is stabilized under these conditions is less clearly understood.

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The carboxyl-terminal domain of p53 (CTD) (Figure 1.1) regulates its ability to bind to DNA, and deletion of this domain activates DNA binding constitutively (Cox *et al.*, 1995). Furthermore, binding of the monoclonal antibody Pab421 to this domain stimulates p53 sequence specific DNA binding and triggers the transcriptional activity of p53 *in vivo* (Cox *et al.*, 1995). The mechanism by which the p53 CTD mediates inhibition of p53 sequence specific DNA binding however, is poorly understood. In addition to its role in regulating DNA binding activity, the p53 CTD also contributes to the protein stability because deletion of as few as 16 C-terminal residues dramatically inhibits Mdm2-mediated turnover (Kubbutat *et al.*, 1998). *In vitro* studies have demonstrated that several kinases can phosphorylate specific residues within the p53 CTD (Ko and Prives, 1996). These kinases include protein kinase C (Baudier *et al.*, 1992; Takenaka *et al.*, 1995), cdc2 (Bischoff *et al.*, 1990), casein kinase II (Hall *et al.*, 1996), and the CDK7-cycH-p36 complex (CAK) (Lu *et al.*, 1997). The physiological significance of p53 phosphorylation by these kinases however, remains unclear. In one study, protein kinase C has been shown to negatively regulate p53 stability in unstressed cells by enhancing p53 ubiquitination and subsequent degradation (Chernov *et al.*, 2001), suggesting that PKC may be required for low steady state p53 protein level. Inhibiting PKC activity by H7 was shown to increase p53 protein level without affecting p53-Mdm2 interaction, suggesting a negative regulatory role for PKC on p53 that is independent of Mdm2 (Chernov *et al.*, 2001). It is possible however, that PKC activity was required for the ability of Mdm2 to ubiquitinate or target p53 for degradation.

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Translational and transcriptional regulation of p53

Although posttranslational modification clearly plays a role in regulating p53 function in response to various stimuli, it is less clear how p53 levels are regulated in normal cells in the absence of stress stimuli. Ubiquitination and subsequent proteolytic degradation of p53 negatively regulate p53 protein level under unstressed conditions, but positive regulation of the p53 protein and mRNA is poorly understood. The p53 mRNA contains two repressible elements in its 5' (Mokdad-Gargouri *et al.*), and 3' UTR (Fu *et al.*, 1999), suggesting that p53 can be regulated translationally. In fact, γ irradiation was shown to elevate p53 levels at least partially, due to enhanced protein translation (Fu *et al.*, 1999), implying that under normal conditions the p53 mRNA is kept under translational repression that may contribute to the low steady state levels of p53 protein. Translational regulation of the p53 protein however, remains largely unclear, and its significance to p53 normal function as a tumor suppressor has yet to be determined.

Whereas control of p53 stability, degradation, and posttranslational modification have been extensively studied, little is known about transcriptional regulation of the *p53* gene. Understanding transcriptional regulation of the *p53* gene is of utmost importance since some human tumors with wild type *p53* genes do not express detectable levels of p53 and in many instances this has been attributed to low levels of p53 mRNA (Prokocimer *et al.*, 1986; Stuart *et al.*, 1995). More recently, the p53 mRNA levels were shown to be low in a large proportion of breast tumors and this has been correlated with decreased expression of the HOXA5 mRNA (Raman *et al.*, 2000). Moreover, the level of p53 mRNA changes dramatically during the cell cycle. It was long demonstrated for

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example, that p53 mRNA is induced prior to DNA synthesis upon mitogenic stimulation of resting murine (Milner and Milner, 1981), and human (Reed *et al.*, 1986) lymphocytes, or serum stimulation of resting fibroblasts (Reich and Levine, 1984; Braithwaite *et al.*, 1990; Ginsberg *et al.*, 1990; Mosner *et al.*, 1995). In this regard, it was recently reported that the basal level of p53 is regulated at the transcriptional level by a mechanism involving the Ras-MAP kinase pathway (Agarwal *et al.*, 2001), commonly activated by serum and other growth stimulatory or mitogenic signals. Agarwal et al., showed that when introduced into REF52 cells, the activated *ras* allele caused 5-8 fold increase in the basal p53 mRNA level, and that interfering with the Map-kinase pathway by the MEK inhibitor U0216, dramatically reduced the basal level of p53 protein without affecting the protein's ability to accumulate in response to various stimuli (Agarwal *et al.*, 2001).

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Protein Kinase C and Phorbol esters

The protein kinase C (PKC) family of serine/threonine kinases is involved in many cellular processes including metabolism, migration, differentiation, proliferation, tumor suppression and apoptosis. At least 11 PKC isoforms have been described and these include conventional cPKCs (isoforms α, β, and γ), novel nPKCs (isoforms δ, ε, η, θ, and μ) and atypical aPKC (isoforms λ, and ζ) (Hofmann, 1997). Conventional and novel PKCs are lipid dependent and utilize diacyl-glycerol (DAG) as a cofactor, but only cPKCs bind calcium (Figure 1.3).

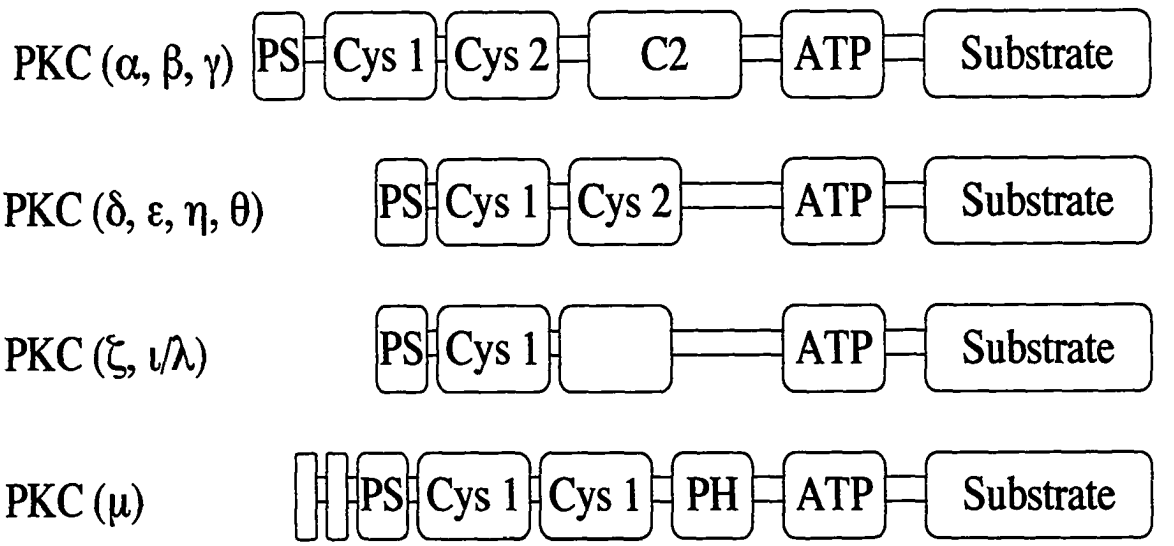

Figure 1.3 Schematic representation of the structural domains of the various PKC isoforms

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Increasing evidence from *in vitro* and *in vivo* studies points to PKC as a key regulator of critical cell cycle transitions (Castellot *et al.*, 1989; Tsuji *et al.*, 1991; Faria and Armelin, 1996; Ashton *et al.*, 1999). The significance of PKC as a critical player in cell cycle regulation came from the discovery that it is the major cellular receptor for the tumor promoting phorbol esters, which can substitute for DAG in activating the enzyme (Castagna *et al.*, 1982), thereby directly linking PKC signaling to mitogenesis and tumor promotion. Accumulating evidence suggests that each PKC isoform has a distinct role in the regulation of cellular proliferation. PKCα for example inhibits proliferation of melanoma cells (Gruber *et al.*, 1992), whereas PKCε enhances growth and proliferation of NIH3T3 fibroblasts (Mischak *et al.*, 1993). Moreover, PKCδ inhibits growth and proliferation of NIH3T3 fibroblasts (Mischak *et al.*, 1993), and induces differentiation of a variety of other cell lines (Han *et al.*, 1995; Fukumoto *et al.*, 1997; Perletti *et al.*, 1999). Little is known however, about the mechanism by which the various PKC isoforms suppress or enhance cellular growth and proliferation. A key to understanding these diverse cellular responses may be that individual PKC isoforms play specific and specialized or, perhaps even opposing roles in cell signaling. Further more, the distinct characteristics of the various PKCs suggest that the qualitative and quantitative composition of PKC isoforms within a cell type should greatly affect cellular responses to PKC agonists such as the tumor promoter phorbol esters.

Phorbol esters are the best-studied class of tumor promoters, and TPA (12-*O*-tetradecanoylphorbol-13-acetate) has been used extensively to investigate the role of PKC in cell signaling. Although phorbol esters and other PKC agonists activate PKC,

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they also down-regulate the enzyme following activation (Bindels *et al.*, 1993; Chen, 1993). Thus, it is unclear whether activation or depletion of PKC is important for the tumor-promoting activities of phorbol esters. Recently, at least some of the tumor-promoting effects of TPA have been attributed to depleting rat fibroblasts of the PKCδ enzyme (Lu *et al.*, 1997). By virtue of its ability to deplete PKCδ, TPA was able to transform rat fibroblasts over-expressing the tyrosin kinase c-Src, but not parental fibroblasts, suggesting that depleting PKCδ by itself is insufficient to derive cellular transformation, but requires additional mitogenic signals not provided by treating rat fibroblasts with TPA alone (Lu *et al.*, 1997). The transforming effect of TPA on c-Src over-expressing rat fibroblasts was mimicked by rottlerin, a PKCδ specific inhibitor (Gschwendt *et al.*, 1994) as well as transient transfection with a dominant negative mutant of PKCδ (Lu *et al.*, 1997), and was reversed by co-treatment with bryostatin 1 which selectively prevent PKCδ down-regulation by TPA (Szallasi et al., 1994; Lu et al., 1997). How down-regulating PKCδ may contribute to the transforming ability of TPA remains unclear. TPA is also known to significantly enhance transformation of Balb3T3 cells transfected with activated *ras* oncogene (v-Ha-ras) (Sasaki *et al.*, 1988). It is therefore possible that the effect of TPA (by down-regulating PKCδ) in cooperating with oncogenic Ras or c-Src is similar to the cooperation between Ras and the SV40 T-antigen (which inactivates the tumor suppressing cell cycle regulatory proteins Rb and p53) in deriving transformation of primary rodent fibroblasts (Land *et al.*, 1983), both requiring an activated mitogenic pathway and a deregulated cell cycle checkpoint (Figure 1.4).

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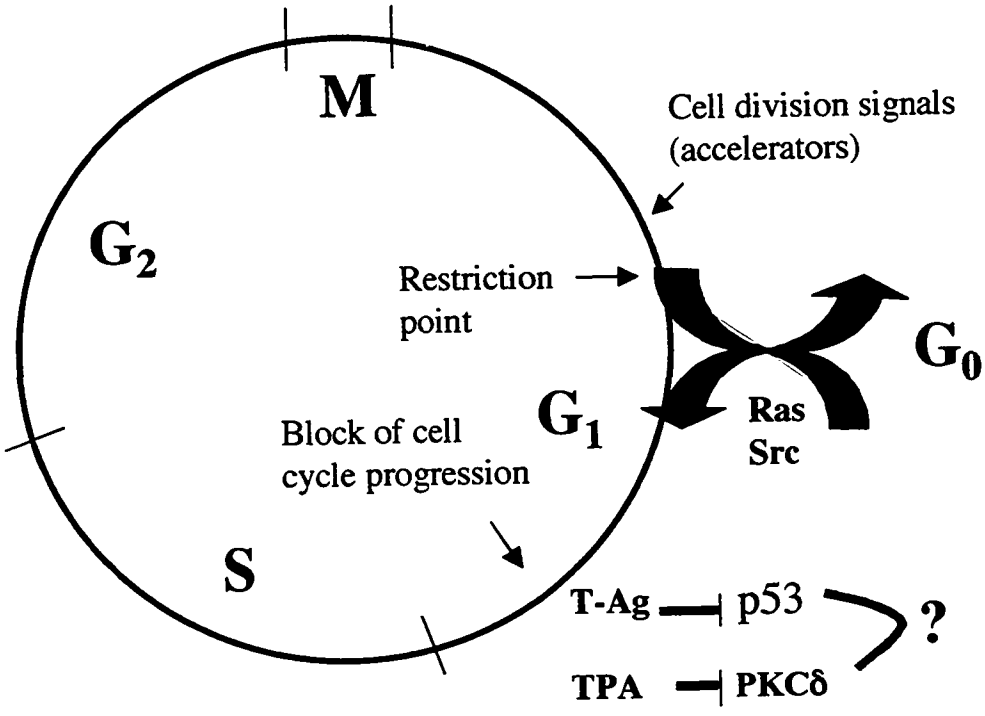

Figure 1.4 Regulation of the cell cycle by 53 and PKCδ

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Chapter 2

Materials and Methods

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Reagents:

Camptothecin, propidium iodide, the calpain inhibitor N-Acetyl-leu-leu-Norleu-al (LLnL/ALLN), MTT assay reagent, and anti-actin were purchased from Sigma. Etoposide, rottlerin, actinomycin D, Gö6976, bisindolylmaleimide II, and TPA were purchased from Calbiochem. Zeocin was purchased from Invitrogen. PSI was purchased from Peptides International. RPMI 1640 and fetal bovine serum (FBS), DMRIE-C and lipofectamine 2000 transfection reagents were purchased from Invitrogen. D. M. Vyas (Bristol-Myers Squibb Co.) supplied MC. 2,7-DAM and DMC were synthesized from MC as previously described (Kim and Rockwell, 1995; Kumar *et al.*, 1996). Trizol reagent was purchased from Gibco. Bryostatin1 was purchased from LC Laboratories. The *p53*-p1-promoter construct (Reisman *et al.*, 1993) was a generous gift from David Reisman. The dominant negative AKT-1 mutant (DN-AKT) and the constitutively active AKT-1 mutant (CA-AKT) constructs were purchased from upstate biotechnology (Cat # 21-152, & 21-151 respectively). The renilla luciferase expressing plasmid was purchased from Promega. The dominant negative mutant pGFP-PKCδ construct was a generous gift from Kufe D. The GFP- PKCδ (K-R) expressing plasmid is a kinase-negative mutant in which the lysine residue at position 378 in the putative ATP-binding site has been substituted with arginine by sit-directed mutagenesis and cloned into pEGFP-C1 (Clontech). The si-RNA constructs directed against PKCδ and GFP were purchased from Qiagen and duplexes were annealed according to manufacturers instructions.

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Cell Culture:

The ML-1 cells were a generous gift from Michael Kastan. The K562 erythro-myeloid leukemia cell line was purchased from the American Type Culture Collection (ATCC) and does not contain p53 (Law *et al.*, 1993). Both cell lines were grown in RPMI 1640 with 10% FBS and 5% CO_2. Both cell lines were seeded at a density of 2.5 x 10^5/ml and exponentially growing cells were used in all experiments. MCF-7 cells were obtained from the American Type Culture Collection (ATCC), and were grown in DMEM with 10% Calf serum, and sub-confluent cultures were used in all experiments. The H1299 and H460 cell lines were generous gift from Arnold J. Levine. Both cell lines were grown in RPMI 1640 with 10% FBS and 5% CO_2, and maintained by regular splitting. Sub-confluent cultures were used for all experiments, except when used for transfection with siRNA where 50% confluent cultures were used.

Flow Cytometry:

FACS analysis was carried out on a Beckton Dickinson FACS Scan. Cells were spun down at 2300 rpm for 7 minutes, washed twice with ice cold phosphate-buffered saline (PBS; 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4) and re-suspended in 20 ml of PBS containing 2% BSA and 0.1% NaN_3. Ethanol (9 ml) was then added drop wise while vortexing. Propidium iodide staining and RNAse treatment were carried out at 37 ^oC for 30 minutes 24 hours prior to flow cytometry.

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MTT cytotoxicity assay:

ML-1 or K562 cells were grown in 1X RPMI supplemented with 10% FBS. Exponentially growing cells were seeded at 2.5 X 10^5/ml in 24 well plates and either left untreated or treated with graded dosages of MC, DMC, or 2,7-DAM, for 24 hrs. Cells were spun down and re-suspended in 0.5 ml of MTT containing media (0.5 mg/ml), and incubated at 37 ^{o}C for one hour. Cells were spun down and re-suspended in 1 ml of 0.04 N HCl in isopropanol to lyse the cells. After 5 minutes incubation at room temperature, samples were spun down and 250 ul aliquots were used for absorbance measurements. Cell viability was measured as the difference in the absorbance at 550 and 620 nm. The assay detects metabolically active cells. Data are expressed as a percent of the control untreated growing strain.

Nuclear and cytoplasmic Extract Preparation:

Nuclear extract was prepared using a variation of the Dignam Protocol (Dignam *et al.*, 1983). Cells were spun down and re-suspended in 5 packed cell pellet volumes of buffer A, (10 mM HEPES pH. 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT). They were then incubated on ice for 10 minutes prior to centrifugation for 10 minutes at 2000 rpm. The pellet was re-suspended in 2 packed cell pellet volumes of buffer A (volume prior to the initial wash). The cells were run through a 25 gauge- needle twice and nuclei were then spun down at 2000 rpm for 10 minutes followed by an additional 20 minutes spin at 15,000 rpm. The pellet was re-suspended at 10^9 cells per 3 ml of buffer B

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(20 mM HEPES pH. 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) by running it through a 25 gauge- needle twice. The suspension was rocked gently for 30 minutes at 4 ^{o}C. The extract was centrifuged for 30 minutes at 15,000 rpm and the supernatant aliquots were stored at –80 ^{o}C.

Western Blot Analysis:

Protein samples were electrophoresed on a 10% SDS-PAGE and electro-transferred to nitrocellulose. Blots were probed with either a mixture of monoclonal antibodies specific to p53 (pAb1801, pAb240, and pAb421), a monoclonal anti-PARP antibody (Pharminogen), monoclonal anti-nPKCδ antibody (SC-937) (Santa Cruz), anti-p21 antibody (Cell Signaling), or anti-actin (Sigma). The blots were probed with anti-actin to normalize for gel loading. The signals were visualized after incubation with goat anti-mouse or goat anti-rabbit secondary antibody using ECL solutions.

Electrophoretic Mobility Shift Assays:

Synthetic oligonucleotides were purchased for this study from Operon. The super-consensus site (SCS) contained three adjacent p53 half sites (Halazonetis *et al.*, 1993). The sequence of this oligonucleotide was the following: 5'-TCG AGC CGG GCA TGT CCG GGC ATG TCC GGG CAT GTC-3'. Labeling of this oligonucleotide was performed with the large fragment of DNA polymerase I, and [α–^{32}P] dCTP. EMSA experiments (30 ul) were carried out in reaction mixtures with 150 pmols of [α–^{32}P]

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oligonucleotide. 10 ug of nuclear extract was added and the reaction was incubated for 20 minutes at room temperature in a reaction buffer containing 20 mM HEPES, pH 7.8, 100 mM KCl, 1 mM EDTA, pH 8.0, 1 mM DTT, 1 ug sheared salmon sperm DNA, and 10% glycerol. Samples were separated by 4% polyacrylamide gel electrophoresis (gels were pre-run at 100 V for 15 minutes at 4 ^{o}C) at 200 V for 3-3.5 hours. Gels were dried for 1 hour at 55 ^{o}C and autoradiography was performed. For gel shift experiments using the NFκB, and the AP1 (c-Jun) oligonucleotides, oligonucleotides were end-labeled according to the manufacturer (Promega) using [γ-^{32}p]-ATP. Their sequences are:

For NFκB: 5'-AGT TGA GGG GAC TTT CCC AGG C-3'

For AP1 (c-Jun); 5'-CGC TTG ATG AGT CAG CCG GAA-3'

Five ug of nuclear proteins were incubated in DNA-binding reaction according to manufacturer (Promega), and prepared and analyzed as above.

RNA Extraction and Northern Blot Analysis:

Total RNA was extracted using the Trizol reagent (Gibco) according to manufacturer. Total RNA (10 ug) was resolved on a denaturing formaldeyde gel, and electro-transferred overnight on a nylon membrane. The membrane was incubated with 10 ml of pre-hybridization buffer (Amersham Biosciences) for 1.5 hr and radio-labeled probes were added directly to the pre-hybridization buffer for 24 hrs. The membranes were then washed twice in 2X SSC buffer at 65 ^{o}C with shaking for 5 min each, followed by another wash with 1X SSC buffer at 65 ^{o}C with shaking for 15 min and twice in 0.1X

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SSC buffer at 65 °C with shaking for 5 min each. The signal was visualized by autoradiography.

Quantitative real-time PCR with Molecular Beacon and TaqMan probes:

Reverse transcription (RT): For each sample, 3 ug of cytoplasmic RNA obtained with Trizol (Gibco), was incubated at 65 °C for 10 minutes with 200 pmol oligo(dT)15 primer (Bohringer) in a total volume of 10 ul. After cooling on ice, 10 ul of RT mix was added: 2X AMV buffer, 12.5U AMV (Roche), 5 mM dNTP, 40U RNase inhibitor RNasin (Promega). Samples were incubated for 1 hour at 37 °C. Samples were heated at 95 °C for one minute to stop the reactions. Sample volumes were then adjusted to 100 ul with H_2O and were stored at –80 °C. PCR primer pairs were designed to anneal to their target at the same temperature (55 °C) and to amplify DNA fragments of approximately 100 bp as described previously (Xiao *et al.*, 2000). For PCR with molecular beacons, 10 ul of the diluted RT products were used. The reactions were carried out under the following conditions: 1X TaqMan Buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 250 uM dNTP, 15 pmol of each primers, 2.5 U AmpliTaq Gold polymerase (Perkin-Elmer) and 125 ng of the appropriate molecular beacon. Forty cycles of amplification (94 °C denaturation for 30 sec, 55 °C annealing for 1 min and 72 °C elongation for 30 sec) were carried out in sealed tubes in an Applied Biosystems 7700 Prism spectro-fluorometric thermal cycler (Perkin-Elmer). Fluorescence was measured during the annealing step and plotted

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automatically for each sample. The primer pairs used for the PCR reactions were synthesized by Operon and were the following:

P21, Forward primer: 5'-ACCTTCCAGCTCCTGTAACATACT-3'; Antisense primer: 5'-GTCTAGGTGGAGAAACGGGAA-3'.

Gadd45, Forward primer: 5'-CCATGCAGGAAGGAAAACTATG-3'; Antisense primer: 5'-CCCAAACTATGGCTGCACACT-3'.

GAPDH, Forward primer: 5'-AGAGCACAAGAGGAAGAGAGAGACC-3'; Antisense primer: 5'-AACTGTGAGGAGGGGAGATTCAG-3'.

The sequences of the molecular beacons were the following:

P21, 5'-CGCTGCAGGACACATGGGGAGCCGAGCAGCG-3'.

Gadd45, 5'CGCTGCAGAATGGTTGAGTTACATTAAAATAAACCGCAGCG-3'.

GAPDH, 5'-GGACGCGGTGGGGGACTGAGTGTGGCGTCC-3'.

For PCR with the assay on demand probes (human p53, p21, and GAPDH), one cycle of 50 ^{o}C UNG incubation for 2 min and 94 ^{o}C priming for 10 min, and forty cycles of 94 ^{o}C denaturation for 15 sec and 60 ^{o}C annealing for 1 min, were carried out in sealed tubes in an Applied Biosystems 5700 prism spectro-fluorometric thermal cycler (Perkin-Elmer). Fluorescence was measured during the annealing step and plotted automatically for each sample. The primer pairs (human GAPDH, p21, and p53) used for the PCR reactions were synthesized by Applied bioscience-assay on demand.

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Transient and stable transfection:

K562 cells were maintained at a concentration of 2.5 X 10^5 cells/ml. Cells were spun down for 5 min at 1850 rpm at RT. Cell pellet was re-suspended to the final concentration of 1 x 10^7 cells/ml. For electroporation, 0.5 ml of cells was then transferred into sterile electroporation cuvettes. Cuvettes were placed on ice, and DNA was added as indicated. After adding the DNA, cuvettes were shaken carefully so that the DNA was mixed with the cells and the cuvettes were then placed on ice for an additional 10 minutes. The Gene pulser (Bio-rad) was adjusted to the high capacitance mode and set at a voltage of 0.5 KV and capacitance to 900 uF. Cells were pulsed for 3 seconds and then placed on ice for 10 minutes before transferring the electroporated cells into the corresponding flask with 20 ml of RPMI supplemented with 10% FCS. The flasks were swirled to make sure all the cells are distributed evenly and incubated for 48 hrs. All samples were done in duplicates. For lipofectamine-mediated transfection: K562 cells were spun down at 2000 rpm for 5 min and re-suspended in fresh serum-free RPMI medium at a concentration of 1x10^7 cells/ml. 100 ul of cells (1x10^6 cells) were then plated in each well in 12 well plates that contains diluted DMRIE-C reagent (Invitrogen) and DNA according to the manufacturer. After 4 hours of incubation at 37 ^{o}C, 1ml of growth medium with 15% FBS is added to each well and incubated for 36-48 hrs. For transient transfection of the H1299 cell line, sub-confluent cultures were transfected with the various DNA constructs using the lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer. Stably transfected K562 cells were prepared in the same way as described above, except that cells were spun down 48h following transfection and re-suspended in 20 ml of RPMI medium supplemented with 15% FBS

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and 750 ug/ml G418 (Geneticin, Life Technologies). Two weeks after, the cultures were carefully diluted with fresh RPMI medium containing 15% FBS and 750 ug/ml G418 and plated in 96 well plates so that each well contains 1-5 cells and incubated at 37 ^{o}C and 5% CO2 for several weeks. Several single colonies were then transferred into a 25-ml tissue culture flasks with 10 ml of fresh RPMI supplemented with 10% FBS and 750 ug/ml G418. After about one week another 10 ml of medium was added to these cultures for another week and propagated afterwards by regular splitting. For transfection of siRNA constructs, 50% confluent H460 cultures were transfected with the various siRNA construct (si-GFP, si-PKCδ constructs -Qiagen) using the lipofectamine 2000 (Invitrogen) transfection reagent according to manufacturer. RNA and proteins are then extracted from transfected cells, as previously described 48-72 hrs post-transfection.

Luciferase Activity assay:

Transiently transfected K562 cells were spun down at 2000 rpm (4 ^{o}C) for 7min and the supernatant was poured off (transiently transfected H1299 cells were washed and harvested in 6-well culture plates directly). After a single wash with ice cold 1X PBS, 0.6 ml of 1X reporter lysis buffer (Promega) was added to each sample, and transferred to eppendorf tubes. Samples were rocked for 30 minutes at RT and vortexed for 30 sec. at maximum speed. The tubes were then micro-centrifuged for 2 minutes at maximum speed. Supernatants were transferred to clean eppendorf tubes and stored at –80 ^{o}C. Protein concentrations were determined and luciferase assays were carried out according to the manufacturers indications (Promega) using Luminoskan reader.

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Chapter 3

TPA inhibits initiation of transcription from the *p53* gene via down-regulation of protein kinase C delta (PKCδ)

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Introduction:

The p53 protein regulates normal responses to DNA damage and other forms of genotoxic stress, and is a key element in maintaining genomic stability (Vogelstein *et al.*, 2000). Mutations in the *p53* gene are frequently associated with the formation of human cancer, and mice engineered to have the *p53* gene knocked out develop tumors at an increased rate (Donehower *et al.*, 1992). In addition to mutations in the *p53* gene, p53 protein inactivation by viral onco-proteins, and functional inactivation of the p53 protein due to *hdm2* gene amplifications, the p53 pathway is also be abrogated by numerous oncogenic proteins (Oren *et al.*, 2002). The pivotal role p53 plays in tumor suppression suggests that agents inhibiting the p53 pathway can have a tumor promoting affect. Abrogation of the p53 protective responses may not only inhibit the ability of cells to respond to DNA damage by inducing growth arrest or apoptosis, but also render them more susceptible to further genetic mutations and genomic instability; the hallmark of cancer.

The phorbol ester tumor promoting agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is known to promote tumor formation in a variety of mice and tissue culture models, and this has been associated with its ability to down-regulate Protein Kinase C (Hansen *et al.*, 1990). Recently, it was demonstrated that some of the tumor promoting activities of TPA are attributed at least in part, to down-regulating PKCδ (Lu *et al.*, 1997; Lu *et al.*, 1998; Hornia *et al.*, 1999; Zhong *et al.*, 2002). Moreover, transgenic mice over-expressing PKCδ in their epidermis are resistant to tumor formation by TPA (Reddig *et*

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al., 1999). Increasing evidence suggests that PKCδ is a key player in DNA damage response pathways. In response to DNA damage, PKCδ is activated by tyrosine phosphorylation and is cleaved to yield a 40 kD constitutively active catalytic fragment (Ren *et al.*, 2002). Moreover, down-regulation of PKCδ expression by inhibitors and short-interfering RNAi (siRNA) has been associated with the attenuation of the DNA damage-induced response (Basu *et al.*, 2001; Yoshida *et al.*, 2002). Furthermore, a number of studies using protein kinase inhibitors have suggested that PKCδ may act as a regulator of the p53 pathway (Ghosh *et al.*, 1999; Heit *et al.*, 2001). More recently, it was shown that mice engineered to have their *PKCδ* gene knocked down show increased proliferation of B cells without increased spontaneous tumor formation (Mathis and King, 2002; Miyamoto *et al.*, 2002), suggesting a possible cell cycle inhibitory role for PKCδ rather than a typical tumor suppressor role. We have carefully examined signaling to and from the p53 protein in human cells treated with the tumor promoter phorbol ester TPA. Although very little attention is usually placed on the transcriptional regulation of *p53*, we present data demonstrating that this is a critical regulatory step in tumor suppression.

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Results:

TPA treatment inhibits DNA damage-induced apoptosis in human cells with wild type p53, but not in human cells lacking p53.

It has been demonstrated that TPA provides a PI3 kinase-independent survival signal that prevents apoptosis induced by the withdrawal of serum by virtue of its ability to down-regulate PKDδ protein (Zhong *et al.*, 2002). To investigate whether the survival signals generated by TPA involved p53, we examined the effect of TPA upon DNA-damage induced apoptosis in human cancer cell lines with wild type p53 (ML-1), or lacking p53 (K562). Treatment of the human myeloid leukemia ML-1 cells with the DNA-damaging agents camptothecin (CPT), mitomycin C (MC), and 10-decarbomyl mitomycin C (DMC) induced cleavage of the caspase 3-substrate poly-ADP ribose polymerase (PARP), commonly used as an indicator of apoptosis (Lazebnik *et al.*, 1994). Co-treatment of these cells with TPA and the various DNA damaging agents did not have any noticeable effect on apoptosis or p53 induction (data not shown). However, when the cells were pretreated with TPA for 21 hours prior to the addition of the various DNA damaging agents, PARP cleavage induced by the DNA damaging agents was almost completely inhibited (Figure 3.1 A). Similar results were obtained using flow cytometric analysis, where the CPT-induced appearance of cell populations with sub-genomic DNA was prevented by TPA pretreatment (Figure 3.1 B). Pretreatment of ML-1 cells with TPA not only inhibited apoptosis mediated by the various DNA damaging agents, but also induced a clear G1/S and G2/M cell cycle arrest (Figure 3.1 B, lower panel) and was accompanied by elevated levels of the p21 protein (data not shown).

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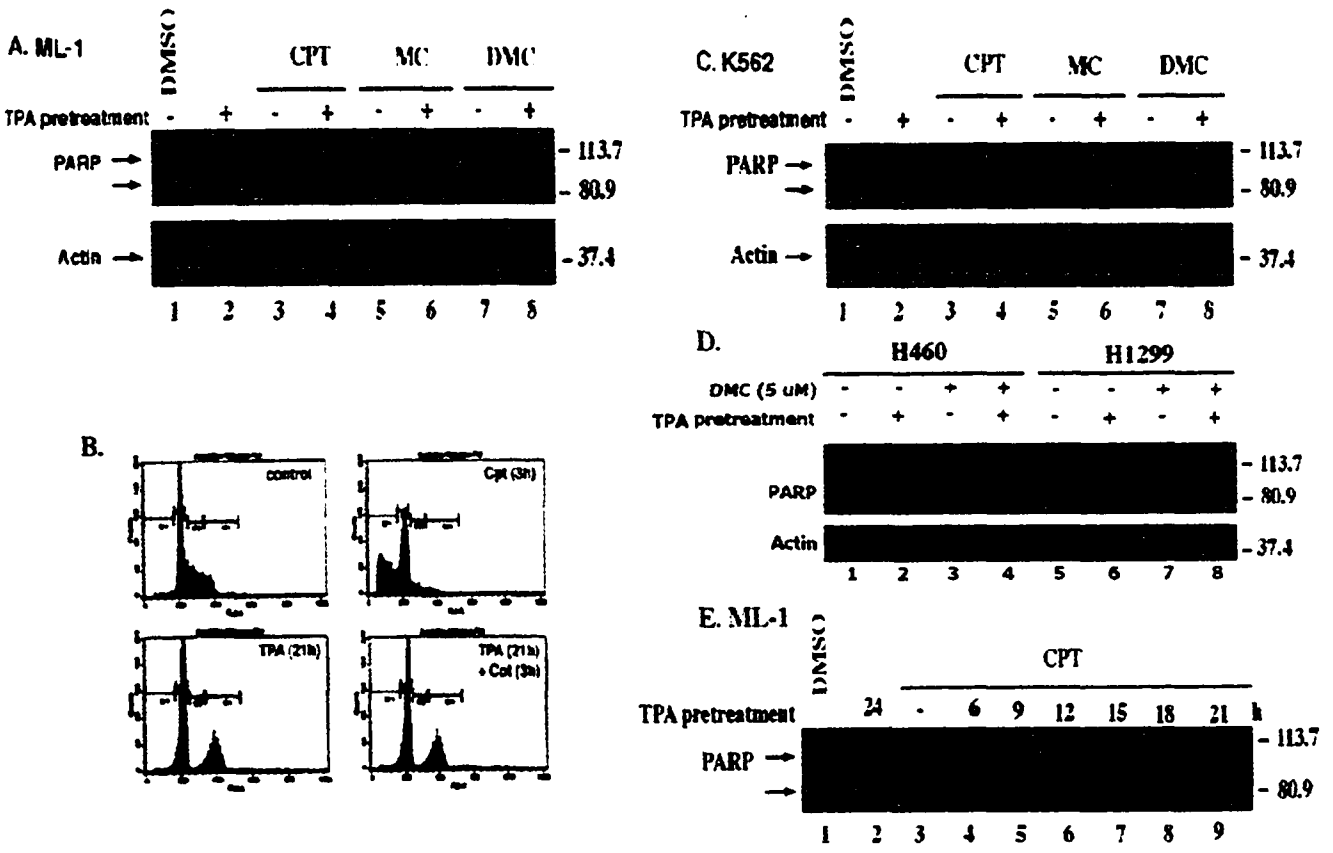

Figure 3.1 Long-term TPA treatment of cells specifically protects against p53-dependent apoptosis.

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Figure 3.1 Long-term TPA treatment of cells specifically protects against p53-dependent apoptosis.

A, B, & C, TPA inhibits DNA damage-induced apoptosis in ML-1 cells with wild type p53 (A, & B), but not in K562 cells lacking p53 (C). ***(A, & C),*** Exponentially growing cells, grown as described in materials and methods were either treated with CPT (0.5 uM), MC (5 uM), or DMC (5 uM), or treated with the solvent DMSO alone for 24 hours. Where indicated, cells were pretreated with TPA (10 nM) for 21 hrs prior to the addition of the various DNA damaging agents. 25 ug of nuclear proteins were resolved by electrophoresis on a 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the anti-human PARP antibody or anti-actin antibody to normalize for loading. ***(B),*** FACS analysis of ML-1 cells. ML-1 cells were grown as in A and were either left untreated, or treated with CPT (0.5 uM) for 3 hrs, or pretreated with 10 nM TPA for 21 hrs followed by CPT for another 3 hrs. Cells where then fixed with 30 % ethanol, stained with propidium iodide for 24 hrs and analyzed by flow cytometry cell sorting. ***D,*** DMC-mediated and *p53-independent apoptosis is not prevented by TPA pre-treatment in H460 (with wild type p53), or in H1299 (lacking p53).* Exponentially growing cells, grown as described in materials and methods were either left untreated or treated with DMC (5 uM) for 24 hrs. Where indicated, cells were pretreated with TPA (10 nM) for 21 hrs prior to the addition of DMC. Proteins extracted and analyzed as in A. ***E,*** *Time course of the ability of TPA to inhibit CPT-mediated, and p53-dependent apoptosis.* ML-1 cells were either left untreated (lane 1), or treated with TPA (10 nM) for 24 hrs (lane 2), CPT (5 uM) for 3 hrs (lane 3), or pretreated with TPA (10 nM) for various hours (lanes 4-9) prior to addition of CPT (5 uM) for 3 hrs. Proteins extracted and analyzed as in A.

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While CPT and MC require functional p53 in order to mediate significant apoptosis, the MC analogue 10-decarbamoyle MC (DMC) is able to induce robust apoptosis even in the absence of p53 (Abbas et al., 2002; also see chapter 5). If a similar experiment was performed with K562 cells, which lack p53, the DNA damaging agent DMC still induced significant PARP cleavage, however TPA pretreatment had no inhibitory effect upon PARP cleavage in these cells (Figure 3.1 C). Modest PARP cleavage was also induced by CPT and MC in the K562 cells (also see chapter 5), and this cleavage was similarly unaffected by TPA pretreatment (Figure 3.1 C). These data suggest that long-term TPA treatment inhibits apoptosis in cells with wild type p53, but not in cells lacking p53. To further show that TPA anti-apoptotic activity is limited to its ability to block p53-dependent, but not p53-independent apoptosis, two other cell lines (H460 with wild type p53, and H1299 without p53) were treated with DMC for 24 hours in the presence or absence of TPA pretreatment. DMC treatment induced substantial apoptosis in both of these cell lines, and TPA pretreatment had no effect on the ability of DMC to induce apoptosis (Figure 3.1 D), suggesting that the anti-apoptotic activity of TPA is limited to its ability to inhibit p53-dependent cell death. We next examined the kinetics with which TPA protects against CPT-induced and p53-dependent apoptosis in ML-1 cell, and as shown in figure 3.1 E, inhibition of CPT-induced PARP cleavage can begin to be detected between 9 and 12 hours.

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TPA inhibits the ability of DNA damaging agents to induce the stabilization of p53, and depletes cells of protein kinase C delta (PKCδ).

Data in figure 3.1 show that the phorbol ester tumor promoting agent TPA, blocks p53-dependent but not p53-independent apoptosis. To further our investigation of the influence of TPA on the p53 pathway, DNA damage-induced p53 accumulation was compared in the presence and absence of TPA. ML-1 cells were pretreated with TPA for increasing times and the influence on the induction of p53 by CPT was observed by Western blot analysis. Treatment of ML-1 cells with 0.5 uM CPT rapidly induced nuclear accumulation of the p53 protein (Figure 3.2 A, compare lanes 1 and 2). Pretreatment of ML-1 cells with 10 nM TPA for 6 hours or longer inhibited CPT-induced p53 accumulation in a time dependent manner (Figure 3.2 A, lanes 4-9). The kinetics with which TPA inhibited p53 protein stabilization correlated with its ability to inhibit DNA-damage induced apoptosis (Figure 3.1 E). TPA treatment of cells initially activates PKCδ , but over time depletes cellular PKCδ levels via the ubiquitin-proteosome pathway (Lu et al., 1997; Lu et al., 1998). As shown in figure 3.2 B, PKCδ is largely depleted by 9 hours of TPA treatment in the ML-1 cells. The kinetics of PKCδ depletion in response to TPA paralleled, or slightly preceded the disappearance of PARP cleavage (in the presence of CPT) observed in figure 3.1 E, consistent with the anti-apoptotic effects of TPA being due to the depletion of PKC δ. Also shown in figure 3.2 B, is the CPT-induced appearance of the 40 kD active catalytic fragment of PKCδ that is also associated with apoptosis (Ren et al., 2002), and similarly depleted by TPA pretreatment (Figure 3.2 B, lanes 3-5). Pretreatment of ML-1 cells with TPA for 21 hours also inhibited the DNA damage-mediated induction of p53 by MC, DMC and Zeocin (Figure

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3.2 C), and similarly down-regulated PKCδ under these conditions (Figure 3.2 D), suggesting that TPA was able to block different DNA damage sensor pathways signaling to p53. Similar results were obtained in the lung carcinoma cell line H460 (Figure 3.2 E, & F), suggesting that the effects of TPA on p53 or PKCδ are not cell or tissue-specific. The inhibition of nuclear accumulation of p53 in response to TPA was not due to decreased nuclear shuttling or enhanced cytoplasmic shuttling of p53 since cytoplasmic p53 protein was not detected under these conditions (data not shown). Collectively, data in figures 3.1 and 3.2 suggest that the ability of TPA to suppress DNA damage-induced apoptosis is due to suppression of p53 accumulation and that this effect correlates with the ability of TPA to down-regulate PKCδ.

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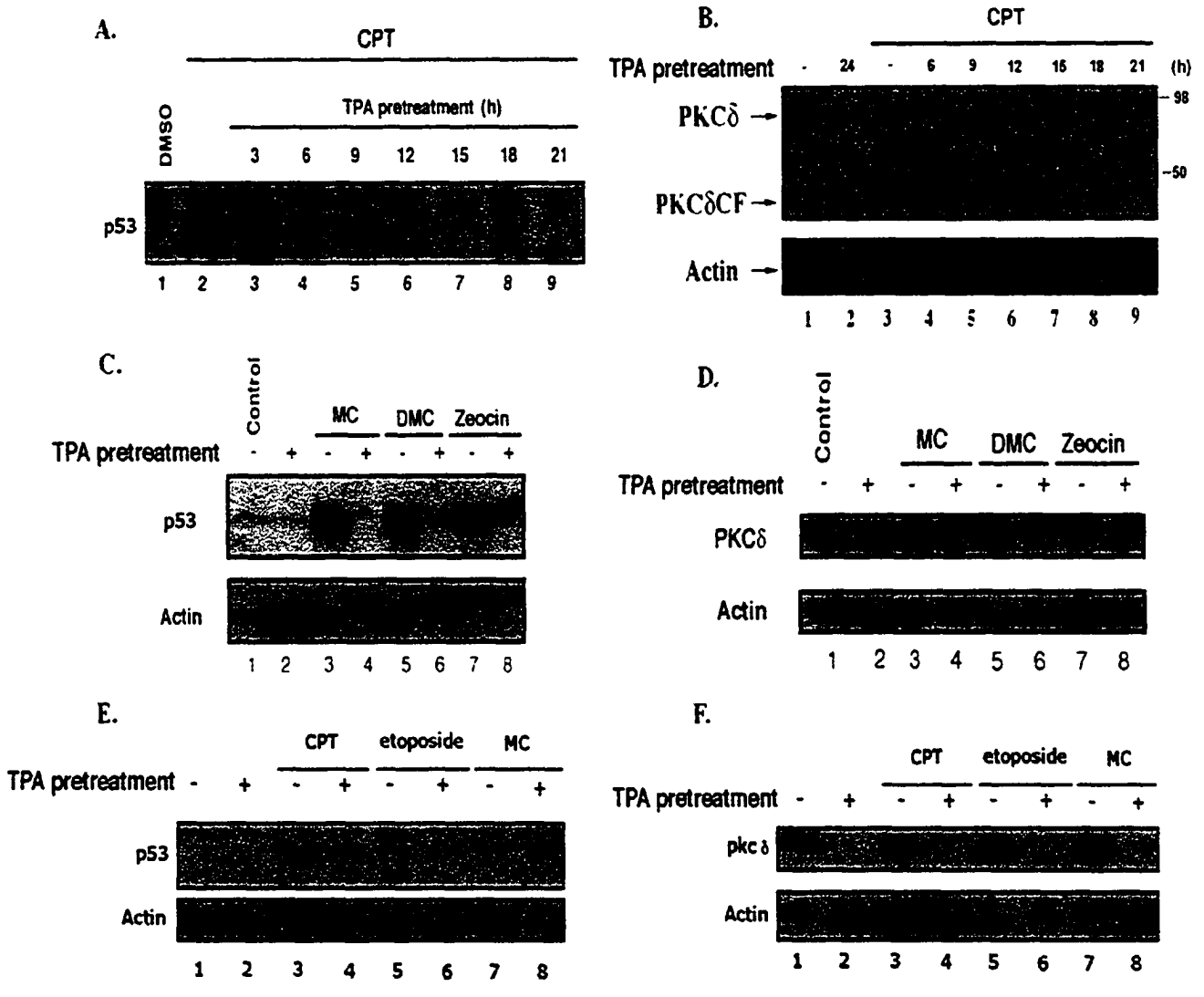

Figure 3.2 TPA inhibits p53 protein stabilization in response to a variety of DNA damaging agents, and depletes cells of PKCδ.

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Figure 3.2 TPA inhibits p53 protein stabilization in response to a variety of DNA damaging agents, and depletes cells of PKCδ.

A, TPA time dependently inhibits p53 stabilization in response to CPT. Western blot analysis of p53 nuclear proteins. ML-1 cells were either left untreated or treated with 10 nM TPA for various hours, followed by treatment with 0.5 uM CPT for 3 hours. 25 ug of nuclear protein was resolved by electrophoresis on a 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a mixture of p53-specific monoclonal antibodies (pAb240, pAb421, and pAb1801). ***B, TPA treatment of ML-1 cells time dependently depletes cytoplasmic PKCδ.*** Western blot analysis of ML-1 cytoplasmic protein extracts. Cytoplasmic extracts of ML-1 cells were resolved by electrophoresis on a 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-human PKCδ antibody or anti-actin. ***C, D, E & F, TPA inhibits p53 protein stabilization induced by various DNA damaging agents in ML-1 (C) and H460 (E)*** cells, *and depletes cells of cytoplasmic PKCδ (D, & F respectively).* ML-1 ***(C, & D)*** and H460 ***(E, & F)*** cells were either left untreated, or treated with MC (5 uM), DMC (5 uM), zeocin (50 ug/ml), CPT (0.5 uM) or etoposide (8 uM) for 4 hrs. Where indicated, cells were pretreated with TPA (10 nM) for 21 hrs prior to the addition of the DNA damaging agent. Nuclear proteins ***(C, & E)*** were analyzed as in figure 3.1 and probed with a mixture of p53-specific monoclonal antibodies (pAb240, pAb421, and pAb1801), or anti-actin. Cytoplasmic proteins ***(D, & F)*** were analyzed as in 3.2 B, and probed with anti-PKCδ or anti-actin antibodies.

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The inhibitory effects of TPA on p53 and the TPA anti-apoptotic activity are due to its ability to deplete cells of PKCδ.

Since the ability of TPA to inhibit p53 nuclear accumulation in response to the various DNA damaging agents correlated with depletion rather than activation of PKCδ, and since TPA is known to deplete other isoforms of protein kinase C (Bindels *et al.*, 1993; Chen, 1993), we needed to know whether the effects of TPA on p53 and apoptosis were in deed due to its ability to specifically down-regulate PKCδ, and were not due to other effects of TPA. To that end, we took advantage of bryostatin 1, which has been shown to specifically prevent the down-regulation of PKCδ by TPA (Szallasi et al., 1994; Lu et al., 1997). Li H., a member of Dr. Foster's laboratory examined the effect of TPA upon DNA damage-induced apoptosis in the MCF-7 human breast cancer cell line, which also has wild type p53 (Ramet et al., 1995). As shown in figure 3.3 A, CPT strongly induced PARP cleavage, which was completely inhibited by TPA pretreatment for 24 hours. Also shown in figure 3.3 A, the stabilization of p53 in response to CPT and that TPA pretreatment also inhibited p53 stabilization in this cell line as well. Importantly, the inhibitory effects of TPA on p53 as well as on apoptosis were rescued by bryostatin1 co-treatment (Figure 3.3 A), and were due to its ability to prevent PKCδ down-regulation by TPA (Figure 3.3 B). Thus, the effects of TPA on p53 stabilization and apoptosis induced by CPT in the MCF-7 cells are likely due to the down-regulation of PKCδ. Surprisingly, treatment of ML-1 cells with bryostatin 1 (with a concentration as high as 0.5 uM) could not prevent the down-regulation of PKCδ by TPA (Figure 3.3 C). In fact, treatment of ML-1 cells with bryostatin 1 alone strongly depleted cytoplasmic PKCδ

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(Figure 3.3 D), but consistent with our hypothesis, it strongly inhibited the DNA-damage induced p53 accumulation (Figure 3.3 C), and apoptosis (data not shown).

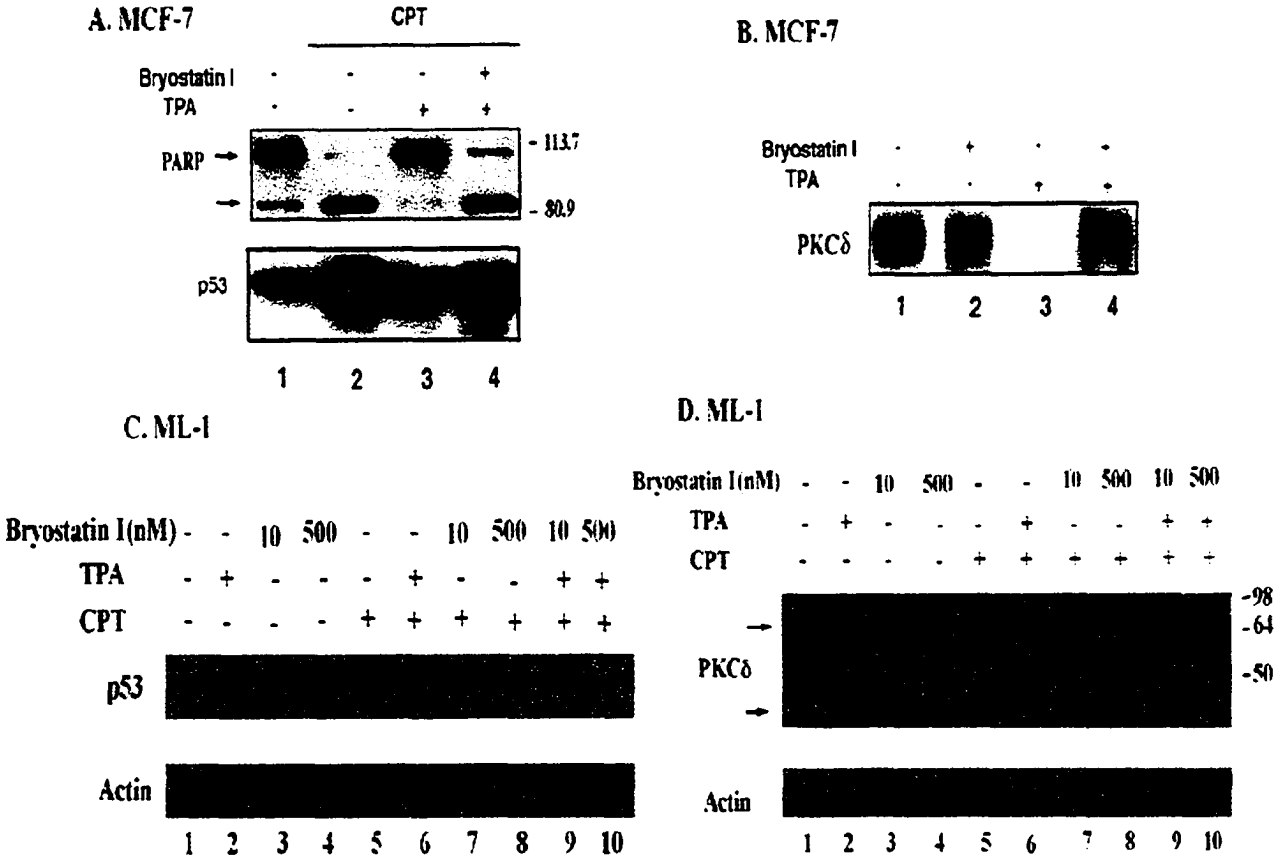

Figure 3.3 TPA's ability to inhibit p53 accumulation and p53-dependent apoptosis is due to its ability to specifically down-regulate PKCδ.

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Figure 3.3 TPA's ability to inhibit p53 accumulation and p53-dependent apoptosis is due to its ability to specifically down-regulate PKCδ.

A, & B. *Bryostatin 1 prevents the effects of TPA on p53 accumulation and apoptosis induction by CPT by preventing the TPA-induced down-regulation of PKCδ in the MCF-7 human breast cancer cells.* Western blot analysis of MCF-7 total protein extracts. MCF-7 cells were either left untreated or treated with CPT for 24 hours. Where indicated, cells were pretreated with TPA (400 nM) or TPA and bryostatin 1 (100 nM) prior to the addition of CPT. Protein extracts were prepared and analyzed as described in materials and methods, and membranes were probed with human anti-PARP antibody, a mixture of p53-specific monoclonal antibodies (pAb240, pAb421, and pAb1801) **(A)**, or human anti-PKCδ antibody **(B).** ***C, & D.*** *Bryostatin 1 depletes ML-1 cells of cytoplasmic PKCδ and inhibits the ability of CPT to induce accumulation of p53.* Western blot analysis of ML-1 nuclear **(C)** and cytoplasmic **(D)** protein extracts. ML-1 cells were either left untreated or treated with TPA (10 nM) for 24 hrs, bryostatin 1 (10 nM, or 0.5 uM) for 24 hrs, or CPT for 3 hrs. Where indicated, ML-1 cells were pretreated with TPA alone, bryostatin 1 alone, or both TPA and bryostatin 1 for 21 hrs prior to the addition of CPT for 3 hrs. Nuclear **(C)** and cytoplasmic **(D)** proteins were prepared and analyzed as described previously and membranes were probed with either a mixture of p53-specific monoclonal antibodies (pAb240, pAb421, and pAb1801) **(C),** or human anti-PKCδ antibody **(D).** Actin was used as a loading control.

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To directly examine the effect of PKCδ on p53 protein stabilization, we examined the effect of rottlerin, a compound that has been widely used to inhibit PKCδ (Gschwendt et al., 1994; Basu et al., 2001; Yoshida et al., 2002) on the ability of ML-1 cells to respond to the various DNA damaging agents. As can be seen in figure 3.4 A, direct inhibition of PKCδ with rottlerin dramatically inhibited the ability of CPT, etoposide, zeocin, or MC to induce the nuclear accumulation of p53, and similar to TPA that was not due to compromised cytoplasmic to nuclear shuttling since cytoplasmic p53 was not detectable under these conditions (Figure 3.4 B). Interestingly, unlike TPA, rottlerin inhibited the accumulation of p21 protein (Figure 3.4 A, & B) in response to CPT or etoposide under these conditions and inhibited CPT (Figure 3.4 C), and etoposide (data not shown) -mediated apoptosis. The inhibitory effect of rottlerin on p53 accumulation was dose dependent and effective at a low concentration as low as 1 uM (IC_{50} 3uM) (Figure 3.4 D). Furthermore, rottlerin consistently inhibited p53 protein accumulation in a variety of human (ML-1, Manca, and LNCap) and mouse (MEFs) cell lines (data not shown). Treatment of ML-1 cells with the conventional PKC (cPKC) isotype specific inhibitor Gö6976, or bisindolylmaleimide II had no effect on the p53 protein accumulation induced by DNA damaging agents (data not shown and figure 3.4 E), indicating that down-regulation of cPKC isoforms did not play a role in the TPA effects observed here.

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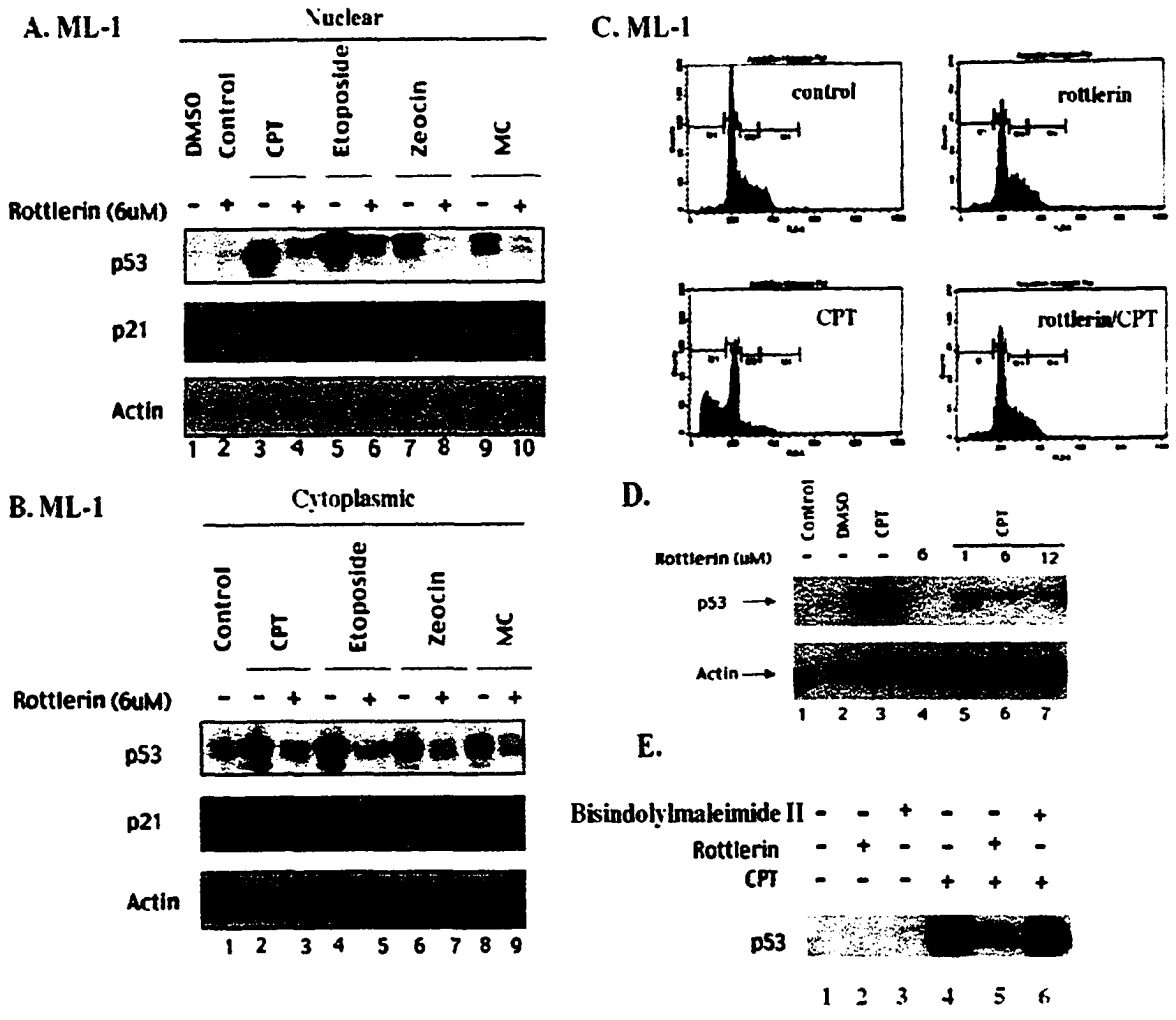

Figure 3.4 Pharmacological inhibition of PKCδ inhibits p53 protein accumulation and p53-dependent apoptosis.

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Figure 3.4 Pharmacological inhibition of PKCδ inhibits p53 protein accumulation and p53-dependent apoptosis.

A, & B, Pharmacological Inhibition of PKCδ inhibits p53 accumulation in response to a variety of DNA damaging agents. Western blot analysis of ML-1 nuclear ***(A),*** and cytoplasmic ***(B)*** protein extracts. Exponentially growing cells were either left untreated, or treated with 0.5 uM CPT, 8 uM etoposide, 50 ug/ml zeocin, or 5 uM MC for 4 hours in the presence or absence of 6 uM rottlerin. Nuclear **(A),** or cytoplasmic **(B)** proteins were extracted and analyzed as described in figure 3.1 and membranes were probed with a mixture of p53-specific monoclonal antibodies (pAb240, pAb421, and pAb1801), anti-p21, or anti-actin antibody. ***C, Inhibition of PKCδ blocks CPT-mediated and p53-dependent apoptosis in ML-1 cells.*** FACS analysis of ML-1 cells. ML-1 cells were either left untreated or treated with 0.5 uM CPT in the presence or absence of 6 uM rottlerin for 4 hours. Cells were fixed with 30% ethanol, stained with propidium iodide for 24 hours, and analyzed by flow cytometry cell sorting. ***D, Rottlerin, dose dependently inhibits the ability of p53 to accumulate in response to DNA damage.*** ML-1 cells were either left untreated, or treated with 6 uM rottlerin, 0.5 uM CPT, or with 0.5 uM CPT in the presence of increasing concentrations of rottlerin for 4 hours. Nuclear proteins were extracted and analyzed as described in figure 3.1 and membranes were probed with a mixture of p53-specific monoclonal antibodies (pAb240, pAb421, and pAb1801), or anti-actin antibody. ***E, Rottlerin, but not bisindolylmaleimide II inhibits the ability of p53 to accumulate in response to DNA damage.*** ML-1 cells were grown as described in materials and methods and were either left untreated, or treated with 6 uM rottlerin, 10 nM bisindolylmaleimide II, 0.5 uM CPT or 0.5 uM CPT in the presence of rottlerin (6 uM), or bisindolylmaleimide II (10 nM) for 4 hrs. Nuclear proteins were extracted and analyzed as described in figure 3.1 and membranes were probed with a mixture of p53-specific monoclonal antibodies (pAb240, pAb421, and pAb1801).

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Stabilization of p53 by proteosome inhibitors is blocked by TPA pretreatment and pharmacological inhibition of PKCδ.

The data shown above suggest that TPA treatment of human myeloid, or epithelial cells or pharmacological inhibition of PKCδ interferes with the ability of p53 protein to accumulate and induce apoptosis. Down-regulation of PKCδ is unlikely interfering with a particular kinase activated by DNA damage that is required for p53 stabilization, since long term TPA treatment or pharmacological inhibition of PKCδ blocked p53 accumulation in response to various DNA damaging agents with various metabolic activities. The increase in the level of p53 protein after DNA damage has been demonstrated to occur at the posttranscriptional level, although some regulation of p53 at the level of transcription has been observed (Maltzman and Czyzyk, 1984; Kirch *et al.*, 1999; Raman *et al.*, 2000). The ubiquitin proteosome pathway is involved in the degradation of p53 and after DNA damage the p53 protein is post-translationally modified so that it is no longer targeted for degradation (Ashcroft *et al.*, 2000). The levels of p53 protein can therefore be increased chemically using inhibitors of the proteosome pathway (Shieh *et al.*, 1997; Siliciano *et al.*, 1997; Chernov *et al.*, 2001). We reasoned that if TPA pretreatment of cells was inhibiting stabilization of p53 through increased degradation of the protein then chemical inhibition of the proteosome pathway would be able to increase p53 levels even after the cells were pretreated with TPA. However, surprisingly we observed that TPA pretreatment of ML-1 cells inhibited the ability of the proteosome inhibitor LLnL to stabilize p53 (Figure 3.5 A).

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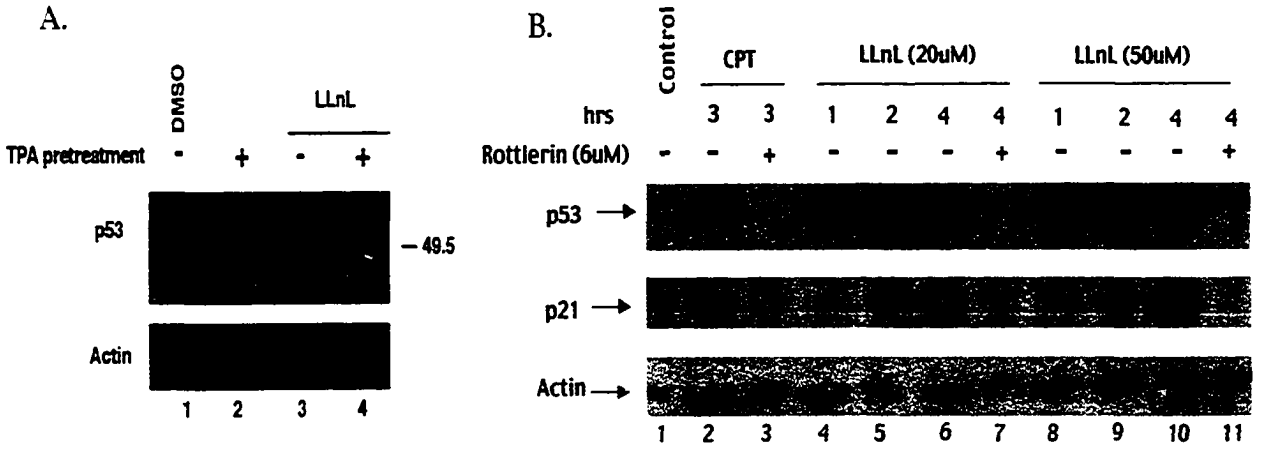

Figure 3.5 Down-regulation of PKCδ by TPA long-term treatment or pharmacological inhibition inhibits p53 protein accumulation at the level of protein synthesis.

TPA pretreatment or pharmacological inhibition of PKCδ inhibits the stabilization of p53 by proteosome inhibitors. Western blot analysis of ML-1 cells nuclear protein extract. ML-1 cells were treated with the proteosome inhibitor LLnL (20 uM or 50 uM) for 3 hours ***(A)*** or for various hours as indicated ***(B)***. Where indicated, ML-1 cells were either pretreated with TPA (10 nM) for 21 hours ***(A)***, or simultaneously treated with rottlerin (6 uM) ***(B)***. Proteins were separated on a 10% SDS-PAGE and electro-transferred on a nitrocellulose membrane and probed with either a mixer of p53 specific antibodies (pab1801, pab240, & pab421), anti-p21 or anti-actin antibodies.

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Similar results were obtained when the proteosome inhibitors PSI, or MG321 were used (data not shown). Therefore the inhibitory effect of TPA on p53 protein accumulation was apparently not due to enhanced p53 degradation by a proteosome mediated proteolysis pathway. Rottlerin was also able to prevent p53 accumulation induced by proteosome inhibition (Figure. 3.5 B), further implicating PKCδ down-regulation as critical for the effect of TPA upon p53 accumulation. Treatment of ML-1 cells with the proteosome inhibitor LLnL not only stabilized p53, but also resulted in the accumulation of the p21 protein after 4 hours of treatment (Figure 3.5 B). Importantly, rottlerin but not TPA was able to block p21 protein accumulation in response to LLnL (Figure 3.5 B, and data not shown) consistent with the finding that TPA induces p53-independent induction of p21 (Park *et al.*, 2001). Taken together, these data suggested that down-regulation of PKCδ blocked signaling to p53 and blocked p53-dependent apoptosis and gene trans-activation by a mechanism involving inhibition of the p53 protein synthesis.

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Basal steady state level of p53 mRNA is inhibited by TPA treatment and inhibition of PKCδ.

The data provided in figure 3.5 indicated that the ability of TPA to suppress p53 accumulation was not due to enhancement of p53 ubiquitination and degradation. We therefore investigated whether TPA was down-regulating p53 mRNA. ML-1 cells were treated with TPA for increasing times and RNA was extracted. Northern blot analysis of total RNA using a p53 cDNA probe was then carried out. A clear reduction in the basal level of p53 mRNA transcript was evident with increasing time of TPA treatment beginning after 8 hours (Figure 3.6 A). The low steady state levels of p53 mRNA was maintained during camptothecin and etoposide drug treatment in ML-1 cells pretreated with TPA (data not shown). This data was further confirmed by quantitative RT-PCR showing that TPA treatment of ML-1 cells for 8 hours resulted in about 60% reduction in p53 mRNA levels (Figure 3.6 B). The TPA effect on p53 mRNA level was specific, since TPA caused about 9 fold increase in the p21 mRNA levels (Figure 3.6 C) consistent with our previous results and the TPA's ability to up-regulate p21 mRNA in a p53-independent manner (Park *et al.*, 2001). The kinetics of TPA mediated inhibition of p53 mRNA were concurrent with the disappearance of PKCδ observed in figure 3.2. David W., a graduate student in our laboratory have showed that the proteosome inhibitor LLnL did not affect the level of p53 mRNA, whereas the PKC δ inhibitor, rottlerin inhibited p53 mRNA level, and maintained this reduction of p53 mRNA in the presence of LLnL (Figure 3.6 D). Inhibition of p53 mRNA by rottlerin was also associated with inhibition of the p53 downstream target genes *p21* and *hdm2* activated in the presence of LLnL treatment (Figure 3.6 D, compare lanes 2 and 4). The cPKC inhibitor Gö6976 had no

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detectable effect on the level of p53 or p21 proteins induced by DNA damaging agents (data not shown), indicating that inhibition of cPKC isoforms did not play a role in the effects of TPA observed here. TPA is known to activate PKC prior to depletion to occur (Bindels *et al.*, 1993; Chen, 1993). We therefore examined the effect of TPA short-term treatment of ML-1 on p53 mRNA level. Short-term treatment of ML-1 cells with TPA had no detectable effect on p53 mRNA (data not shown), suggesting that although PKCδ is apparently required for maintaining basal p53 mRNA steady state level, it is not sufficient for enhancing the p53 mRNA level under these conditions.

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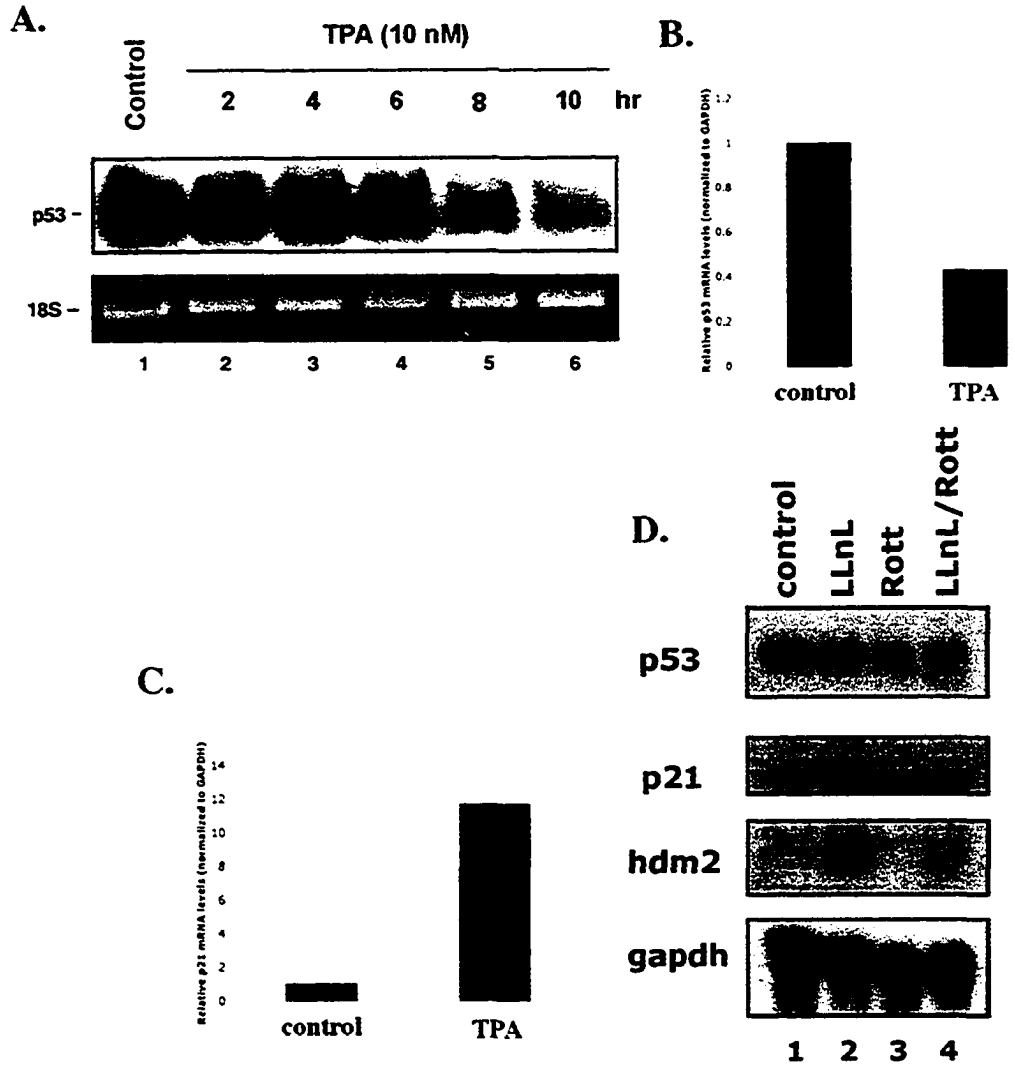

Figure 3.6 TPA or inhibition of PKCδ specifically inhibits the basal steady state level of p53 mRNA.

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Figure 3.6 TPA or inhibition of PKCδ specifically inhibits the basal steady state level of p53 mRNA.

A, & B, TPA inhibits the steady state levels of p53 mRNA in ML-1 cells. A. Northern blot analysis of ML-1 total RNA extracts. ML-1 cells were either left untreated (lane 1), or treated with 10 nM TPA for various hours as indicated. 10 ug of total RNA was resolved on a denaturing formaldehyde gel, transferred electro-phoretically on a nylon membrane and probed for p53. 18S r-RNA was used as loading control. ***B.*** Quantitative RT-PCR analysis of the p53 mRNA. Ml-1 cells were either left untreated (control) or treated with 10 nM TPA for 8 hours. cDNA was then prepared from total RNA as described in materials & methods, and PCR reaction was then performed using specific primers for the human *p53* gene. ***C, TPA increases the steady state levels of the p21 mRNA independent of p53.*** Quantitative RT-PCR analysis of the p21 mRNA. Ml-1 cells were either left untreated (control) or treated with 10 nM TPA for 8 hours. cDNA was then prepared from total RNA as described in materials & methods and PCR reaction was then performed using specific primers for the human *p21* gene. ***D, Pharmacological inhibition of PKCδ inhibits p53 gene transcription, and the p53-down-straem target genes p21 and hdm2.*** Northern blot analysis of ML-1 total RNA. Exponentially growing ML-1 cells were either left untreated (lane 1), or treated with 20 uM LLnL for 4 hrs in the presence (lane 4) or absence (lane 2) of 6 uM rottlerin. 50 ug of total RNA was resolved on a denaturing formaldehyde gel, transferred electro-phoretically on a nylon membrane and probed for p53, p21, hdm2, or GAPDH.

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To directly examine the effect of PKCδ down-regulation on the endogenous p53 mRNA level, we transiently trasfected the human H460 lung carcinoma cells with short interfering RNA (siRNA) directed against PKCδ. Using this strategy, we were able to down-regulate the basal level of PKCδ to about 40% of its normal levels (Figure 3.7 A). Significantly, this down-regulation of PKCδ was associated with the down-regulation of p53 mRNA to about 40 % of its basal level as measured by quantitative RT-PCR (Figure 3.7 B). In this study, siRNA directed against GFP as a negative control had no effect on either PKCδ protein or p53 mRNA (Figure 3.7 A, & B).

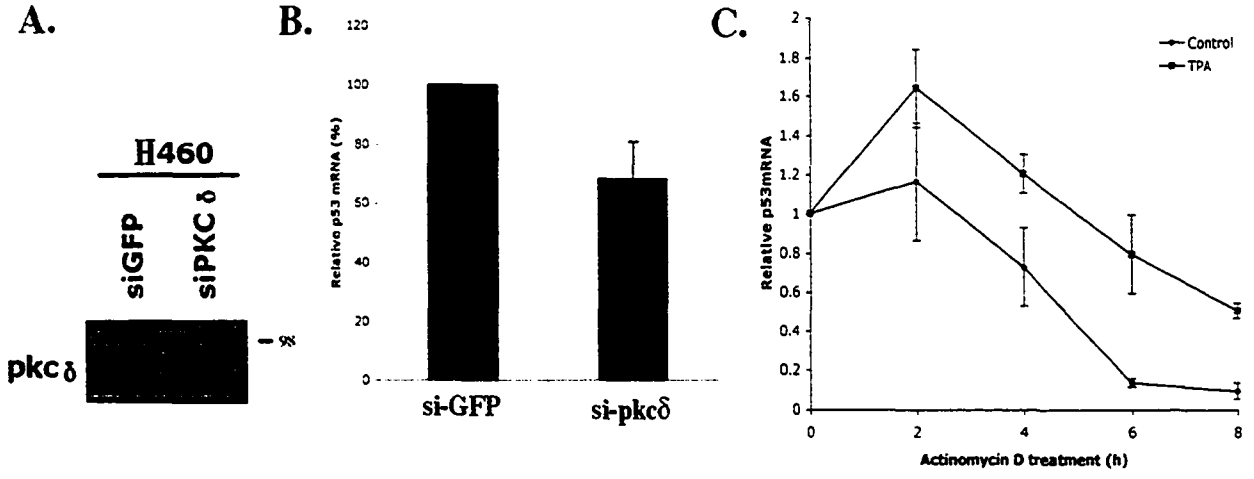

Figure 3.7 Down-regulation of PKCδ inhibits the steady state p53 mRNA level, and TPA does not interfere with the stability of p53 mRNA.

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Figure 3.7 Down-regulation of PKCδ inhibits the steady state p53 mRNA level, and TPA does not interfere with the stability of p53 mRNA.

A, & B, *Targeted down-regulation of PKCδ inhibits the steady state level of endogenous p53 mRNA in H460 cell line.* Western blot analysis of PKCδ as well as quantitative RT-PCR analysis of p53 mRNA in H460 cell line transfected with siRNA against PKCδ. H460 cells were grown as described in materials & methods and transfected with either siRNA against the *GFP* mRNA or PKCδ mRNA. ***A,*** Western blot analysis of total H460 proteins prepared and analyzed as described in materials & methods, and membrane was probed with anti-PKCδ antibody. ***B,*** *Quantitative RT-PCR. RNA* was extracted and cDNA was prepared and analyzed as previously described (data is representative of 4 independent experiments). ***C.*** *TPA does not interfere with the stability of p53 mRNA in the ML-1 cells.* Quantitative RT-PCR analysis of the p53 mRNA. ML-1 cells were either left untreated (control), or treated with TPA (10 nM) for 5 hours (time 0 hr). Control and TPA-treated cells were then treated with actinomycin D (10 ug/ML) and aliquots were harvested after 2, 4, 6, and 8 hours treatment with actinomycin D (data is representative of 4 independent experiments). Total RNA was extracted and analyzed as previously described.

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TPA has been reported to affect the mRNA stability of many genes including c-Myc among others (Wang *et al.*, 1994). In order to test the possibility that TPA was indeed inhibiting the stability of p53 mRNA as well, we treated ML-1 cells with TPA for 5 hours (the time point at which we observed dramatically reduced p53 mRNA by Northern blot analysis-Figure 3.6 A) followed by treatment with the general transcriptional initiation inhibitor actinomycin D for various hours, and compared the levels of p53 mRNA in ML-1 cells in the presence or absence of TPA pretreatment. As can be see in figure 3.7 C, the level of p53 mRNA was reduced with similar kinetics in cells that were either treated with TPA or those that were not. These data suggest that TPA was not affecting the stability of p53 mRNA, and suggest that the effect of TPA on p53 mRNA was not at the posttranscriptional level.

Down-regulation of PKCδ inhibits transcriptional initiation from the human p1-p53 promoter.

We next examined the effect of PKCδ inhibition upon gene expression from the *p53* promoter. Studies on the regulation of *p53* promoter are not plentiful, but have demonstrated that numerous transcription factors influence human *p53* transcription including AP-1, HoxA5, YY1, NFκB, and Myc (Reisman and Loging, 1998). Using a human p1-*p53* promoter luciferase construct containing the region previously shown to be regulated by the above mentioned transcription factors (Raman *et al.*, 2000), we carried out transient transfection experiments in the H1299 lung carcinoma cells that have no endogenous *p53*. Co-transfection of the human H1299 cells with a dominant negative catalytically inactive PKCδ expression plasmid (Lu *et al.*, 1997) dose dependently

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repressed transcription from the human p1-*p53* promoter driving luciferase expression (Figure. 3.8 A). The effect of DN-PKCδ on transcription from the human p1-*p53* promoter was specific, since the DN-PKCδ had no effect on renilla luciferase expression driven by the HSV promoter (data not shown). Further more, DN-PKCδ had no effect on gene expression from a p53-dependent P2-mdm2 promoter (Figure 3.8 B). Co-transfection of the H1299 cells with wild type p53 driven by the CMV promoter (SN3) significantly enhanced expression from the p53-dependent Mdm2 promoter, and under these conditions DN-PKCδ had no inhibitory effects (Figure 3.8 B).

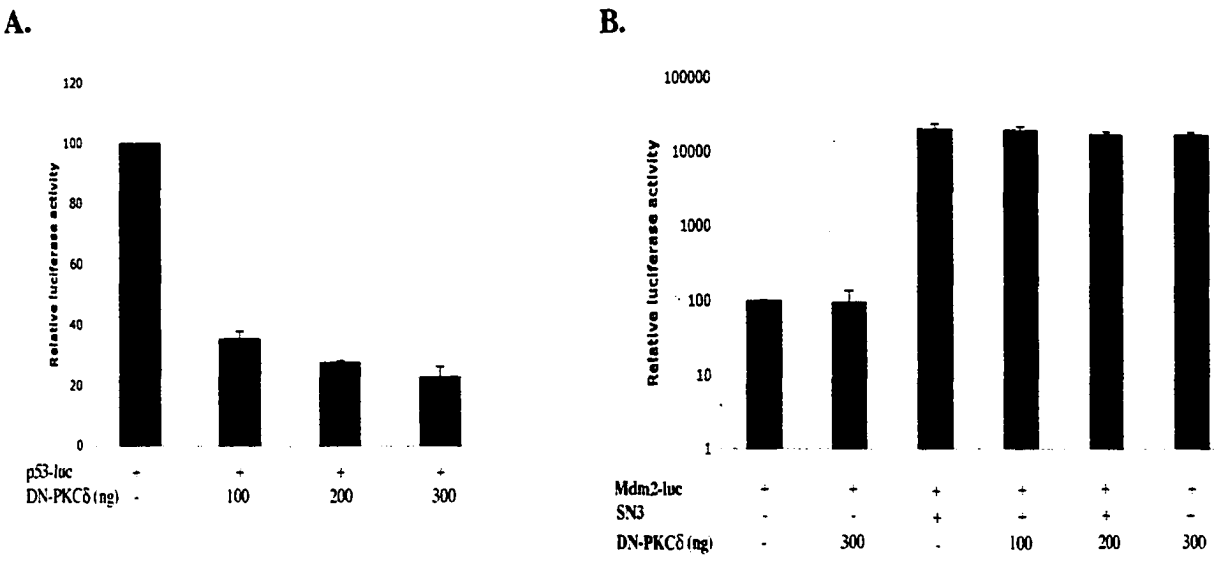

Figure 3.8 Down-regulation of PKCδ specifically inhibits transcription from the human p1-*p53* promoter, and does not affect the p53 protein stability.

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Figure 3.8 Down-regulation of PKCδ specifically inhibits transcription from the human p1-*p53* promoter, and does not affect the p53 protein stability.

A, DN-PKCδ inhibits expression from the human p1-p53 promoter. Luciferase activity assay of H1299 protein extracts. H1299 cells were grown as described in materials & methods and transiently trasfected with a luciferase reporter driven by the human p1-p53 promoter (p53-luc). Where indicated, cells were co-transfected with an increasing concentration of a dominant negative mutant of PKCδ. Proteins were extracted as described in materials & methods and assayed for firefly luciferase expression. Data represent luciferase activity relative to p53-luc transfected cells, and normalized to co-transfected renilla reporter to control for transfection efficiency. ***B, DN-PKCδ does not interfere with the stability of p53 protein.*** Luciferase activity assay of H1299 protein extracts. H1299 cells were grown as described and were either transfected with a luciferase reporter driven by the p2-mdm2 (p53-dependent) promoter (mdm2-luc) alone, or co-transfected with a wild type p53-coding SN3 plasmid in the presence of increasing concentration of DN-PKCδ plasmid. Proteins were extracted as described in materials & methods, assayed for luciferase activity, and presented as luciferase activity relative to Mdm2-luc transfected cells.

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Discussion:

Abrogation of the p53 protective tumor suppressing functions is a major event in the course of malignant transformation. Agents in the environment are often associated with the promotion of tumor formation, however none have been shown to directly inhibit the transcription of *p53* or p53 protein activity. Here, we show that the DNA damage mediated increases in p53 protein and p53-mediated apoptosis are dramatically inhibited by long-term treatment of cells with the tumor promoter phorbol ester TPA. We show that TPA inhibits apoptosis in cells with wild type p53, but not in cells lacking p53 (Figure 3.1). We have noticed that although DMC is capable of inducing p53-independent as well as p53-dependent apoptosis, TPA was able to significantly inhibit DMC-induced PARP cleavage in the ML-1 cell line (Figure 3.1 A, lane 8). This discrepancy can be explained by the hypothesis that TPA may have anti-apoptotic effects that goes beyond its ability to block p53-dependent apoptosis in ML-1 cells, perhaps as have been suggested via activation of the NFκB survival pathways, activated in response to TPA (Dhar et al., 2002; Huang et al., 2003). In fact, as discussed below, the NFκB-DNA binding activity is significantly enhanced by TPA treatment in the ML-1 cell line, but not in the K562 cell line (Figure 4.2 B). Alternatively, it can be explained by the ability of TPA to induce p21 protein in the ML-1 but not in the k562 cells (data not shown). It was recently demonstrated for example, that rapamycin-induced and p53-independent apoptosis was blocked by over-expression of p21 protein, which associates with and inactivates the apoptosis signal-regulating kinase-1 (ASK1), activated in response to rapamycin (Huang *et al.*, 2003).

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In some systems bryostatin 1 (a compound unrelated to TPA) induces some of the same responses as TPA (Lu *et al.*, 1997; Heit *et al.*, 2001), while high concentrations of bryostatin 1 can reverse the effect of TPA, thus resulting in the inability to deplete PKCδ (Lu *et al.*, 1997; Heit *et al.*, 2001). Interestingly, bryostatin 1 treatment (100 nM) of MCF-7 cells prevented the TPA-associated PKCδ down-regulation, and more importantly reversed the inhibitory effect of TPA on p53 accumulation and apoptosis induced in response to CPT, providing an excellent correlation between PKCδ protein level and the ability to signal to p53 (Figure 3.3 A, & B). In ML-1 cells however, a bryostatin 1 concentration as high as 0.5 uM, by itself depleted cells of PKCδ, and consistent with our hypothesis that the effect of TPA is mediated via down-regulation of PKCδ, bryostatin 1 also inhibited CPT-mediated accumulation of p53 (Figure 3.3 C, & D) and apoptosis induction (data not shown). Consistent with the hypothesis that the inhibitory effects of TPA on p53 protein accumulation and apoptosis induction is dependent on its ability to down-regulate PKCδ, pharmacological inhibition of PKCδ also abrogated the ability of various DNA damaging agents to induce the accumulation and stabilization of p53. Significantly, the DNA damage-mediated apoptosis associated with activation of the p53 pathway is inhibited by both TPA and rottlerin treatment of cells containing wild-type p53. This may be particularly significant since malignant cells must overcome apoptotic signals that are generated in the course of malignant transformation in response to inappropriate mitogenic or oncogenic signals. In light of our data, it is tempting to speculate that tumor promoters or similar agents in the environment may provide such survival signals via early transcriptional inactivation of critical checkpoint proteins that would otherwise provide protective apoptotic signals.

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Most significantly, we show for the first time that treatment of cells with TPA or inhibition of PKCδ resulted in the inhibition of the basal steady state p53 mRNA level (Figure 3.9). Targeted silencing of the *PKCδ* gene in the human H640 using siRNA technique significantly lowered the basal p53 mRNA levels demonstrating a requirement for PKCδ in maintaining basal steady state p53 mRNA. We show that the p53 mRNA stability was not affected by TPA treatment since blockade of transcriptional initiation by actinomycin D reduced the p53 mRNA levels with similar kinetics in cells that were either left untreated or in TPA treated cells. Furthermore, our *p53* promoter studies suggest that the initiation of *p53* transcription is blocked by down-regulation of PKCδ. A previous *p53* promoter study demonstrated that transcription from the human *p53* promoter, activated under normal conditions after TPA short-term treatment, requires the binding motifs for AP-1, NKκB, and Myc/Max (Kirch *et al.*, 1999). These transcription factors synergistically activate *p53* after mitogenic stimulation. We show that transfection of the human H1299 cells with a dominant negative mutant of PKCδ repressed transcription from the human p1-*p53* promoter. Protein kinase C delta (PKCδ) itself is not a transcription factor, but may very well be a regulator of one or more of the above mentioned transcription factors regulating expression from the human p1-*p53* promoter, and therefore the effect of PKCδ on p53 expression we believe to be an indirect one. Although we clearly show that there is a requirement for PKCδ in maintaining basal transcription from the *p53* gene, we found that activation of PKCδ via short-term TPA treatment is insufficient to activate transcription from the endogenous human *p53* gene (data not shown).

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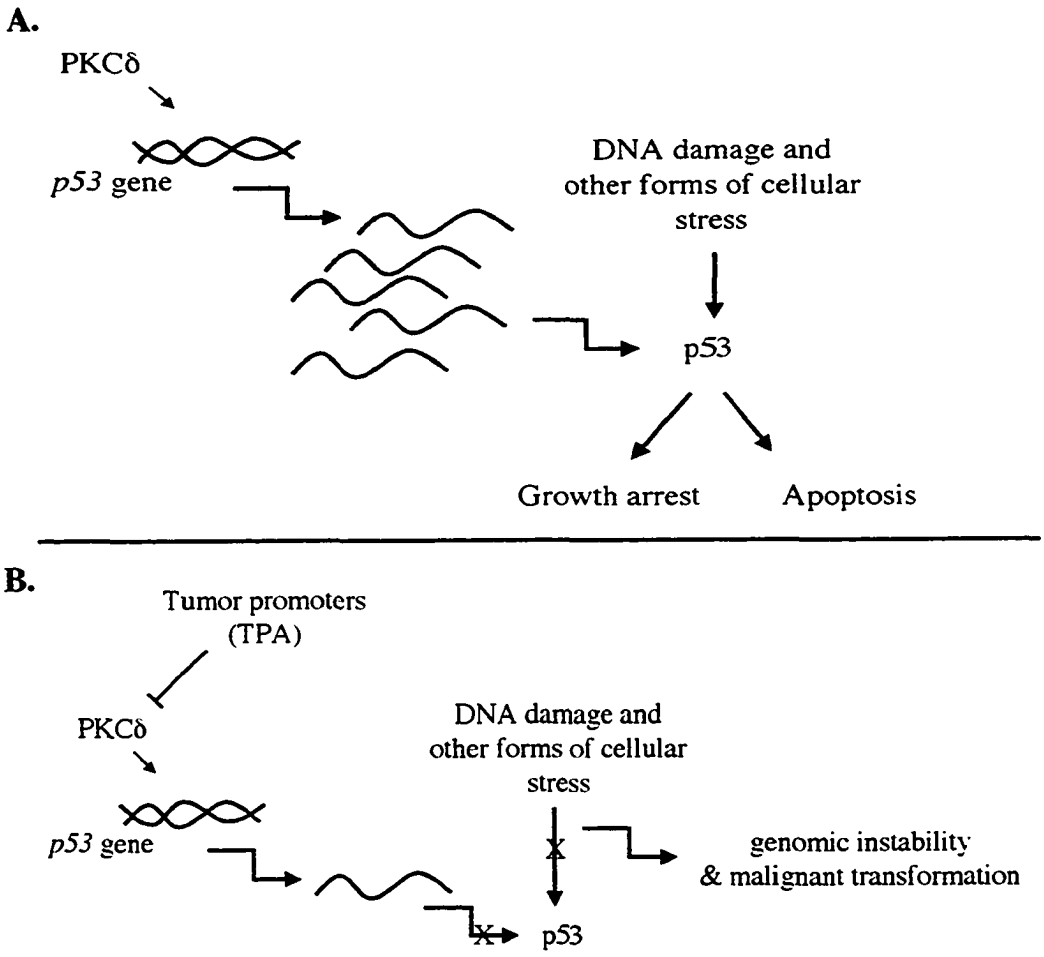

Figure 3.9 Regulation of the *p53* gene by PKCδ

A, Regulation of the p53 tumor suppressor pathway under normal conditions. The p53 protein is stabilized in response to various cellular stresses and protects the cells via inducing growth arrest, DNA repair or apoptosis. ***B, Abrogation of the p53 protective responses via transcriptional inhibition in response to tumor promoters or other factors that down-regulate, or inhibit PKCδ.*** In the absence of PKCδ, the p53 mRNA is expressed at very low levels insufficient of driving normal tumor suppressing activities. In the absence of p53 tumor suppressor activities, cells are rendered susceptible to environmental insults leading to genomic instability and malignant transformation.

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In light of our finding that long-term TPA treatment is associated with inhibition of transcription from the *p53* gene, it is interesting that *p53* mutations are not found in papillomas formed in the skin of mice in response to TPA. However, mutations in the *p53* gene are found in late carcinogenic lesions formed in these animals (Zhang *et al.*, 1998). Furthermore, Weinberg et al. showed that genetic deletion of *p21/Waf* enhanced epidermal hyperplasia and papilloma formation but not malignant conversion induced by TPA (Weinberg *et al.*, 1999), suggesting that tumor suppressing functions of p53 other than its ability to up-regulate p21 are necessary to inhibit malignant transformation induced by TPA. These findings, and our results suggest that the TPA's ability to inhibit the steady state level of p53 mRNA and protein is perhaps sufficient for initiating the process of malignant transformation, but *p53* gene inactivation is a later step that may be a result rather than a cause of genomic instability due to compromised p53 function by TPA or other tumor promoters (Figure 3.9).

Although we clearly show that TPA and down-regulation of PKCδ inhibit transcription of *p53*, it is less clear whether TPA or down-regulation of PKCδ is associated with reduced translation or stability of the p53 protein. Transient transfection of the human lung carcinoma H1299 cells with a dominant negative mutant of PKCδ had no detectable effect on the expression or DNA binding and trans-activation activities of wild type p53 expressed from the CMV promoter (Figure 3.8 B). In this experiment, the level and DNA binding activity of wild type p53 was monitored by co-transfection of a p53-dependent p2-mdm2 promoter driving luciferase expression. The lack of detectable effect of DN-PKCδ on wild type p53 is unlikely due to the strong expression of p53 from

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the CMV promoter, since the dominant negative mutant of PKCδ is also expressed from the same CMV promoter. More sensitive experiments however, may still be needed to further confirm our findings.

We often examine damaged genes in tumor cells as mechanistic drivers towards oncogenesis. The regulation of endogenous genes, specifically tumor suppressors however, may be a key regulatory mechanism. Our data provides a mechanism for tumor promotion by TPA, and predicts that agents that interfere with PKCδ activity may modulate p53 responses (Figure 3.9). Since activating the p53 apoptotic pathway is a checkpoint used by cells to evade gaining mutations, and is also the target of many chemotherapeutic regimes, agents that could have PKCδ activating properties may provide synergistic cytotoxic activity (Delia *et al.*, 1995). Conversely, agents that have PKCδ inactivating potential may drastically reduce chemotherapeutic drug efficacy. Our findings demonstrate that focusing on the transcriptional regulation of *p53* presents a new and novel targeted approach in the war against cancer. In addition, it highlights the possibility that agents in the environment may adversely affect the p53 pathway by inhibiting the genes transcription.

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Chapter 4

Analysis of the effect of TPA and down-regulation of PKCδ on the human p1-*p53* promoter

Preliminary data and future directions

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We have provided data suggesting that the tumor promoter TPA by virtue of its ability to down-regulate PKCδ, inhibits the basal steady-state level of the tumor suppressor p53 mRNA, and thereby prevents p53 protein accumulation in response to cellular stress such as DNA damage. It is possible that this inhibitory effect of TPA on *p53* gene expression contributes to its tumor promoting activity especially in the context of cellular stress. Our actinomycin D experiment (Figure 3.7 C) showed that TPA does not interfere with the stability of the p53 mRNA, and our transient transfection experiments on the p1-*p53* promoter suggest that the effect of TPA and down-regulation of PKCδ is at the level of transcriptional initiation from the *p53* gene. Although our data clearly demonstrates a requirement for PKCδ for maintaining basal transcription of *p53*, we have yet to determine the mechanism by which interfering with PKCδ activity (via inhibition or down-regulation by TPA) inhibits p53 mRNA transcriptional initiation.

Initially, we will perform RNA nuclear run-on experiments to confirm the results obtained from our transient transcription experiments in order to provide clear evidence that the effect of TPA by virtue of down-regulating PKCδ is inhibiting transcriptional initiation from the endogenous *p53* promoter. It is unclear how inhibition or down-regulation of PKCδ inhibits transcription from the *p53* promoter. One possibility is that PKCδ kinase activity is required for recruiting a transcription factor or cofactor to the *p53* promoter. For example, it is possible that PKCδ phosphorylates one or more of the transcription factors regulating transcription from the *p53* gene, and that this phosphorylation event is required for the nuclear translocation or proper alignment of

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such factor on the *p53* promoter. It is also possible that PKCδ phosphorylates a factor(s) that is required for the appropriate chromatin remodeling necessary for transcriptional initiation from the *p53* gene. Furthermore, it is possible that the effect of PKCδ on *p53* promoter is not dependent on its kinase activity, but rather on its ability to regulate protein-protein interaction. As discussed above (and shown in Figure. 4.1 A, & B), there are many different transcription factor binding sites on the human *p53* promoter used in our study.

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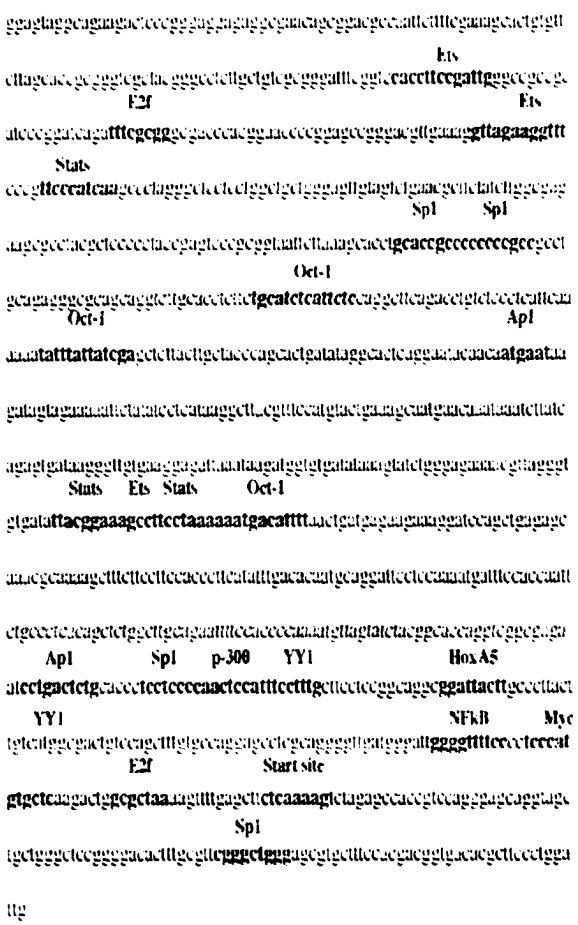
A.



B.

Transcription factor binding site	Copy number
ETS	3
E2F	2
STATS	3
SP1	4
OCT	3
AP1	2
P-300	1
YY1	1
HOXA5	1
NFKB	1
MYC	1

Figure 4.1 The human p1-*p53* promoter

A, DNA sequence of the human p1-*p53* promoter highlighting the various known transcription factor-binding sites. ***B***, List of the various putative transcription factors that can bind to the human p1-*p53* promoter and their copy numbers.

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We will use site-specific oligonucleotide sequences in electro-mobility gel shift assays (EMSA) in an attempt to test whether the DNA-binding activities of any of the candidate transcription factors regulating *p53* transcription are compromised in nuclear extracts of cells treated with TPA or rottlerin. It is however, equally possible that inhibiting PKCδ may modulate the affinity of such transcription factor(s) to its binding site on the *p53* promoter rather than compromising its DNA-binding activity altogether. We will therefore, carefully use different concentrations of competitive and noncompetitive oligonucleotide sequences in our EMSAs that will allow us to analyze and compare the DNA-binding affinity of these transcription factors in the presence or absence of TPA. Our preliminary data show that the DNA binding activities to the binding sites of AP-1 (c-Jun) and the NFκB transcription factors are not inhibited in cells treated with TPA (10 nM) for 24 hours (Figure 4.2 A, & B). Nuclear extracts of ML-1 cells incubated with AP-1 oligonucleotides showed no clear band on an EMSA gel (Figure 4.2 A, lanes 3-5), however upon TPA treatment a clear DNA binding band that corresponded to the activated AP-1 protein-DNA binding (Figure 4.2 A, lane 2) appeared. This band was specific since it was competed by using non-radio-labeled AP-1 oligonucleotide (Figure 4.2 A, lane 7) but not with a nonspecific NFκB oligonucleotide (Figure 4.2 A, lane 8). The binding to the Ap-1 binding site was activated in response to TPA in K562 cells as well (Figure. 4.2 A, lanes 9-14). Unlike the binding to the AP-1 oligonucleotides, the binding to the NFκB oligonucleotides was activated in ML-1 (Figure 4.2 B, lanes 3-8), but not in K562 (Figure 4.2 B, lanes 9-14) in response to TPA. The activation of NFκB binding in the ML-1, but not in the K562 cells may explain the discrepancy discussed above regarding the ability of TPA to protect against DMC-induced and p53-independent

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apoptosis in ML-1 cells but not in K562 cells (Figure 3.1, and discussed in Chapter 6). Although the data described above does not show inhibition of AP-1 or NFκB binding in nuclear extracts of ML-1 cells, it does not exclude or rule out the possibility that either of these transcription factors is in fact responsible for the inhibitory effect of TPA or down-regulated PKCδ on *p53* transcription. For example, it is possible that PKCδ may phosphorylate and activate NFκB or AP-1 transcription factors leading to increased DNA binding activity in such a way that it has now higher affinity for some promoters that compete with the *p53* promoter for these factors. In fact, based on the observation that TPA treatment of ML-1 showed a clear new AP-1-DNA (Figure 4.2 A) band that was not present in untreated ML-1 cells, suggest that the AP-1 transcription factor is a strong candidate that its activity may have been modulated in response to TPA in such a way that it is no longer capable of driving transcription from the *p53* promoter. Such a possibility can be examined by employing chromatin immuno-precipitation (CHIP) assays. If the hypothesis that AP-1 transcription factor binding affinity to the *p53* promoter is inhibited in cells treated with TPA is correct, then amplification from the *p53* gene using PCR in such case will be inhibited as well in these extracts. This hypothesis can be further examined by utilizing inhibitors of the various MAPK kinase components leading to activation of AP-1 and investigating their effect on *p53* transcription. We will carry out similar DNA binding reactions using oligonucleatide sequences known to bind to other candidate transcription factors shown in figure 4.1.

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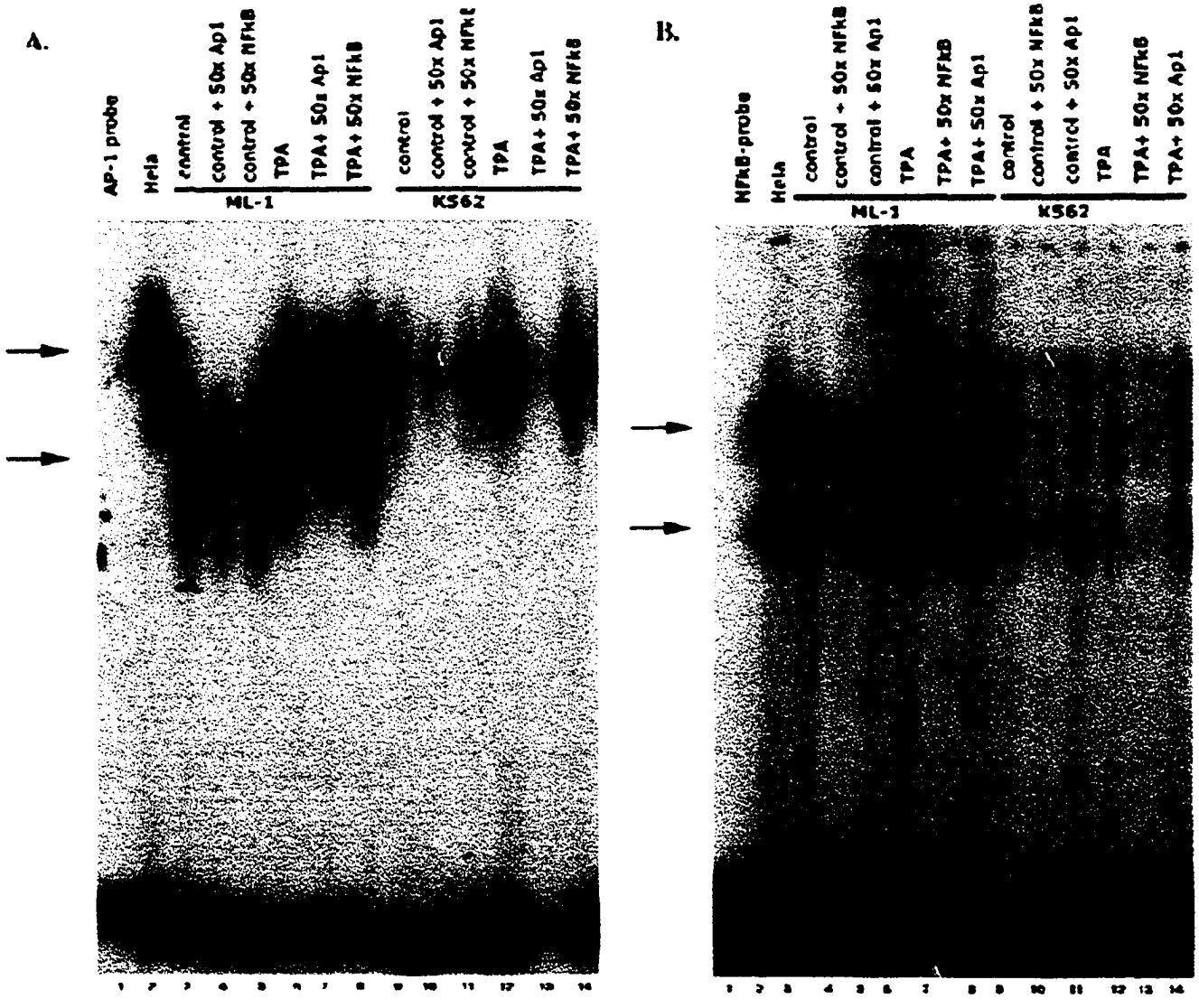

Figure 4.2 TPA activates the DNA-binding activity of the AP-1 and the NFκB transcription factors in ML-1 cells and only AP-1 in K562 cells.

Electro-mobility shift assay (EMSA) analyzing of the DNA binding activity of the transcription factors AP1 (**A**), or NFκB (**B**) in nuclear protein extracts of ML-1, and K562 cells that were either left untreated or treated with TPA (10 nM) for 24 hours.

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Another approach that we may utilize is to try to introduce site-specific mutations in the human *p53* promoter construct, and see which one of these mutant constructs have similar transcriptional activity in the presence and absence of DN-PKCδ. It has been shown that multiple transcription factors work synergistically to activate transcription from an exogenously expressed *p53* promoter (Kirch *et al.*, 1999). Because of the complex nature of TPA's activity (short-term activation versus long-term inhibition of various PKC isoforms), we will not utilize TPA in this kind of study, since it is anticipated that a single site-specific mutation in any of the known transcription factor-binding site will inhibit the ability of TPA to activate transcription from the *p53* promoter. If down-regulation of PKCδ (by co-transfection of DN-PKCδ or siRNA directed against PKCδ) inhibits transcription from the *p53* promoter by inhibiting the binding of a transcriptional activator to the *p53* promoter or by decreasing the affinity of a transcriptional activator to the *p53* promoter as suggested and described above, a mutation to such site to which a transcriptional activator binds will have similar effects to co-transfection of DN-PKCδ or siPKCδ. If down-regulation of PKCδ on the other hand leads to activation of a transcriptional repressor rather inhibition of a transcriptional activator, mutation of the site to which such putative repressor binds is anticipated to enhance transcriptional activity regardless the presence or absence of DN-PKCδ, and co-transfection with a DN-PKCδ is anticipated to have no further effect on the transcriptional activity of such promoter. Foot-printing experiments can also be performed to carefully analyze the site to which such putative repressor binds. DNA can be extracted from cells that are treated with TPA or si-RNA directed against PKCδ and digested with DNAase. In the case a repressor is activated by TPA or si-PKCδ, a region

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of the *p53* promoter will be protected from the enzymatic digestion only in cells treated with TPA or si-PKCδ, but not in control cells.

Although our study clearly shows that TPA or PKCδ down-regulation inhibits the transcription from the *p53* gene, our results cannot rule out the possibility that TPA and/or PKCδ inhibition may have additional effects on p53 protein stability or translation. In fact, using Western blot analysis, our preliminary data suggest that TPA and rottlerin, the pharmacological inhibitor of PKCδ, elevate the level of the p53 negative regulator Mdm2 protein in the nuclei of ML-1 cells, but not of K562, a cell line that lacks p53 (Figure 4.3 A and data not shown). Since p53 mRNA and protein are down-regulated in cells treated with TPA, we propose that TPA in addition to its effects on the transcription of p53, it may also destabilize the p53 protein by up-regulating Mdm2 in a manner that is independent of p53 trans-activation activity. In fact, David W. showed that Northern blot analysis of RNA extracted from TPA treated Ml-1 cells do not show elevated levels of Mdm2, suggesting that the effect of TPA on Mdm2 observed in ML-1 cells is occurring at the post-transcriptional level (Figure 4.3 B).

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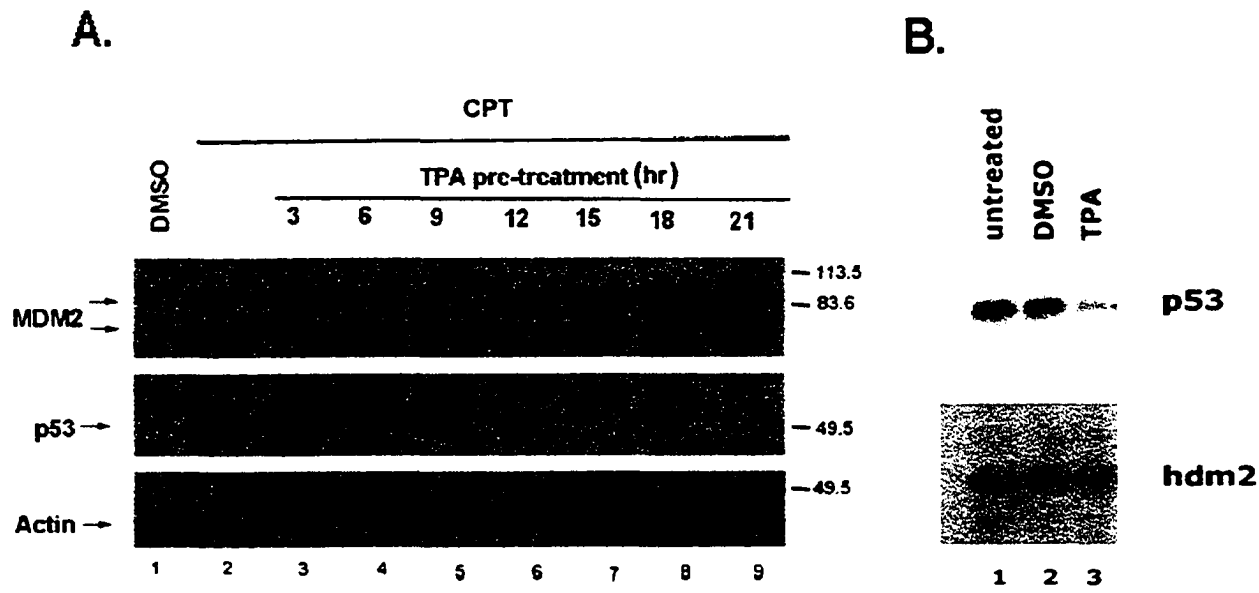

Figure 4.3 TPA elevates Mdm2 protein levels in ML-1 cells by a post-transcriptional mechanism.

***A**, TPA elevates Mdm2 protein levels in ML-1 cells.* Western blot analysis of ML-1 nuclear protein extracts. ML-1 cells treated and analyzed as described previously and membranes were probed with the anti-Mdm2 antibody D7. ***B**, TPA does not affect the transcription of Mdm2 in ML-1 cells.* Northern blot analysis of total RNA extracted from ML-1 cells that were either left untreated, or treated with TPA for 24 hrs.

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It has been shown that the pro-survival protein AKT/PKB is phosphorylated and activated in response to multiple mitogenic signals (Johnson *et al.*, 2002; Grey *et al.*, 2003). More recently, AKT has been shown to directly phosphorylate Mdm2 (Ashcroft *et al.*, 2002; Zhou and Hung, 2002), and that this phosphorylation induces Mdm2 translocation to the nucleus (Mayo and Donner, 2001). It is therefore possible that the effect of TPA on Mdm2 protein may be mediated via activation of the PI3/AKT (PKB) survival pathway. We have stably transfected the human myeloid ML-1 cell with a dominant negative mutant of AKT in order to carefully examine the role AKT may be playing in mediating the TPA effect on MDM2. We will also investigate whether or not TPA treatment of cells is associated with Mdm2 phosphorylation at this site. We will use various rodent as well as human cell lines and see whether or not the TPA effect on Mdm2 is universal or limited to the ML-1 cell line. The fact that TPA had no effect on the Mdm2 protein level in K562 (data not shown) suggests that the status of p53 may be detrimental to Mdm2 response to TPA or AKT activation. We will utilize short interfering RNAi (siRNA) directed against PKCδ in order to directly asses the role PKCδ may be playing in mediating the TPA effect on Mdm2 and/or AKT.

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Chapter 5

Differential induction of p53 by the various adducts of mitomycin C

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Introduction:

MC (MC; Figure 5.1) is a natural antibiotic and cytotoxic chemotherapeutic agent and is used in the clinical treatment of several human malignancies (Verweij, 1990). In cells, MC is enzymatically reduced, yielding reactive species that are capable of producing radicals through redox cycling. Furthermore, MC induces DNA damage in the form of DNA cross-links and mono-functional DNA alkylation products in various bacterial and mammalian cells and tissues (Szybalski, 1964; Bizanek *et al.*, 1993; Pan *et al.*, 1993; Warren *et al.*, 1998; Palom *et al.*, 2000; Warren *et al.*, 2001). Six major adducts are formed as shown in mouse mammary tumor cells and their precise molecular structures have been elucidated (Palom *et al.*, 2000).

Recently, the MC derivative 10-decarbamoyl mitomycin C (DMC; Figure 5.1) was shown to generate an array of DNA cross-links and DNA-mono-adducts in EMT6 mouse mammary tumor cells which had similar or identical structures to those formed with MC (Palom *et al.*, 2002). In contrast, 2,7-diaminomitosene (2,7-DAM-Figure 5.1), the major metabolite of MC in tumor cells (Chirrey *et al.*, 1995), forms only DNA mono-adducts (Palom *et al.*, 1998).

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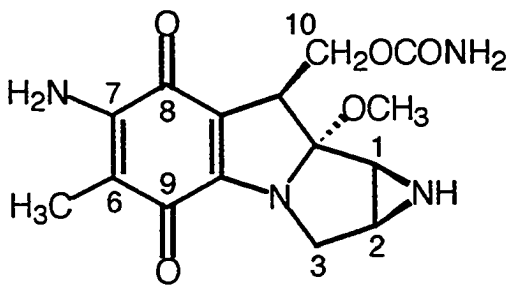
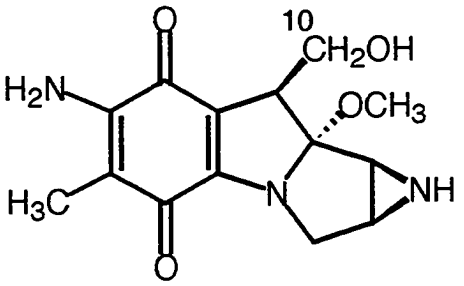
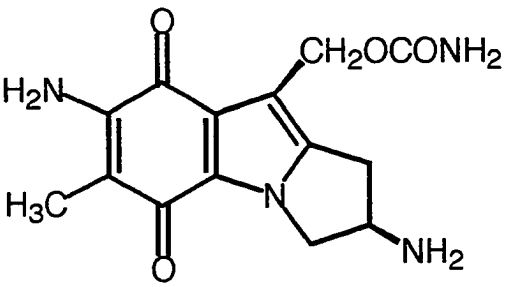


mitomycin C
(MC)



10-decarbamoyl mitomycin C
(DMC)



2,7-diaminomitosene
(2,7-DAM)

Figure 5.1 Structure of the various mitomycins

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A comparison of the three drugs with respect to their DNA cross-linking and mono-alkylating activities and their specific DNA adducts formed in the EMT6 cells is summarized in Table 5.1 and the corresponding DNA adducts are shown in Figure 5.2.

Drug	Monoadduct frequency (mol/mol DNA-nucleotide)	Cross-link frequency (mol/mol DNA-nucleotide)	Specific DNA adducts formed (Fig. 5.2) 1 2 3 4 5 6 7
MC	1.4×10^{-6}	4.8×10^{-7}	+ + + + + + -
DMC	5.3×10^{-6}	3.4×10^{-7}	+ - + - - - +
2,7-DAM	9.5×10^{-6}	0	- - - - + + -

Table 5.1 Frequencies of DNA mono-adducts and cross-links formed in EMT6 mouse mammary tumor cells treated with MC, DMC, or 2, 7-DAM at 10 uM concentration

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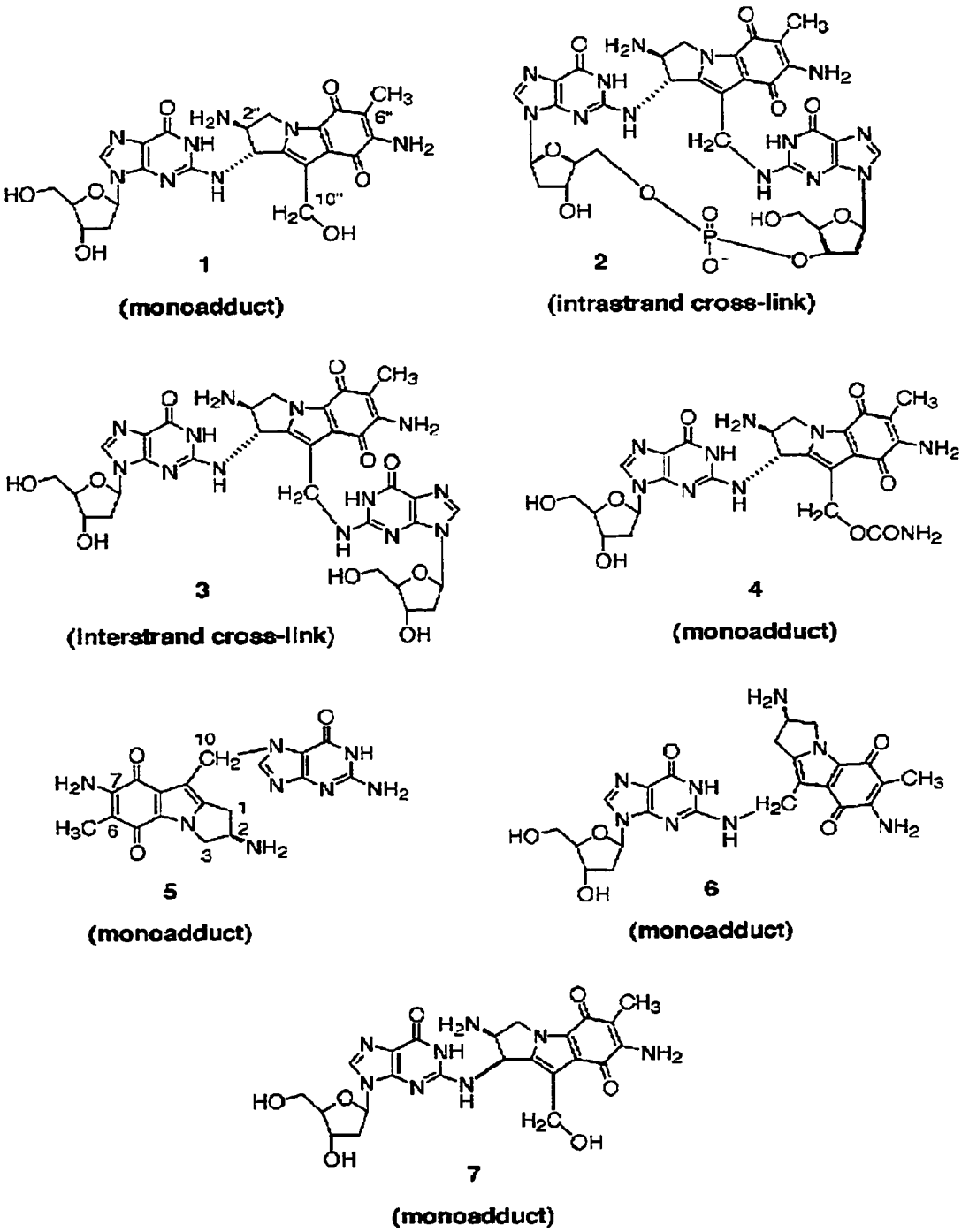

Figure 5.2 The major DNA-adducts formed in mitomycins-treated mouse mammary tumor cells

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Comparative studies of the cytotoxicity of the three mitomycins indicated that MC and DMC have closely similar cytotoxicities (Carrano *et al.*, 1979; Hoban *et al.*, 1990; Kim and Rockwell, 1995), while 2,7-DAM is essentially non-cytotoxic (Palom *et al.*, 1998). Since the cytotoxicity of these drugs correlates with the frequencies of the DNA cross-link adducts but not with the DNA mono-adducts, these findings point to cross-links as being the lesions primarily responsible for the cytotoxicities of the mitomycins (Palom *et al.*, 2002). It is therefore apparent that the specific structures of the various DNA adducts play a differential role in the cytotoxicity of the various mitomycins. However, the basis for this differential cytotoxic activity of the individual DNA adducts formed by these drugs remains unclear.

The ability of chemotherapeutic drugs to be cytotoxic correlated in many instances with p53 induction (Lowe *et al.*, 1994). Although it is known that treatment of mammalian cells with MC causes an increase in the cellular p53 protein level (Kastan *et al.*, 1991), the outcome on p53 induction by DMC and 2,7-DAM had not been investigated. Furthermore, studies focused on the cytotoxic activities of MC, DMC, or 2, 7-DAM have not addressed the possible involvement of p53 in mediating the cytotoxic or apoptotic responses. It is unclear how particular DNA adducts can activate a signal transduction program that culminates in p53 accumulation. It has been hypothesized that the cytotoxic effect of MC may be mediated by the tumor suppressor protein p53 (Kastan *et al.*, 1991; Inoue *et al.*, 2001). The ability of DNA adducts to induce p53 has been implicated by the fact that agents that generate multiple type DNA adducts also cause the accumulation of p53 (Ramet *et al.*, 1995; Khan *et al.*, 1998). However, it has been

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difficult to recognize a general correlation between cytotoxicities and the induction of p53 thus far (Zamble *et al.*, 1998). The lack of cytotoxicity of 2,7-DAM in contrast to MC and DMC may be explained by the hypothesis that 2,7-DAM -DNA mono-adducts are unable to induce p53 accumulation. We tested this hypothesis by comparing the induction of p53 in a wild-type p53 containing human myeloid leukemia cell line (ML-1) by MC, DMC and 2,7-DAM. Our data show a dramatic differential p53 response to treatment with MC, DMC and 2,7-DAM. We further show that the human myeloid leukemia k562 cell line, lacking functional p53 is resistant to the cytotoxic and apoptotic effect of MC but not DMC.

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Results:

MC and DMC, but not 2, 7-DAM can induce p53 nuclear accumulation.

The non-cytotoxic effect of 2, 7-DAM suggested that the DNA mono-adducts produced in cells after treatment with this compound (adducts 5, & 6, Figure 5.2) were unable to induce a signal transduction pathway that culminated in the induction of p53. We tested this hypothesis by treating the ML-1 cell line with MC, DMC, and 2, 7-DAM, as well as two other drugs previously shown to induce p53 (Kastan *et al.*, 1991). Treatment was carried out with the mitomycins at a concentration of 5 uM for 3 and 6 hours, and nuclear extracts were prepared and analyzed by Western blot as shown in figure 5.3. Both MC and DMC induced a robust stabilization of p53 (Figure 5.3, lanes 2-5) at both time points, while no p53 stabilization was detected in cells that had been treated with the same concentration of 2, 7-DAM (Figure 5.3- lanes 6, & 7).

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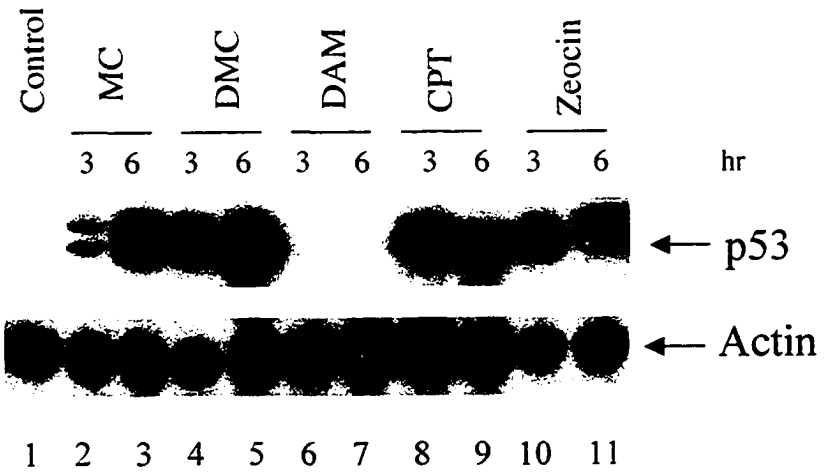

Figure 5.3 Mitomycin C (MC), and 10-decarbamoyl MC (DMC), But not 2, 7-diaminomitosine (DAM) induce p53 nuclear accumulation.

Western blot analysis of ML-1 nuclear protein extracts. Exponentially growing ML-1 cells, grown in 1X RPM1 medium and supplemented with 10% fetal bovine serum were either left untreated or treated with 5 uM MC, 5 uM DMC, 5 uM DAM, 0.5 uM camptothecin (CPT), or 50 ug/ml zeocin for 3 (lanes 2, 4, 6, 8, &10) or 6 (lanes 3, 5, 7, 9, & 11) hours. 100 ug of nuclear protein was resolved by electrophoresis on a 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with either a mixture of p53-specific monoclonal antibodies (Pab-240, Pab-421, and Pab-1801), or anti actin. Results are representative of four independent experiments.

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Higher dosages of 2,7-DAM (50-100 uM) also failed to induce detectable levels of p53 (data not shown). It is highly unlikely that 2,7-DAM was able to induce p53 since the Western blot analysis employed was carried out using a mixture of monoclonal antibodies specific to the central domain as well as the carboxyl and amino-terminus (PAb240, PAb421 and PAb1801 respectively) of p53. Both MC and DMC were able to induce p53 to levels comparable to those induced by treatment of the ML-1 cells with camptothecin and zeocin (a member of the family of bleomycins) (Figure 5.3- lanes 8-11).

MC and DMC treatment, but not treatment with 2, 7-DAM results in the transcriptional activation of p53 target genes.

The tumor suppressor p53 is a transcription factor that activates a multitude of downstream target genes (Zhao *et al.*, 2000). We analyzed the trans-activation of the endogenous *p21* and *Gadd45* genes in the ML-1 cell line in response to the chemotherapeutic drug treatments shown above. The drugs able to result in the stabilization of p53 (MC, DMC, CPT and zeocin) also induced significant trans-activation of the endogenous *p21* and *Gadd45* genes as monitored by RT-PCR with molecular beacons (Figure 5.4 A & B, respectively).

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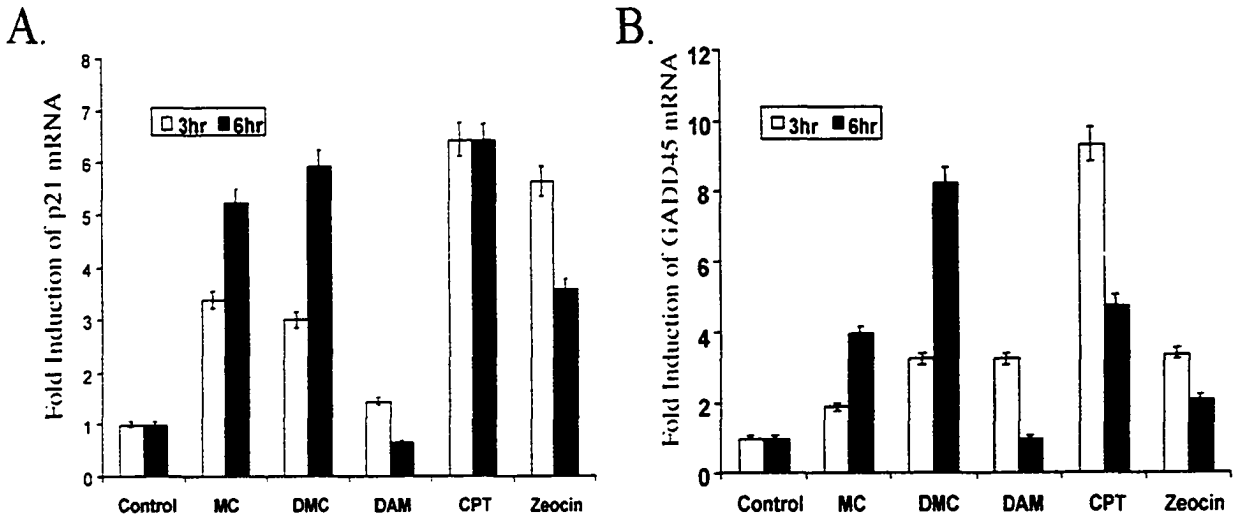

Figure 5.4 MC and DMC treatment, but not treatment with 2, 7-DAM result in the transcriptional activation of p53 target genes.

Quantitative real-time RT-PCR with molecular beacons was used to analyze (**A**), p21/Waf1 and (**B**) Gadd45 mRNA expression. Results were normalized using the control samples and the GAPDH values to give relative units of mRNA induction. Bars, the SD for the fold induction of p21 and GAD45 as shown.

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The induction of these p53 target genes by MC and DMC treatment was dose dependent (data not shown), and for *Gadd45*, was higher with DMC treatment than with MC especially at the 6 hr time point (Figure 5.4 A & B). The ML-1 cells treated with 2, 7-DAM on the other hand showed very limited trans-activation of the endogenous p53 target genes tested and this limited activation decreased at the longer time point (Figure 5.4 A and B).

The p53 induced by MC and DMC can bind to DNA.

Treatment of ML-1 cells with both MC and DMC was able to induce p53 and also resulted in the activation of *p21* and *gadd45* transcription. This suggested that these DNA damaging agents resulted in the stabilization of p53 species that were able to bind to DNA. Sandra H., a member of our laboratory analyzed the DNA binding ability of p53 in nuclear extract derived from ML-1 cells treated with MC, DMC and 2,7-DAM by electro-mobility gel shift assay (EMSA). Although MC and DMC form different but overlapping DNA adducts (Table 5.1), both resulted in an increase in the level of p53 (Figure 5.3) and an increase in the p53 DNA binding ability (Fig. 5.5 A, lanes 2 and 3) when ML-1 cells were treated with a concentration of 5 uM for 6 hour.

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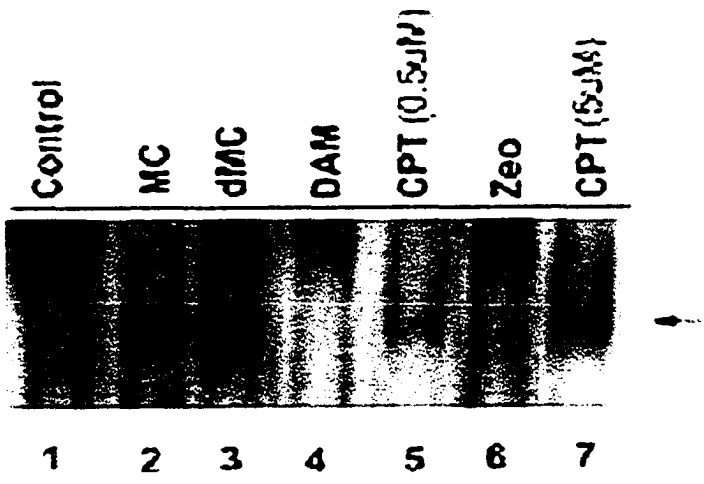

Figure 5.5 MC and DMC-induced p53 exhibits DNA binding activity.

Gel mobility shift assay of p53 super-consensus site with nuclear proteins extracted from ML-1 cells. ML-1 cells were grown in 1X RPMI medium, supplemented with 10% FBS. Exponentially growing cells were either left untreated (lane 1), or treated with 5uM MC (lane 2), 5uM DMC (lane 3), or 5uM DAM (lane 4) for 6 hours. Nuclear extracts were then incubated with ^{32}p-labeled DNA-oligonucleotides corresponding to the p53 super-consensus site (SCS), electrophoresed on a 4% polyacrylamide gel and visualized by autoradiography.

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As expected, no p53 specific gel shift was detected in nuclear extract derived from ML-1 cells treated with 5 uM 2, 7-DAM for 6 hours (Figure 5.5 A, lane 4), as treatment with this drug also did not result in a detectable increase in p53 protein by Western blot analysis (Figure 5.3). The PAb421 induced gel shift species detected in the ML-1 nuclear extract from drug treated cells has been determined to be specific by competition analysis with non-labeled specific and non-specific oligonucleotide (data not shown).

MC and DMC, but not 2, 7-DAM are able to induce apoptosis efficiently in the ML-1 cell line containing wild-type p53.

Because treatment of ML-1 cells with MC and DMC, but not 2,7-DAM, was able to result in the stabilization of p53, we asked if this correlated with the ability of MC and DMC, but not 2,7-DAM, to induce apoptosis. ML-1 cells were treated with MC, DMC or 2,7-DAM at equidose concentration of 5 uM for 24 hours and the cells were then fixed and stained with propidium iodide. The samples were analyzed by FACS and compared to non-drug treated cells to assess the increase in sub-G_1 DNA content (Figure 5.6 A). Both the MC and DMC treated ML-1 cells showed a substantial increase in sub-G_1 DNA content (although not as dramatic as that seen with CPT), indicating that the ML-1 cells underwent programmed cell death (apoptosis) in response to these drugs. ML-1 cells treated with 2, 7-DAM on the other hand showed no increase in sub-G_1-DNA content after 24 hours of treatment, indicating that these cells were in fact not undergoing cell death. Apoptosis in ML-1 cells was also monitored by PARP cleavage (by Western blot analysis) and this further confirmed the induction of apoptosis by MC and DMC but not by 2, 7-DAM treatment (Figure 5.6 D, lanes 4, &6).

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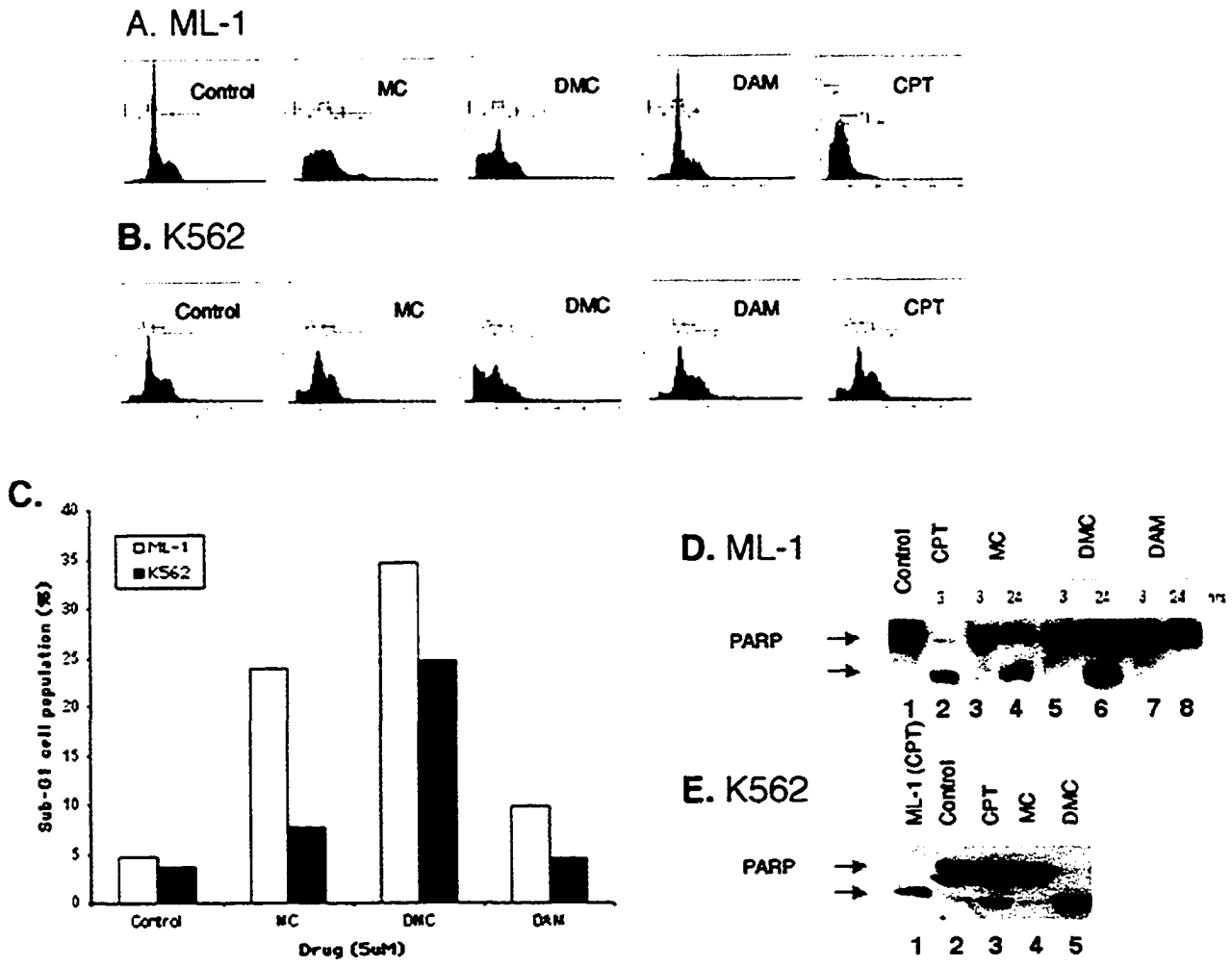

Figure 5.6 MC and DMC, but not 2, 7-DAM induce apoptosis in ML-1 cells, and only DMC still induces significant apoptosis in the absence of p53.

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Figure 5.6 MC and DMC, but not 2, 7-DAM induce apoptosis in ML-1 cells, and only DMC still induces significant apoptosis in the absence of p53.

***A* and *B*,** FACS analysis of ML-1 and K562 cells. Cells were grown in 1X RPMI supplemented with 10% FBS. Exponentially growing cells either were left untreated (control) or treated with 5 uM MC, 5uM DMC, 5uM 2,7-DAM, or 0.5 uM CPT for 24 hours. Cells were fixed with 30% ethanol, stained with propidium iodide for 24 hrs, and analyzed by flow cytometry cell sorting. ***C*,** Quantitative comparison of sub-G_1 DNA content in ML-1 and K562 cells. ***D*,** Western blot analysis of ML-1 nuclear extracts using anti-human PARP antibody. Nuclear proteins extracted from ML-1 cells were resolved by 10 % SDS-PAGE. Drug treatment was for 3 and 24 h except in the case of CPT, which was for 3 h as indicated. Anti-actin was used as a loading control (not shown). ***E*,** Western blot analysis of nuclear extracts of K562 cells untreated or treated with CPT (0.5 uM), MC (5 uM), DMC (5 uM), or 2,7-DAM (5 uM) for 24 h. Extracts were resolved by 10% SDS-PAGE and probed with anti-human PARP antibody.

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In order to address the ability of MC and DMC to induce apoptosis in the absence of p53, the K562 cell line was treated with the same battery of drugs and analyzed in the same way. No substantial increase in sub-G_1 DNA content was observed when the K562 cells were treated for 24 hours with MC, 2, 7-DAM or CPT (Figure 5.6 B, & C). Surprisingly, although K562 cells were largely resistant to the apoptotic effect of MC, K562 cells treated with DMC underwent substantial apoptosis as determined by morphological changes (Figure 5.7), FACS analysis and PARP cleavage in Western blot analysis (Figs 5.6 B, C & E respectively), suggesting that DMC was able to induce p53-independent apoptosis not induced by MC. (Also see chapter 6).

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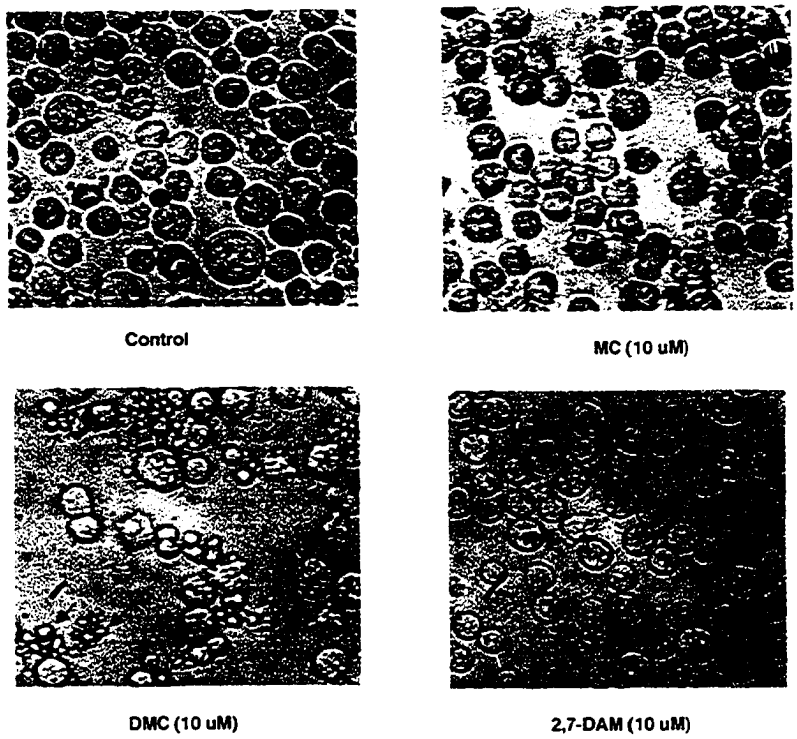

Figure 5.7 DMC, but not MC or 2, 7-DAM is capable of significantly inducing apoptosis in K562 cells lacking p53.

Microscopic examination of K562 cells treated with MC (10 uM), DMC (10 uM), or 2, 7-DAM (10 uM) for 24 h.

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MC and DMC are cytotoxic to ML-1 cells while 2, 7-DAM is not, and only DMC is cytotoxic in the absence of p53.

Cytotoxicity can be observed in the absence of apoptosis (Avantaggiati *et al.*, 1997), and therefore the cytotoxicities of the panel of mitomycins were compared utilizing the ML-1 cells to confirm that 2, 7-DAM were not cytotoxic. Cytotoxicity of the three mitomycins was monitored by the MTT cytotoxicity assay and trypan blue exclusion assay. Cytotoxicity was observed when ML-1 cells were treated with MC and DMC but not when they were treated with 2,7-DAM (Figure 5.8 A).

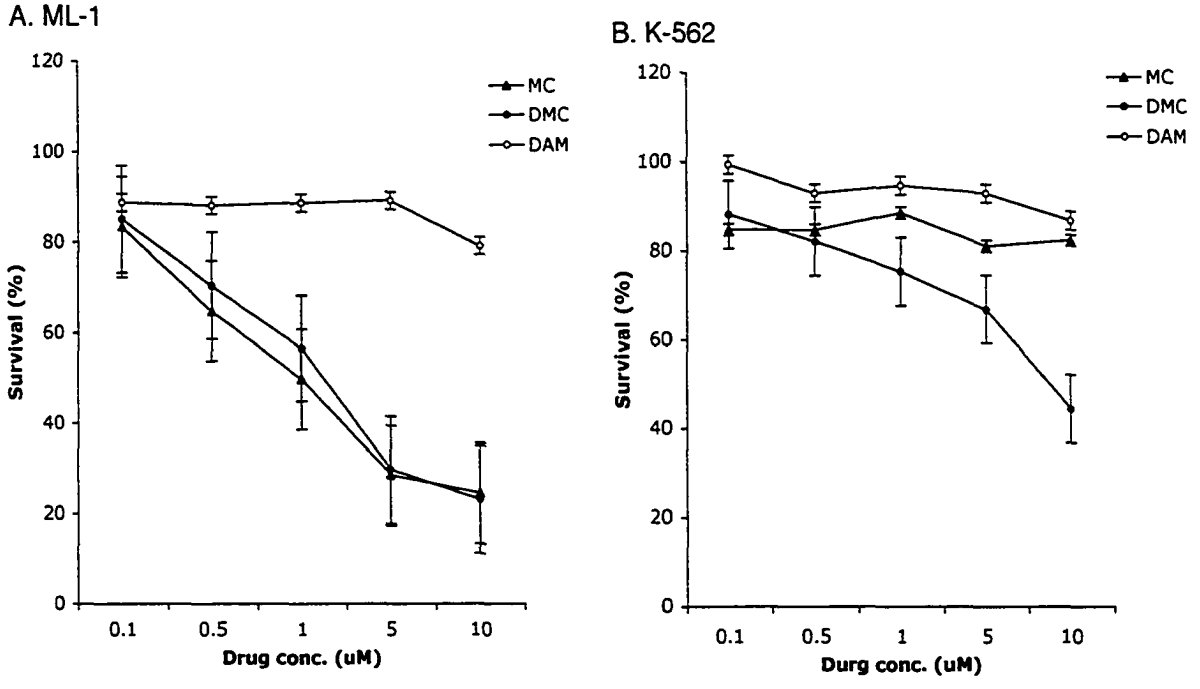

Figure 5.8 MC, and DMC, but not 2, 7-DAM are cytotoxic in ML-1 cells, and only DMC is cytotoxic in K562 cells.

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Figure 5.8 MC, and DMC, but not 2, 7-DAM are cytotoxic in ML-1 cells, and only DMC is cytotoxic in K562 cells.

MTT cytotoxicity assay of ML-1 ***(A)*** and K562 ***(B)*** cells: Exponentially growing ML-1 and K562 cells were either left untreated or treated with graded doses of MC, DMC, or 2, 7-DAM for 24 hrs. Cells were then incubated in MTT containing media (0.5 mg/ml) at 37 °C for one hr. Equal aliquots of cell lysates were used for absorbance measurements. Data are expressed as the difference between absorbance at 550 and 620 nm. Bars represent mean of two independent experiments +/- SD.

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Similar results were obtained when cells were assayed by the trypan blue exclusion assay (data not shown). Therefore 2,7-DAM treatment of ML-1 cells did not induce p53, did not induce apoptosis and was not cytotoxic to the ML-1 cells. In order to address if the cytotoxicity induced by MC and DMC was p53-dependent, we analyzed the cytotoxicity of these drugs in the K562 cells, which lack p53. As expected, when K562 cells were assayed for cytotoxicity using the MTT assay, 2, 7-DAM was observed to be non-cytotoxic, wile MC demonstrated limited cytotoxicity (Figure 5.8 B). Consistent with its ability to induce p53-independent apoptosis, DMC was cytotoxic, and the level of DMC-induced cytotoxicity in k562 was comparable to that induced in ML-1 cells (Figure 5.8 compare A and B).

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Discussion:

DNA adducts do not always activate the p53 pathway.

Normally p53 is present at undetectable levels and specific stimuli are able to elicit signal transduction pathways that allow for the stabilization of p53. Chemotherapeutic drugs that damage DNA function to a great extent by activating pathways that result in p53 stabilization (Kastan *et al.*, 1991). MC is a chemotherapeutic drug whose mechanism of action continues to be investigated. Here we demonstrate that, in contrast to the DNA-adducts induced by MC and DMC, 2,7-DAM, the mono-functional, major metabolite of MC allows for DNA-mono-adducts that is unable to stabilize p53. Thus, this form of DNA damage evades the p53 tumor suppressor pathway and is not cytotoxic.

MC and DMC were previously shown to be equally cytotoxic in various rodent cell lines (Carrano *et al.*, 1979; Hoban *et al.*, 1990; Kim and Rockwell, 1995). Our data extends this finding to human myeloid leukemia cells. The cytotoxicity of MC has been attributed mainly to the generation of lethal DNA cross-links. DMC was initially thought to be devoid of DNA cross-linking activity, based on tests carried out in cell-free systems, which employed chemical reductive drug activation. (Carrano *et al.*, 1979; Tomasz, 1988) Consequently, DMC has traditionally been regarded as the mono-functional counterpart of MC. Using a mouse mammary tumor cell line, it has been recently demonstrated that DMC generates the DNA cross-link adduct **3** (see figure 5.2) at frequencies comparable to those observed with MC under equidose drug treatment

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conditions (Table 5.1). In contrast, the frequencies of mono-adducts generated by both drugs widely differed, with DMC displaying 20 to 50 fold greater mono-adduct frequencies than MC. (Palom *et al.*, 2002) (see Table 5.1 and Figure 5.2). These findings suggest that the DNA cross-links common to both drugs, are the major determinants of the drugs' cytotoxicity and possibly their ability to induce the stabilization of p53. Another common property of MC and DMC is their higher redox potential compared to that of 2,7-DAM and theoretically this could allow them to cause more DNA oxidative damage by quinone redox cycling. However, such oxidative damage has not been detected and is not thought to play a significant role in the cytotoxicity of MC in tumor cells. It is consistent with this hypothesis that 2,7-DAM which is non-cytotoxic does not cause DNA cross-linking (Palom *et al.*, 1998; Palom *et al.*, 2001), and does not cause the stabilization of p53. Although it is clear that DNA mono-adducts formed upon treatment of EMT6 cells with 2, 7-DAM (Palom *et al.*, 1998; Palom *et al.*, 2000) (Table 5.1, & Figure 5.2) can not cause the stabilization of p53, our study does not rule out the possibility that DNA mono-adducts formed upon treatment of EMT6 cells with MC (adducts 1, 2, 4, & 7, Figure 5.2), and not produced by 2, 7-DAM, may have the ability to activate the p53 pathway.

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A relationship exists between the ability to induce p53, the DNA cross-links formed by the mitomycins, and their cytotoxicty.

It has been shown that the 2, 7-DAM DNA-mono-adducts do not contribute to the cytoxicity induced by MC. (Palom *et al.*, 1998; Palom *et al.*, 2001) In keeping with this lack of cytoxicity of 2, 7-DAM we show here that this compound is unable to induce p53, suggesting a connection between MC cytoxicity and the ability of MC to induce p53 in ML-1 cells (human myeloid leukemia). Indeed, of the three mitomycins investigated here, only MC and DMC form DNA cross-links and only these two drugs elicit the accumulation of p53, while 2,7-DAM does not. This indicates a positive correlation between DNA cross-links and p53 induction by the mitomycin drug series, as seen in human myeloid leukemia cells. In this study, we show that the human myeloid leukemia K562 cell line, lacking p53 is largely resistant to the apoptotic and cytotoxic effect of MC, suggesting that the apoptotic and cytotoxic effects of MC require functional p53. It is possible that tumors deficient in p53 and/or its upstream or downstream pathway, similar to K562 cell line are resistant to MC chemotherapy. It will be important to test the generality of this hypothesis by employing additional tumor cell lines in analogous experiments.

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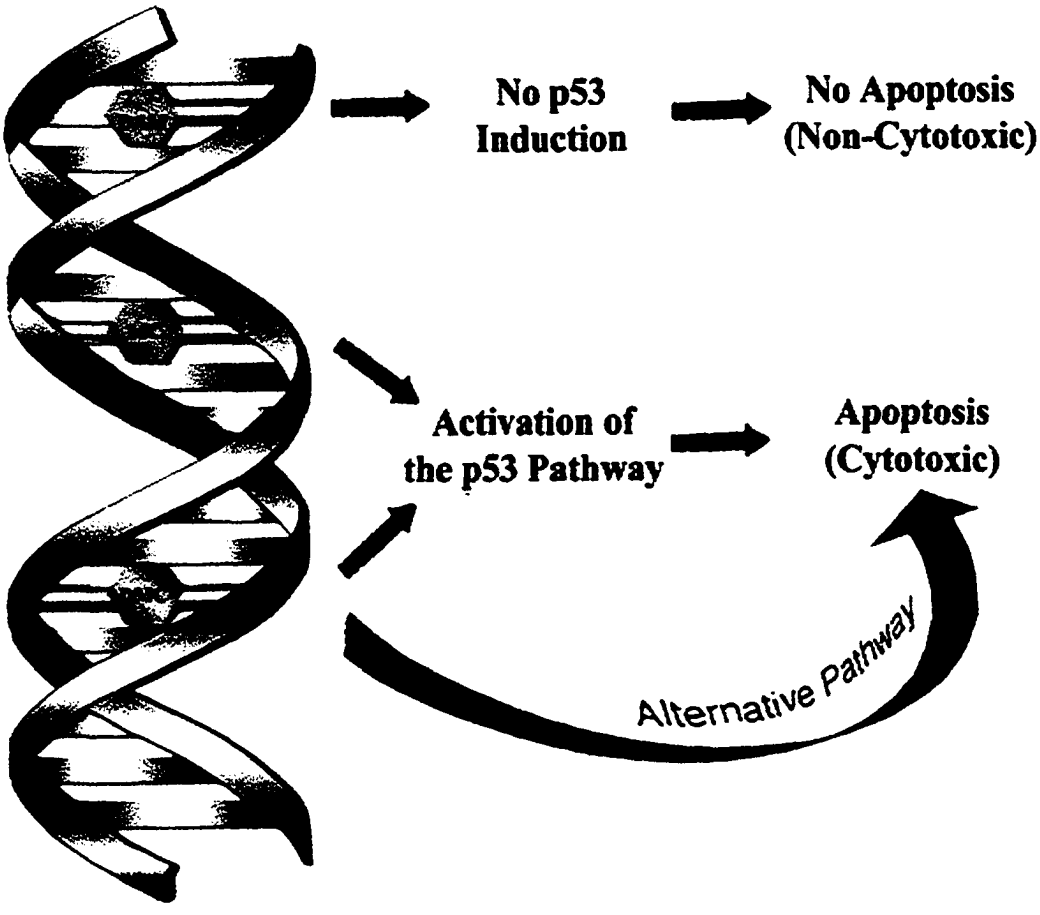

Figure 5.9 Proposed model for the various adducts made by the various mitomycins and their ability to signal to p53 and induce apoptosis.

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In the cell much of mitomycin C is broken down to the mono-functional derivative 2, 7-DAM (Chirrey *et al.*, 1995). The clinical dosage of MC given is high and under such conditions a large proportion of the DNA damage is expected to be in the form of 2, 7-DAM mono-functional DNA adducts (Palom *et al.*, 1998; Palom *et al.*, 2001). The fact that 2, 7-DAM was unable to induce the stabilization of p53 suggests that this damage either goes undetected, or that it is rapidly repaired prior to activating any downstream signal that culminates in the stabilization of p53. If in fact the damage induced by 2, 7-DAM is quickly repaired, then the repair pathway itself does not signal to p53. However, because adducts of 2, 7-DAM with the DNA can be isolated from nuclei of treated cells, they must remain long enough to be detected, and thus the former suggestion appears more likely. DNA-adducts that can be evaded by the p53 pathway appear surprising because we know that multiple types of DNA damage, as well as cellular stress, can activate p53 (Kastan *et al.*, 1991). If the 2,7-DAM-induced damage is evaded by the signal transduction pathways required for p53 activation, then after MC treatment, most of the DNA damage would exist in a form that could not perform a therapeutic outcome. This would ultimately be detrimental for genomic stability.

Our study suggests that DMC may represent a more compelling chemotherapeutic than MC for a number of reasons; firstly, a mechanism exists in cells to evade the cytotoxic potential of MC through reductive conversion of MC to 2, 7-DAM (Palom *et al.*, 1998). Therefore, DMC or other derivatives, or analogues of MC that cannot be metabolized to 2, 7-DAM may be more effective in treating malignancies. Secondly, similar to MC, DMC can induce p53, but unlike MC is capable of inducing apoptosis and

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is cytotoxic even in the absence of p53 (as observed by drug treatment of the K562 myeloid leukemia cell line lacking p53). DMC is able to activate an alternative DNA damage pathway (Figure 5.9), which may utilize either p73 or BRCA1 (Harkin *et al.*, 1999; Chen *et al.*, 2001). This may be clinically relevant given that over 50% of total human cancers have defective or mutated *p53* genes (Nigro *et al.*, 1989). It may be hypothesized that DNA adduct 7 (Figure 5.2), formed upon treating cells with DMC, but not MC is particularly involved in activating a signaling transduction program that culminates in induction of apoptosis, since this DNA mono-adduct is the only adduct formed upon treating EMT6 cells by DMC and not formed upon MC treatment.

Our data show that mono-adducts of DNA by 2, 7-DAM, the major metabolite of MC, are unable to activate the p53 pathway (Figure 5.9). An understanding of the relationship between the metabolic activation of bio-reductive drugs and the death of cancer cells is needed to optimally exploit the drugs for more effective chemotherapy. Reductive metabolism of MC to 2, 7-DAM actually allows the concentration of the active cytotoxic form of MC to be reduced in the cell. 2, 7-DAM may be a compound that potentially will have deleterious effects by damaging the DNA in a way that is evaded by the p53 tumor suppressor pathway and could ultimately result in increased genomic instability and new tumors. Importantly, MC derivatives, such as DMC, have the potential to target tumors with inactive p53, which might be resistant to other drugs signaling exclusively through the p53 pathway. It will be important to consider the target pathways of chemotherapeutic drugs when assessing which drugs should be used for specific types of cancers.

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Chapter 6

10-decarbamoyle mitomycin C (DMC); a novel DNA –alkylating and cross-linking agent with p53-independent cytotoxic activity

Preliminary data and future direction

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*I*n our study, we have shown that similar to MC, the MC derivative 10-decarbamoyl-MC (DMC) is capable of stabilizing the tumor suppressor p53 protein and is similarly cytotoxic. Surprisingly, but significantly utilizing the p53-lacking human leukemia cell line, we found that unlike MC, DMC is highly apoptotic and cytotoxic. Our finding that DMC treatment of human cells allowed for the stabilization of p53 that is capable of trans-activating down-stream target genes and is still quite apoptotic and cytotoxic in cells that lack p53 suggest that DMC has a p53-dependent as well as p53-independent cytotoxic activity (Figure 5.9). The mechanism by which DMC induces p53-independent apoptosis is currently being investigated in our laboratory in collaboration with Dr. Maria Tomasz who will supply the MC and DMC and provide expert opinion on the metabolic activities of these drugs. Our preliminary data show that DMC unlike MC is significantly apoptotic in another p53-lacking lung epithelial cell line (H1299) (Figure 6.1). Surprisingly, DMC was found not to be apoptotic in a Burkett's lymphoma cell line (Manca) that harbors a wild type p53 gene (data not shown). Interestingly, in this cell line we have evidence to suggest that although the p53 is wild type, it is incapable of trans-activating down-stream target genes. Preliminary data generated by Tammy G., a member of our laboratory, suggest that in this cell line, the negative regulator of p53, Mdm2 is constitutively bound to p53 and possibly responsible for inactivating the p53 protein. Recently, it has been shown that Mdm2 can also inactivate the tumor suppressor p73 (Dobbelstein *et al.*, 1999), a major player in mediating DNA damage-induced apoptotic response (Yuan *et al.*, 1999; Chen *et al.*, 2001; Catani *et al.*, 2002; Flores *et al.*, 2002). These data along with our findings as well as the fact that Manca cells are resistant

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to DMC-mediated apoptosis suggest that p73 might be the mediator of DMC-mediated and p53-independent apoptosis. We propose that DMC unlike MC is capable of signaling not only through the p53 protein, but also through its family member p73 tumor suppressor protein.

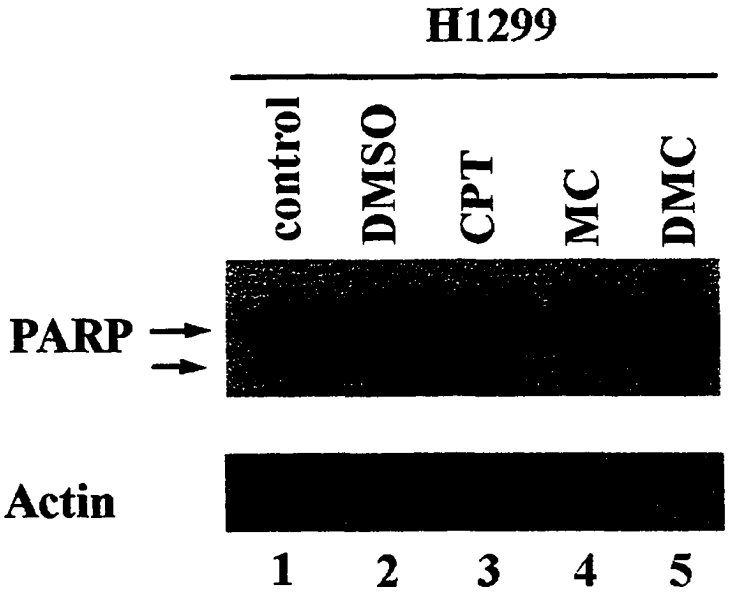

Figure 6.1 DMC, but not MC or CPT induces substantial apoptosis in H1299 cells.

Western blot analysis of H1299 nuclear protein extracts. H1299 cells were grown as described in materials and methods and were either left untreated, or treated with CPT (0.5 uM), MC (5 uM), or DMC (5 uM) or solvent (DMSO) for 24 hours. Proteins were harvested and analyzed as described previously and membrane was probed with human anti-PARP or anti-actin antibodies.

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We will test this hypothesis by investigating the effect of MC and DMC on p53 deficient, p73 deficient, and p53/p73 deficient mouse embryonic fibroblasts. We will further investigate the p73 accumulation and trans-activation activity in response to the various mitomycins in a variety of rodent as well as human cell lines. We would also like to begin to dissect the mechanism by which apoptosis is induced in response to DMC in the context of DNA damage induced and p53-independent apoptosis. We will test the involvement of cytochrome C and activation of the various effecter caspases. We will utilize various biological and biochemical techniques to carefully examine the kinetics of DMC induced and p53-independent cytotoxicity.

The lack of apoptotic effect of DMC in the Manca cell line as compared to K562 and ML-1 can alternatively be explained by our recent finding that Manca cells exhibits a constitutive NFκB activity (data not shown) similar to that seen with other B lymphoma cell lines (Neri *et al.*, 1991; Fracchiolla *et al.*, 1993). Since the primary transcriptional target of NFκB is the well-established pro-survival molecule Bcl2, it is possible that NFκB provides a bcl2 survival signal that opposes a DMC mediated, p53-independent, and p73-dependent apoptotic signal, possibly via the pro-apoptotic Bcl2 family member Bax or a similar molecule. A support for such hypothesis stems from the fact that c-Myc which is highly expressed in Burkett's lymphomas (including Manca cells) selectively binds to the N-terminus of the p73 tumor suppressor protein, thereby inhibiting transcription from the *p21* and *Bax* promoters (Watanabe *et al.*, 2002). Another support for such hypothesis comes from our surprising finding that TPA protects not only against p53-dependent apoptosis, but also against DMC-mediated and p53-independent apoptosis

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in ML-1 cells (Figure3.1 A). In our study, we found that TPA highly activates the NFκB-DNA binding (Figure 4.2 B). In such a setting, we propose that DMC can not induce apoptosis due to functional inactivation of Bax or a related pro-apoptotic protein by Bcl2. Interestingly, K562 cells which lack p53, and are sensitive to the apoptotic activity of DMC do not show similar activation of NFκB-DNA binding activity (Figure 4.2 B) in response to TPA treatment, and they are not protected from the DMC apoptotic activity in the presence of TPA (Figure 3.1 C, lane 7, & 8). The ability of TPA to activate NFκB has been studied in great details and has been suggested to be dependent upon PKC activity. It is unclear why TPA is incapable of activating the NFκB-DNA binding activity in K562 (Figure 4.2 B), but it is possible that this is dependent on the expression pattern of the various PKC isoforms or even on the status of p53 in these cells. Further analysis of the TPA affects on transcriptional regulation of *p53*, and a careful investigation of the DMC p53-dependent and p53-independent cytotoxic activities will greatly enhance our understanding of the mechanisms underlying tumor promotion, and will provide alternative avenues that can be exploited in our war against cancer.

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