DNA Adducts of 10-decarbamoyl Mitomycin C Activate p53-dependent and p53-independent Cell Death

by

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Abstract

Title: DNA Adducts of 10-decarbamoyl Mitomycin C Activate p53-dependent and p53-independent Cell Death

by

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Mitomycin C (MC), a natural antibiotic and DNA cross-linking agent, has cytotoxic activity and is known to activate the tumor suppressor p53 protein. 10-decarbamoyl mitomycin C (DMC), a derivative of MC, has increased cytotoxicity compared to MC. Both MC and DMC induce cellular cytotoxicity in cells with wild-type p53, while only DMC shows significant cell death activity in the absence of wild-type p53. We investigated the difference in MC and DMC cytotoxicity by comparing DNA adduct composition and the cellular regulation of molecular targets in human cancer cell lines with or without wild-type p53. Compared to MC, DMC produced substantially more mitosene-1-β mono and 1-β cross-link adducts in DNA and resulted in abnormal nuclear morphology in human cancer cells with or without p53. Significantly, greater poly(ADP-ribose)polymerase (PARP) activity was observed after DMC treatment in both the presence and absence of wild-type p53. Both MC and DMC induced double strand breaks as indicated by gamma-H2AX foci formation irrespective of the p53 status, suggesting that double strand breaks cannot account for DMC's increased cytotoxicity. In cell lines expressing wild-type p53, both MC and DMC signaled for p53 stability and apoptosis induction resulting in

cleavage of procaspase-3 and -8. Despite the DMC induced cellular cytotoxicity observed in cell lines lacking wild-type p53, cleavage of procaspase-3 or -8 was not observed in these cells. However, we observed an increase in caspase activity. Caspase-2 activation has been suggested as a pathway for p53-independent cell death in the absence of Chk1. Interestingly, Chk1 was depleted following DMC, but not MC treatment in cells with or without wild-type p53. This Chk1 depletion was achieved through the ubiquitin proteasome pathway since chemical inhibition of the proteasome protected against Chk1 depletion. Additionally, gene silencing of Chk1 by siRNA increased the cytotoxicity of MC but not of DMC. DMC treatment also caused a decrease in the level of total ubiquitinated proteins without increasing proteasome activity. This suggests that DMC- mediated DNA adducts facilitate signal transduction to a pathway targeting proteins for proteolysis. In conclusion, we have found that DMC generates significantly more mitosene-1-β stereoisomeric DNA adducts than MC and causes rapid down-regulation of multiple cellular targets. These studies suggest increased mitosene-1-β stereoisomeric DNA adducts more effectively signal for a mode of cell death which does not require a functional p53 protein.

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This thesis is dedicated to my mother, Anna Ekua Howard. Thank you for being the light of inspiration and helping me know and appreciate the true meaning of perseverance and resilience. To my father and step mother, thanks for all your support throughout the years. A special thank you and appreciation to my partner and best friend, Erica, for believing in me. To my two brothers, Eric and Ben, thank you. To my friend, the soon to be Dr. Sarah Dixon, thanks for all the years of listening to me. I will forever appreciate you.

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CHAPTER 1
INTRODUCTION

1.1 Mitomycins

1.1A Family and activation

Since their discovery, quinone-containing alkylating agents have received considerable attention due to their antitumor properties. Mitomycin C (MC), a bioreductive DNA alkylating agent, is a well known antibiotic and chemotherapeutic agent (Palom et al., 2002; Sartorelli et al., 1994; Tomasz and Palom, 1997). Mitomycin C belongs to a group of naturally occurring secondary metabolites composed of mitomycin A, B, C, and porfiromycin (Begleiter, 2000). Hata et al isolated this group of metabolites from *Streptomyces caespitosus* fermentation broth cultures in 1956 (Claridge et al., 1986; Coleman et al., 2004; Hata et al., 1956). Since then, this group has been expanded to include approximately 15 or more other secondary metabolites (Kang et al., 2006; Sitachitta et al., 2007). By far, the only members of this group reported to have any significant antigrowth and chemotherapeutic properties are mitomycin C and porfiromycin (Begleiter, 2000; Fracasso and Sartorelli, 1986).

The mitomycins have three main structural components: quinone, carbamate, and aziridine groups (Figure 1). Unreduced mitomycin is unable to bind to DNA, making reduction a prerequisite for the DNA binding form of the prodrug (Palom et al., 1998a; Tomasz and Palom, 1997). To generate DNA binding electrophilic species, quinone group of mitomycin is reduced to activate its alkylating property (Sartorelli et al., 1994; Siegel et al., 1992; Tomasz and Lipman, 1981). Briefly, the activation of mitomycins antitumor properties requires an initial reduction of the quinone moiety, opening of aziridine ring followed by DNA alkylation (Palom et al., 2001; Sartorelli et al., 1994; Tomasz and Palom, 1997). Thus, reductive state of the quinone group to a semiquinone derivative modulates activity of the alklytaing group (Siegel et al., 1992; Tomasz et al., 1988).

Reduction of the quinone group during activation is mediated through well established mechanism of enzymatic reduction (Palom et al., 2001; Paz et al., 2004; Spanswick et al., 1998; Tomasz and Lipman, 1981). Enzymatic activation is undertaken by a variety of flavoreductases which utilize NADH or NADPH as electron donors (Paz et al., 2001). This group of enzymes includes the flavoreductase enzymes NADPH-cytochrome *c* reductase, xanthine oxidase, NADH-cytochrome b_5 reductase and DT-Diaphorase, NADPH-cytochrome P-450 oxidoreductase (Seow et al., 2004; Suresh Kumar et al., 1997). Interestingly, no single reductase has been shown to have increased binding to MC nor has the absence of an individual reductase completely abrogated drug activation (Palom et al., 2001; Tomasz and Palom, 1997).

Two major factors, oxygen levels and pH, have been shown to modulate the enzymatic activation of the mitomycins (Pan et al., 1993; Tomasz and Palom, 1997; Yu and Pan, 1993). Under aerobic conditions, enzymatic activation is less robust compared to hypoxic conditions which favors increased production of DNA adducts (Palom et al., 2001). The lack of robust DNA adduct production under aerobic conditions has been attributed to increase reoxidation of MC intermediates by O_2 (Siegel et al., 1990; Suresh Kumar et al., 1997). The semiquinone product that is generated during activation of MC can act as an electrophile, which attacks DNA to form adducts or consume O_2 to regenerate MC and form oxygen radicals. The significance with respect to drug sensitivity is observed in cell lines which have acquired resistance to mitomycin C (Hoban et al., 1990). Under hypoxic conditions, these cells lose their resistance and become sensitive to MC. This can possibly be attributed to increased production of DNA adduct under hypoxic conditions. Perhaps, increase production of DNA adducts overwhelms the cellular repair machinery resulting in the activation of cell death pathway.

The pH of the cellular environment has also been proposed to have a significant effect on mitomycin activation and DNA adduct production (Pan et al., 1993). For example, *in vitro* studies using purified cell free extracts indicated increased production of DNA monoadducts and cross-links at acidic pH compared to neutral pH (Yu and Pan, 1993). Similar activity has been observed in tissue culture models where increased cross-links adducts are observed under acidic conditions (Pan et al., 1993; Siegel et al., 1993).

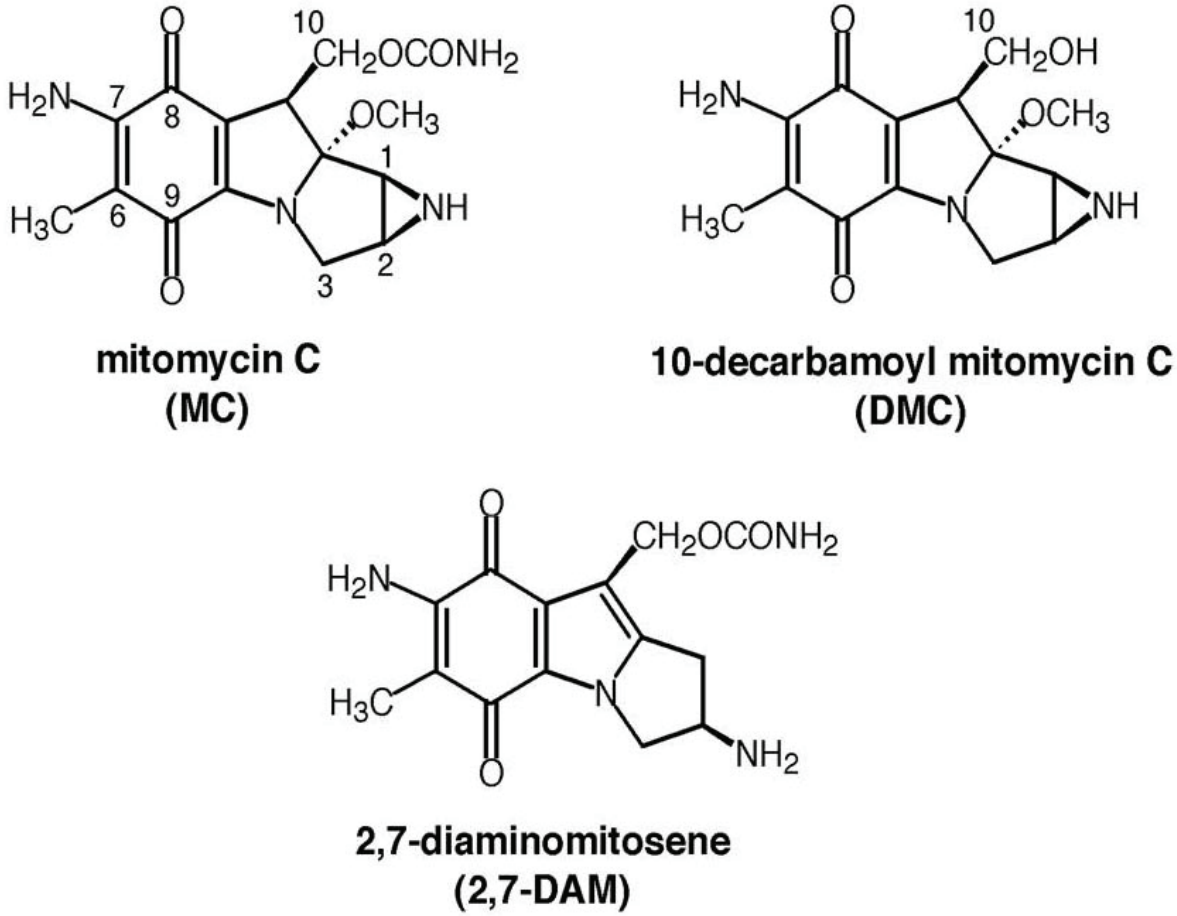


Figure 1. Structure of mitomycin C and its derivatives

1.1B Derivatives and DNA adducts

As a prototype bioreductive agent, activation of MC generates derivatives which have been shown to interact with DNA template and form adducts (Palom et al., 2000; Sartorelli et al.,

1994; Tomasz and Lipman, 1981; Tomasz and Palom, 1997). The known derivatives produced upon activation of MC are, 2,7-diaminomitosene (2,7-DAM) and 10-decarbamoyl mitomycin C (DMC) (Figure 1).

2,7-DAM , the major intracellular metabolite of MC, has been reported to form mainly monoadducts upon its interaction with DNA (Palom et al., 1998a; Subramaniam et al., 2001; Tomasz and Lipman, 1981). These monoadducts have very low cytotoxic properties in both human and murine cell lines (Abbas et al., 2002a; Palom et al., 1998a; Utzat et al., 2005). This cellular outcome is substantiated by lack of markers associated with arrest or death in cell culture models (Abbas et al., 2002a).

Interestingly, DMC forms similar DNA structures as MC and is also reported to have increased biological activity compared to MC in multiple cell lines (Abbas et al., 2002a; Boamah et al., 2007; Palom et al., 2000). Within intracellular compartment, like MC, DMC is metabolized by reductive enzymes to generate reactive DNA binding species and oxygen radicals through redox cycling (Palom et al., 2002). The reactive oxygen species generated during the activation of either mitomycins is not associated with drug cytotoxicity in cell lines (Sartorelli et al., 1994; Sartorelli et al., 1993; Tomasz and Palom, 1997).

Activated mitomycins preferentially bind guanine in a CpG sequences within minor groove (Kumar et al., 1995; Palom et al., 2000; Palom et al., 2001). To form a monoadduct, the C1 aziridine group of a mitomycin molecule interacts with guanine at the N^2-position (Figure 2 and Figure 3). This interaction is terminal and does not form cross-link products. To generate cross-links, a molecule of drug binds the N^2 of guanine using its C1 group and sequentially interacts with the N^2 of another guanine using the C10 group (Palom et al., 2002; Paz et al., 2008; Tomasz et al., 1974). If the second interaction happens with a guanine on the same strand

of DNA, an intrastrand cross-link product is generated; interstrand cross-links are formed when the second guanine interaction is on a different DNA strand (Bizanek et al., 1992; Palom et al., 2002).

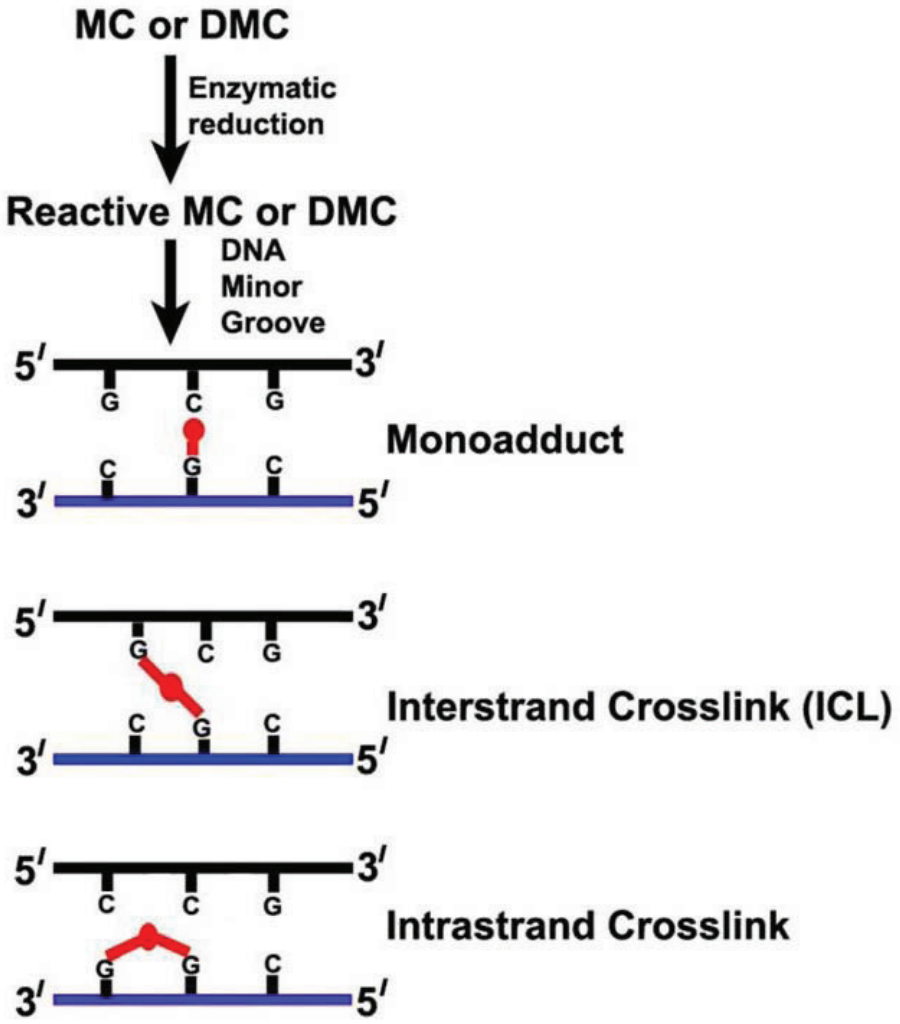


Figure 2. Diagrammatic representation of DNA adducts formed by the mitomycins

The DNA adduct structures formed by mitomycin and its metabolites within the minor groove causes very little disturbance of the native structure of the B form of DNA, however, the structure of the intrastrand cross-link has been reported to cause slight DNA bending (Rink et al., 1996). In the absence of extreme DNA distortions, the ability to induce potent antitumor and

antigrowth properties has been attributed to the interstrand cross-links products (Palom et al., 2002). Specifically, interstrand cross-links exert great anti biological activity because these form of adducts inhibit strand separation during replication and transcription.

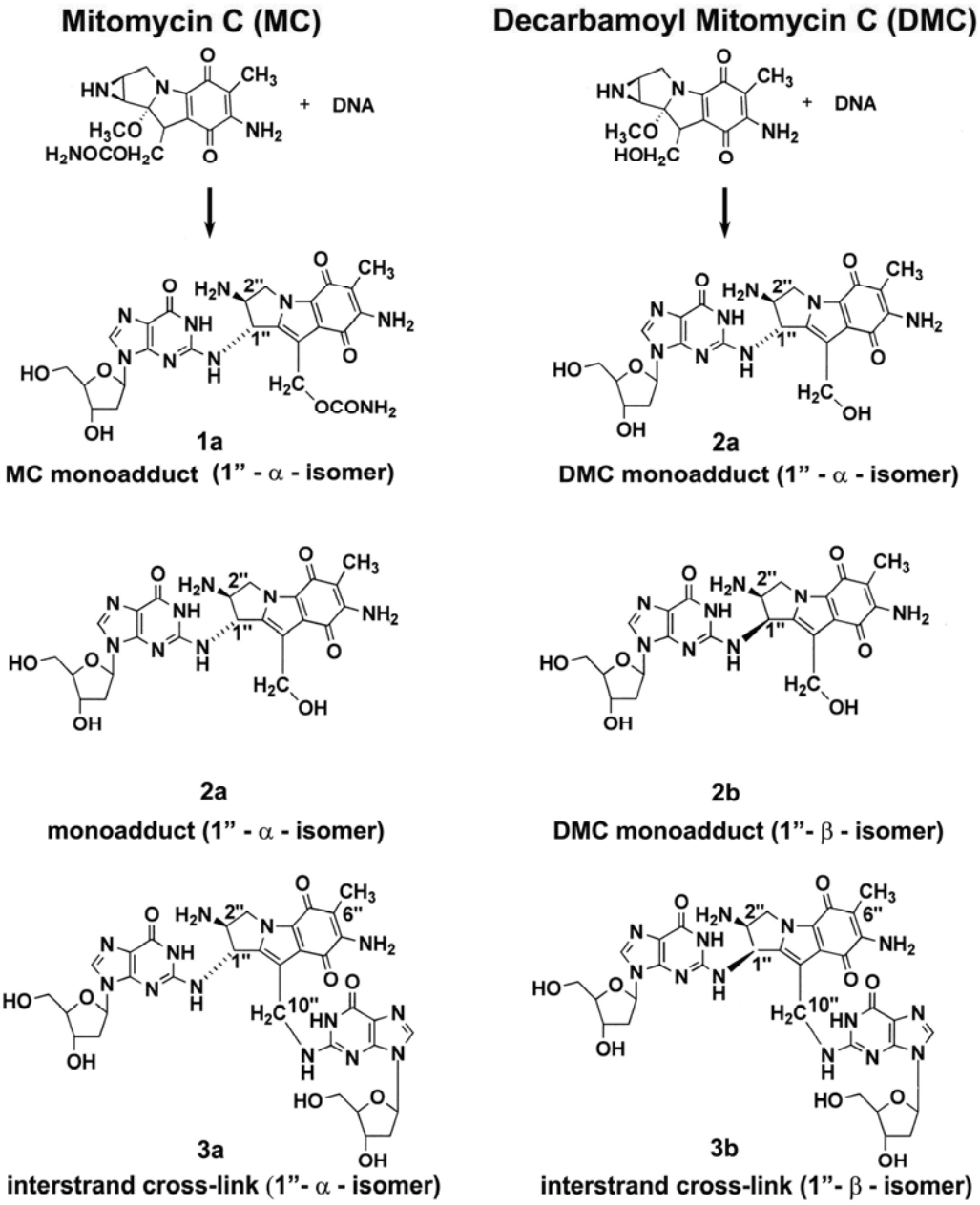


Figure 3. Chemical structures of activated MC and DMC bound to DNA

1.1C Clinical applications

Since its discovery, mitomycin C has been successfully used in treatment of solid tumors such as, head and neck cancer, cervical cancer, superficial bladder cancer, gastric and pancreatic tumor, anal and esophageal carcinomas (Begleiter, 2000; Bradner, 2001). Most solid tumors share a common characteristic: the internal micro-environment have limited blood supply (Fracasso and Sartorelli, 1986). In the absence of adequate blood supply to tumor masses, the internal environment becomes increasingly hypoxic (Cummings et al., 1998) . This condition favors growth of tumor cells by conferring acquired resistance to radiotherapy and other chemotherapeutic treatments (Kennedy et al., 1980a; Kennedy et al., 1980b). In addition to this acquired advantage, the low oxygen environment reduces the biological activity of most chemotherapeutic drugs, rendering these drugs clinically inadequate (Belcourt et al., 1996; Kennedy et al., 1980b).

A unique property of the mitomycins is that, being bioreductive agents, these drugs are activated in hypoxic conditions (Kennedy et al., 1980a; Sartorelli et al., 1994; Tomasz et al., 1974). Furthermore, hypoxia favors the overall ratio of total adducts formed by these mitomycins (Tomasz and Palom, 1997). This makes the mitomycins attractive chemotherapeutic drugs for the treatment of solid tumors.

All existing data on DMC has been generated from laboratory studies in cell culture models to define its biological activity.

1.2 DNA Damage Response (DDR)

Interstrand cross-links (ICLs) DNA adducts have been proposed as the clinically relevant

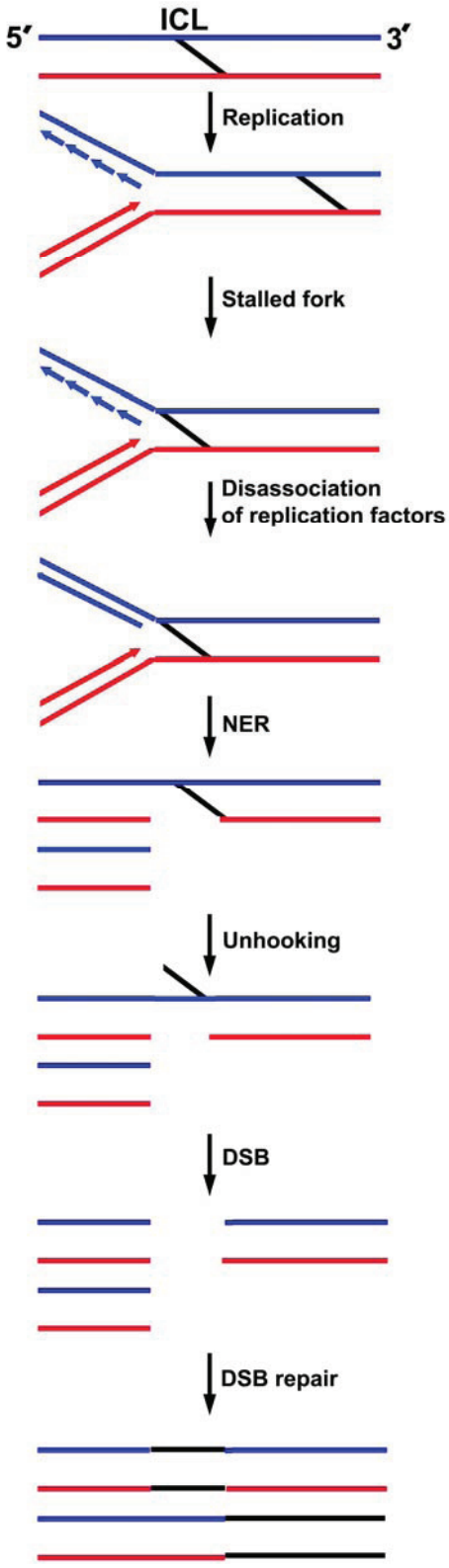


Figure 4. ICLs affect replication by causing polymerase stalling. The stalled polymerase is sensed by the DNA damage repair pathway (DDR). These signals to cell cycle checkpoint regulators and the nucleotide excision repair (NER) pathway. Members of NER pathway are able to unhook the ICL from both strands of the DNA. The gap that is generated after the removal of the ICL is repaired by either homologues recombination (HR) or non-homologues end joining (NHEJ). For a detailed description of each pathway involving ICL excision, please refer to text.

lesion produced upon exposure to MC and DMC (Dronkert and Kanaar, 2001; O'Connor and Kohn, 1990; Palom et al., 2002). However, it should be noted that exact mechanism of ICL induced cytotoxicity is not clearly defined. Also, the role of other DNA adducts, such as the monoadducts, in mitomycin cytotoxicity is also not been clear established.

Examining the role of DNA cross-link repair is paramount to understanding the molecular pathways activated during mitomycin induced cell death (Figure 4). The intrinsic ability of cellular machinery to repair these ICL lesions from the genome and activate cell death when genomic integrity is beyond repair underlies sensitivity of multiple cell lines to DNA cross-linking agents.

Interestingly, detection of ICLs by the cellular machinery has lead to the proposal of two primary mechanism of ICL recognition in mammalian cells. They are nucleotide excision repair (NER) dependent and replication dependent pathway (Dronkert and Kanaar, 2001; Naegeli, 1995). These two pathways initiate recognition of ICLs and activate downstream molecular events that determine sensitivity to ICL inducing agents.

1.2A Nucleotide excision repair (NER) pathway

The molecular mechanism(s) of ICL repair in mammalian cells has not been well characterized (Wang et al., 2001; Zhang et al., 2007). A simple model proposed in bacteria and yeast suggests an initial role of nucleotide excision repair (NER) in the removal of ICL from both strands of DNA (Wang et al., 2001). Most studies done to investigate NER in mammalian cells used Chinese hamster ovary (CHO) cells mutant in individual NER proteins (Telleman et al., 1995). By far, the existing data suggest recruitment of multiple cellular factors, during removal and repair of ICL (Figure 4). In fact, most of the reported data suggest NER pathway as

the primary mechanism of removing ICLs in both unicellular and multicellular organism (Barber et al., 2005; Wang et al., 2001).

The NER pathway is composed of more than 20 proteins amongst which are DNA damage recognition proteins (XPA, XPC and XPE), helicases (XPB and XPD) which are responsible for localized DNA unwinding, and the endonucleases (XPF and XPG) (Evans et al., 1997). Although ICL repair is initiated by members of NER pathway, it is still unclear exactly how ICL lesions are recognized in the genome (Zheng et al., 2006). While NER pathway has been proposed to be utilized for excision of ICL, there is some evidence suggesting a role in the recognition of ICL (Zheng et al., 2006). It has been proposed that XPC, hHR23b (the human homologue of yeast Rad23) proteins, XPA and RPA (replication protein A) are among the early set of proteins that recognizes these lesions and activate downstream events (McHugh et al., 2001). These protein complexes recognize ICL and signal to other factors involved in NER to unhook ICL from the genome.

NER mediated unhooking of ICL proceeds when the endonucleases, ERCC1 and XPF form a heteromeric complex (De Silva et al., 2000; van Vuuren et al., 1993). The ERCC1-XPF endonuclease complex has been shown to have critical role in mammalian ICL repair (Brookman et al., 1996; Sijbers et al., 1996). XPA binds to the genomic region containing the ICL and recruits ERCC1-XPF endonuclease complex to the ICL region (Park and Sancar, 1994). However, the catalytic activity of the ERCC1-XPF complex is only stimulated by the localization of XPG endonuclease at the ICL region (Niedernhofer et al., 2004). ERCC1-XPF cut the DNA at the 5' region while XPG cuts at the 3' region adjacent to the ICL lesion, releasing a short 27-29 base oligonucleotide containing the ICL (Clingen et al., 2007; Fisher et al., 2008; Kuraoka et al., 2000). ERCC1 and XPF mutants are highly sensitive to agents that

induce ICL, while other mutants of the NER pathway show very moderate sensitivity to these DNA damaging agents (Andersson et al., 1996; Hoy et al., 1985; Thompson and Schild, 2001; Zhang et al., 2007). Sensitivity of ERCC1 and XPF mutants has been attributed to inability to unhook ICL while others have also suggested that perhaps these proteins have NER independent function in ICL repair (Damia et al., 1996; Thompson, 1996).

1.2B Stalled replication machinery

DNA replication and transcription machineries are potential ICL recognition factors (Friedberg, 1996; Selby and Sancar, 1993; Tornaletti and Hanawalt, 1999). At the region where ICL is located, cross-linked base pairs will interfere with strand unwinding (Dronkert and Kanaar, 2001). This generates Y-shaped DNA structures at the opposite end of the replication fork (Figure 4). These Y-shaped structures block fork progression and signal to the DNA damage response pathway. Interestingly, replication dependent ICL recognition happens during the S-phase of cell cycle, a stage where cells are actively replicating their DNA (Dronkert and Kanaar, 2001).

Both NER and polymerase stalling will activate checkpoint regulators to ensure that cell cycle is temporarily halted while genetic defects, in this case DNA adducts, are removed.

1.2C Activators of Checkpoints

During ICL repair, cell cycle arrest is induced to prevent cells from cycling into the next phase with damaged DNA content (Figure 5). There are different pathways activated in response to DNA damage, however, the nature of the damage determines which factors are recruited to regulate cell cycle. Since ICLs disrupt polymerase extension, the presence of this aberrant DNA structures will activate cellular pathways sensitive to replication blockers. This is primarily due to production of ssDNA, which is proposed to originate due to lack of coordination between the

activity of helicases and the polymerase. It has been proposed that, ssDNA is generated when MCM replication helicases unwind DNA ahead of the polymerases. Using yeast as a model for DNA replication arrest, it was observed that when polymerase stalls, helicases unwind DNA past the stalling point which results in the generation of ssDNA (Katou et al., 2003). These ssDNA are sensed by the DDR response network (Figure 5). Generation of ssDNA past ICL will activate RPA, which binds exposed DNA structures (Zou et al., 2003). Binding of RPA will recruit ATR, through ATRIP, its binding partner (Figure 5).

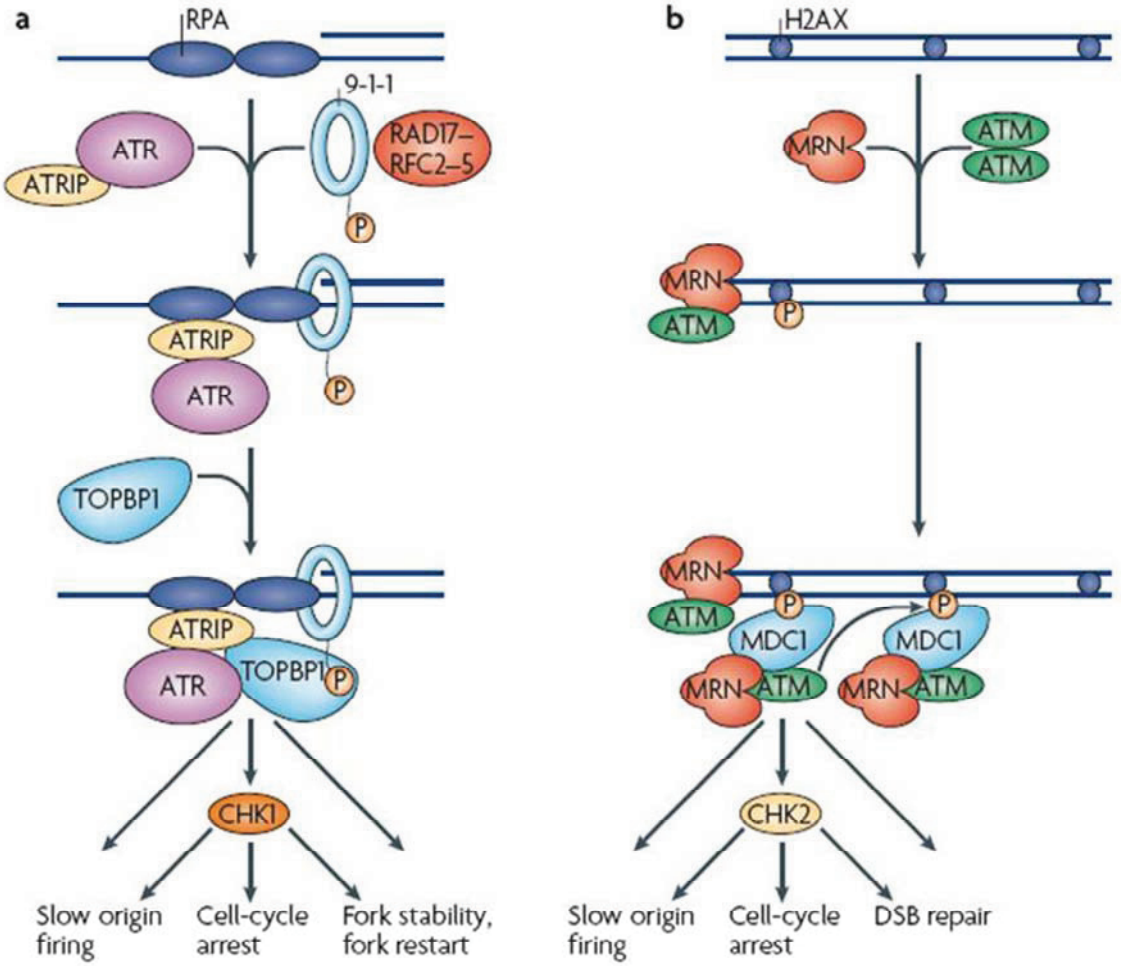


Figure 5. ATR (a) and ATM (b) checkpoint pathways are activated in response to DNA damage. The mitomycins form ICL DNA adducts which have been shown to cause polymerase stalling and activation of the ATR pathway. Excision of ICL from the genome results in double strand breaks and activation of ATM kinase. Borrowed from Cimprich KA and Cortez D. ATR: an essential regulator of genome integrity. Nat Rev Mol Cell Biol. 8, 616-27 (2008)

ATR recruitment to ssDNA causes its activation, leading to phosphorylation and recruitment of Rad 17 (Parrilla-Castellar et al., 2004). Rad 17 is a clamp loader which loads the PCNA-like complex, Rad9-Rad1-Hus1 (9-1-1) onto the DNA (Bermudez et al., 2003; Parrilla-Castellar et al., 2004; Rauen et al., 2000). ATR phosphorylates Rad17, 9-1-1complex, and TopBp1 during activation of replication block checkpoint pathway. Additionally ATR has been shown to phosphorylate multiple targets (see figure 5) including the checkpoint kinase, Chk1 (Wang et al., 2006). Activation Chk1 results in cell cycle arrest and replication fork stabilization (Osborn et al., 2002).

After processing ICLs and generating DSB, Mre11-Rad50-Nbs1 (MRN) complex is activated to induce cell cycle arrest and facilitate gap repair (Lee and Paull, 2005; Paull and Lee, 2005). MRN complex recruits and activate ATM (Lee and Paull, 2004; Lee and Paull, 2005). ATM exists in cells as inactivated dimers (Bakkenist and Kastan, 2003; Lee and Paull, 2007). Monomerization and autophosphorylation of ATM activates the protein (Figure 5). Activated ATM also phosphorylates Nbs1, which is part of the MRN complex, and other targets such as H2AX, which acts a scaffold protein to recruit other factors to the region of DNA damage (Lim et al., 2000; Rogakou et al., 1998; Rothkamm and Lobrich, 2003; Wu et al., 2000; Zhao et al., 2000). Phosphorylated H2AX interacts with the BRCA-1 C-terminal repeats of MDC1 and recruits the protein to sites of DNA damage (Lee et al., 2005; Stewart et al., 2003; Stucki et al., 2005). MDC1 interacts with MRN complex and enhances the recruitment and activation of ATM downstream targets such as 53BP1, Chk2, and p53 (Goldberg et al., 2003; Lou et al., 2006). p53 activation results in cell cycle arrest through activation of p53 downstream targets.

After halting the cell cycle due to the presence of deleterious lesions within the genome, the cellular repair pathway is initiated. Repair of DNA gaps, generated after excision of the ICL,

utilizes two established cellular mechanism, homologues recombination and non-homologues end joining (Ferreira and Cooper, 2004).

1.2D Homologues recombination (HR)

Niedernhofer et al, recently suggested that resolution of ICL from the genome produces double strand breaks as indicated by positivity to γ-H2AX foci formation in time dependent manner (Niedernhofer et al., 2004). After removing the DNA region containing the ICL by NER, the gaps that are generated are repaired by protein complexes involved in homologues recombination pathway. This mechanism of DNA repair occurs when cells are in S phase because that is the cell cycle point where the two sister chromatids align and facilitate recombination (Hendrickson, 1997; Richardson et al., 1998; Saleh-Gohari and Helleday, 2004; Symington, 2002).

HR is considered to be an error free mechanism of DNA repair. Briefly, DSB repair is initiated by 5'-3' degradation of damaged DNA at the region which allows for formation of a 3' single strand DNA overhangs (Symington, 2002). Binding of RPA, a single strand DNA binding protein, to exposed DNA overhangs, recruits RAD51 protein and its paralogs, RAD52 and RAD54 to site of damage (Baumann et al., 1996; Baumann and West, 1997; Eggler et al., 2002; Sigurdsson et al., 2001). Rad51 binds the exposed 3'end to initiate pairing and strand invasion with sister chromatid. The DSB gaps are then repaired by DNA synthesis and ligated (Llorente et al., 2008).

1.2E NHEJ (non-homologues end joining)

Unlike homologues recombination, NHEJ occurs throughout the cell cycle and it has been proposed to be a major pathway for DSB repair (Ma et al., 2005; Ma et al., 2004). Activation of NHEJ can result in loss or gain of nucleotides making this process highly error

prone (Daley et al., 2005; Lieber et al., 2004)

Ku70/Ku80 complex bind to DNA ends and initiates repair of DSB by NHEJ. Binding of Ku70/Ku80 complex recruits another protein complex, Artemis/DNA-PKc (Singleton et al., 1999; Suwa et al., 1994). Artemis/DNA-PKc binding dislocates Ku70/Ku80 complex from DNA ends (Lieber, 2008). Binding to the DNA ends at the DSB region leads to the autophosphorylation of DNA-PKc, which in turn phosphorylates Artemis(Hammarsten et al., 2000). Phosphorylation of Artemis activates its 5'- or 3'- endonuclease activity at overhangs (Lieber, 2008). Resectioning of DNA ends allows for the binding of DNA polymerase γ and μ (Mahajan et al., 2002; Nick McElhinny and Ramsden, 2004). These polymerases extend the ends to fill the gap generated at the region of DSB. After filling the gap, DNA ligase IV-XRCC4 complex ligates the ends of the gap (Nick McElhinny et al., 2000).

ICLs, and the different DNA states produced during their processing (ssDNA and DSB) can signal for cell death if these structures persist for prolonged periods due to difficulties associated with their repair. ICLs are highly cytotoxic lesions which are not easily repaired, especially when they are produced at alarming frequency. It has been shown that a single ICL per genome is sufficient to kill bacteria (Szybalski and Iyer, 1964). These data shows the extent of the cytotoxicity and the potency associated with ICL. Although both mitomycins can produced ICLs, DMC has continuously shown increased DNA adduct formation in cell lines (Palom et al., 2002).

As seen with cytotoxic chemotherapeutic agents used in clinical studies, often the extent of damage is beyond the capabilities of the cellular repair machinery. In this case, cell death is activated as default pathway in the absence of repair.

While different pathways can be activated during cell death, most often, the activated

pathway signals for the phosphorylation and activation of the tumor suppressor protein, p53. p53 has a unique ability to induce both arrest and cell death during prolonged exposure to DNA damaging agents. The fact that this protein is often mutated in cancer cells shows its importance in tumor suppression, which is mediated partly through its ability to activate apoptotic cell death.

1.3 Cell Death

1.3A p53: structure, regulation, and modifications

A brief description of p53 regulation and post translational modification after exposure to DNA damaging agents, such as the mitomycins, is relevant to understanding the tumor suppressor activity of this protein. The *Tp53* gene is located on the short arm of chromosome 17, band 13 and is approximately 20kb in length (Baker et al., 1989; Greenblatt et al., 1994; Nigro et al., 1989a). The intermediate gene product is an mRNA transcript of 2.8kb long which encodes for a 53 kDa phosphoprotein composed of 393-amino acid. The N-terminus of p53 contains a transcriptional and a proline rich domain followed by a DNA binding domain which forms the central core of the protein .Within the carboxy terminus is an oligomerization domain, which mediates p53 tetrameric conformation, flanked at both ends by nuclear localization domains.

In the absence of stress, p53 protein levels and activity is highly regulated to prevent aberrant cell cycle arrest or induction of apoptosis. The negative regulatory feedback loop, involving p53 and Mdm2, has been well studied and defines the main known mechanism of p53 regulation in the absence of stress (Michael and Oren, 2003; Oren et al., 2002). The p53-Mdm2 relationship is unique because, Mdm2 is a downstream target gene of p53 and also regulates p53 levels and transcriptional activity under normal cellular conditions (Barak et al., 1993). With respect to transcription regulation, Mdm2 has been shown to colocalize with p53 on certain

target genes (Arva et al., 2005; White et al., 2006). This has been proposed to affect the ability of p53 to recruit its binding partners and thus effectively signal for target gene transcription. Using chromatin immmunoprecipitation assay, our lab have shown that Mdm2 colocalizes with p53 on specific target genes, to inhibit its transcriptional activity (White et al., 2006). This data supports the proposed mechanism of Mdm2 mediated inhibition of p53 transcription in the absence of stress.

The primary mechanism of p53 regulation by Mdm2 is through its E3-ubiquitin ligase activity mainly because this method of p53 regulation has been under intense investigation. Mdm2 mediates p53 monoubiquitination and subsequent degradation through the proteasome and thus maintains low levels of p53 in the absence of stress (Fuchs et al., 1998; Honda et al., 1997; Rodriguez et al., 2000).

When DNA damage occurs, p53 undergoes multiple post translational modifications (PTMs) to activate and stabilize the protein (Harris and Levine, 2005). Some of these PTMs stabilize p53 protein by disrupting its interaction with Mdm2. Several studies have shown that the N-terminal transcriptional domain of p53 undergoes multiple modifications during protein activation (Unger et al., 1999). Phosphorylation of ser15 within has been shown to be critical for p53 stability and transcriptional activation (Shieh et al., 1997; Siliciano et al., 1997).

Adding to this plethora of regulators are the PI3-Kinase proteins, ATM and ATR, and their respective downstream targets, Chk2 and Chk1, which have also been reported to phosphorylate and stabilize p53 after DNA damage (Canman et al., 1998; Hirao et al., 2000; Shieh et al., 2000; Tibbetts et al., 1999). All together, these damaged induced post translational modifications serve to, 1) increase the half life of p53 from approximately 6-20min and, 2) allow about 3-20 fold induction of the protein (Harris and Levine, 2005).

To be able to transcribe target genes, p53 protein is further modified by the addition of different groups. Acetylation is another PTM required to induce p53 transcriptional activity. For example, acetylation within the C-terminus of p53 by p300/CBP and PCAF, is a prerequisite for p53 transcriptional competency (Avantaggiati et al., 1997; Gu and Roeder, 1997; Liu et al., 1999; Luo et al., 2000; Scolnick et al., 1997). Additionally, recent developments suggest a role of methylation in regulating p53's transcription (Huang et al., 2006; Huang et al., 2007).

Upon its activation by DNA damaging agents, stabilized p53 activates target genes involved in either cell cycle arrest or apoptosis. We and others have shown that treatment with either MC or DMC can induce p53 dependent apoptotic cell death (Abbas et al., 2002b; Boamah et al., 2007; Kang et al., 2006; Pirnia et al., 2002).

1.3B p53-dependent cell death

When cellular stress is sustained for a prolonged period of time, p53 as a master regulator induces apoptotic cell death. Apoptosis, a regulated form of cell death, is characterized by chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies (Wyllie, 1980). Apoptotic cell death can either originate from loss of mitochondrial membrane potential (intrinsic cell death), or through activation of cell surface death receptors (extrinsic cell death) (Figure 6). p53 protein can initiate either of these two cellular pathways through its ability to upregulate targets involved in each of the individual pathways.

1.3C Intrinsic apoptotic cell death

The mitochondria lumen acts as a storage site for proteins which, when released into the cytoplasm, induce caspase activation resulting in proteolytic degradation of cellular structures (Green, 2005). The term "intrinsic cell death" refers to a form of apoptotic cell death originating from the mitochondria.

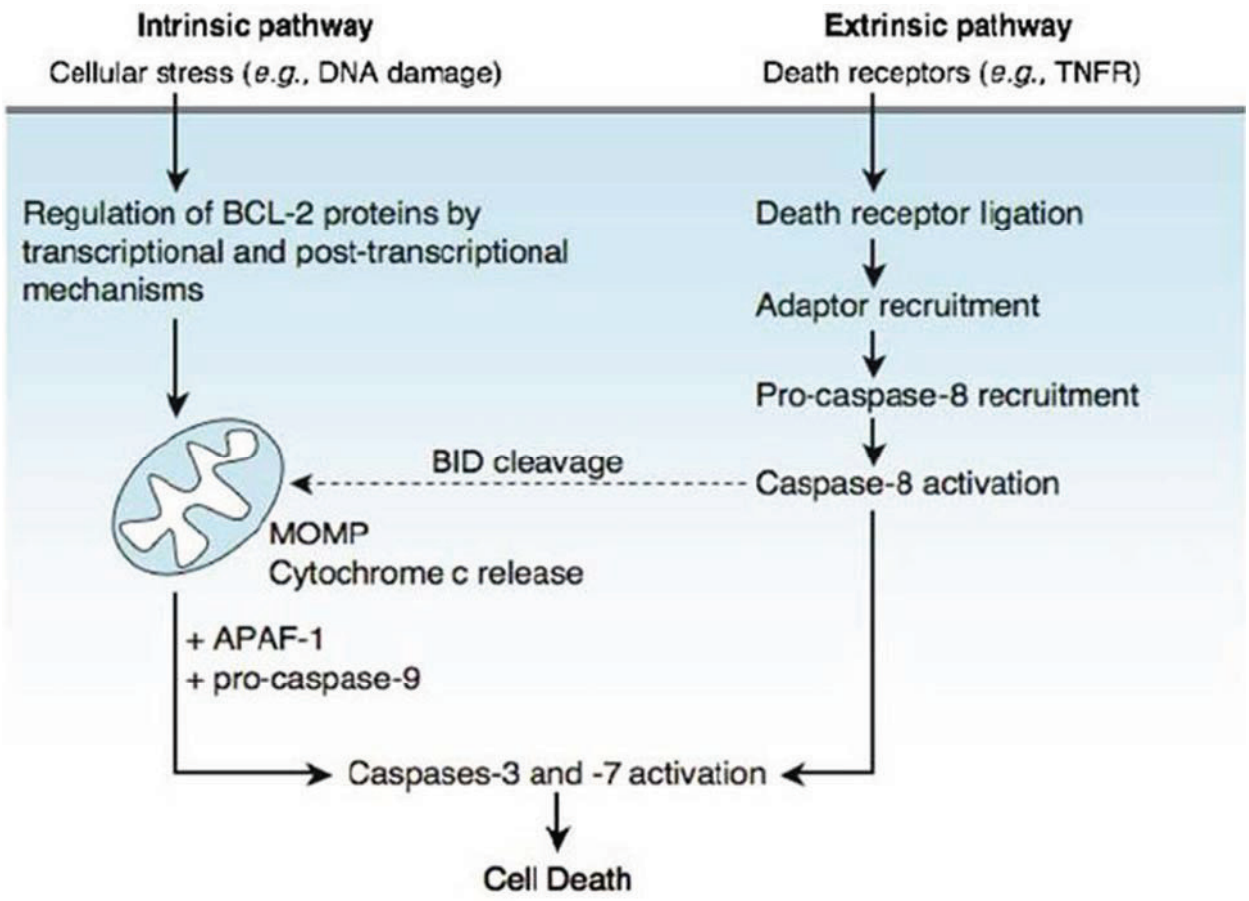


Figure 6. Chemotherapeutic DNA damage activates cell death pathways. In cells expressing wild-type p53, cell death can be induced through the activation of either the mitochondrial or death receptor pathway. p53 activates transcription of target genes involved in either of these cell death pathways. Borrowed from Chipuk, J.E. and Green, D.R. Dissecting p53-dependent apoptosis. *Cell Death and Differentiation*. 13, 994-1002, (2006).

Upstream of loss of mitochondria membrane integrity are a group of proteins called BCL-2 family which are pivotal in the initiation, and inhibition of apoptosis (Mitchell, 2001). The BCL-2 family proteins are classified into three distinct groups, based on the conservation of a BCL-2 homology (BH1-4) domain (Gelinas and White, 2005). The three groups are: 1) has members of the anti-apoptotic proteins such as BCL-2, BCL-x_L; 2) proteins have multidomain pro-apoptotic proteins represented by BAX and BAK; 3) BH3-only pro-apoptotic proteins including BID, BAD, BIM, PUMA, and NOXA (Gelinas and White, 2005; Oda et al., 2000) .

In the absence of cellular stress, BCL-2 proteins that inhibit apoptosis (BCL-2, BCL-x_L) heterodimerize with pro-apoptotic BCL-2 family members (BAX) (Cheng et al., 2001; Kuwana et al., 2002). In order for BAX and its associated proteins to execute release of mitochondrial proteins, inhibitory ability of anti-apoptotic proteins have to be disrupted (Gelinas and White, 2005).

When activated by DNA damaging agents, p53 disrupts the inhibitory heteromeric complex formed by anti-apoptotic proteins through transcriptional upregulation of target genes or by physical interaction (Chipuk et al., 2004). One of the many transcriptional targets of p53 upregulated during apoptotic cell death is PUMA (p53 upregulated modulator of apoptosis). PUMA functions as an inhibitor of BLC-2 anti-apoptotic family members through its ability to physically interact with these proteins (Nakano and Vousden, 2001; Yu et al., 2001).

When released from inhibitory interaction during the induction of apoptosis, BAD and BAX localize at the outer mitochondrial membrane (Figure 7). BAX and BAD function as channels to allow for cytosolic translocation of inner mitochondrial resident proteins (Kuwana et al., 2002). This results in the loss of mitochondrial membrane potential (MOMP) and the release of a critical mediator of apoptotic cell death, cytochrome c (Baptiste and Prives, 2004).

Cytochrome c is one of the proteins released into the cytoplasm through the mitochondrial membrane channels generated by BAX and BAD during apoptotic cell death (Wei et al., 2001). In the cytoplasm, cytochrome c initiates the formation of the apoptosome, a complex of APAF1, dATP, cytochrome c and caspase-9, which cleaves and activates the effector caspases, -3 and -7 (Hu et al., 1999; Li et al., 1997; Liu et al., 1996; Zou et al., 1997). Activated caspase-3 and -7 initiate proteolytic degradation of intracellular components to generate the observed apoptotic phenotype (Zou et al., 1997).

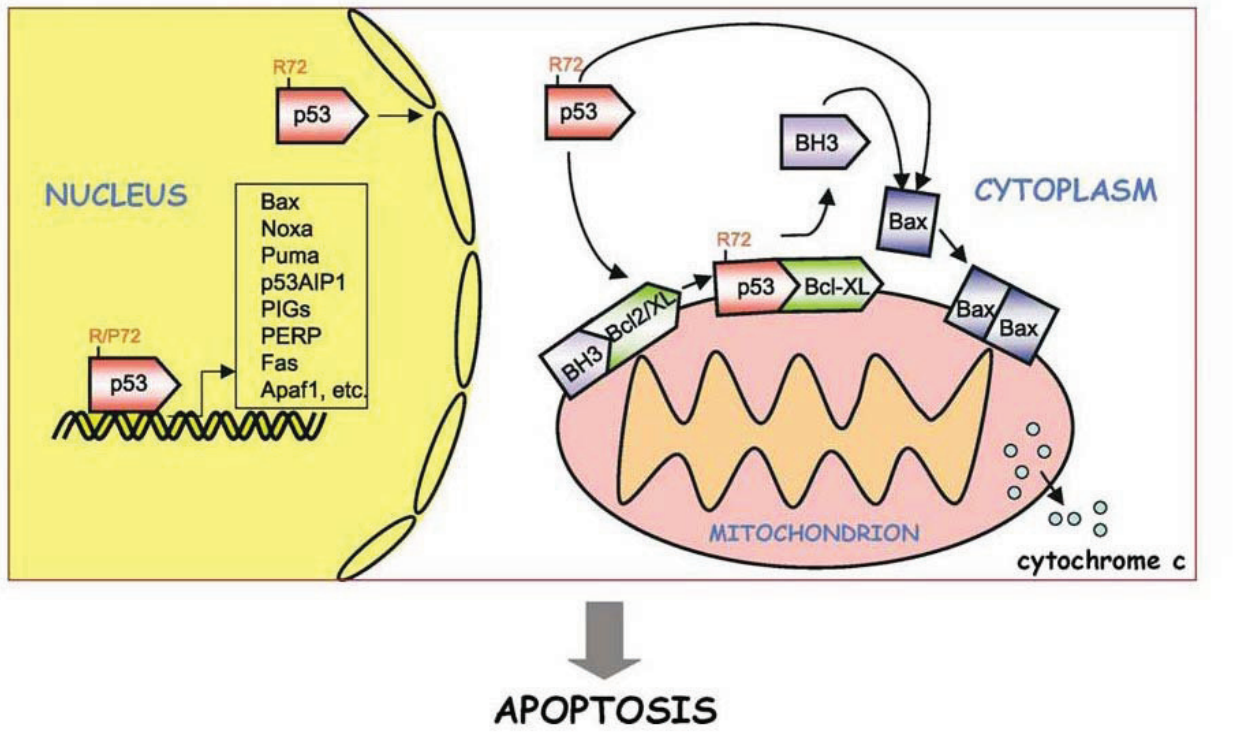


Figure 7. p53 targets induce loss of mitochondrial membrane potential and cytochrome c release. Borrowed from Baptiste, N and Prives, C. p53 in the Cytoplasm: A question of overkill? *Cell*. 116, 487-489, (2004).

1.3D Extrinsic apoptotic cell death

The extrinsic cell death pathway is initiated when a respective ligand bind to any of the surface tumor necrosis factor death receptors (TNF-R) superfamily, which includes TNF-receptor1 (TNF-R1), FAS/CD95, and death receptor (DR) 3 (Chinnaiyan et al., 1996a; Chinnaiyan et al., 1996b; Locksley et al., 2001; Peter et al., 1999).

Ligand binding to death receptors induces aggregation of intracellular adaptor proteins at the cytoplasmic membrane to form a death inducing signaling complex [DISC] (Kischkel et al., 1995). This DSIC complex cleaves and activates pro-caspase 8 (Kischkel et al., 2000; Peter, 2000; Varfolomeev et al., 1998). Caspase 8 activates the effector pro-caspase-3 and -7, which causes the morphological and biological changes associated with apoptosis(Liu et al., 2005).

Caspase 8 can also cleave cytosolic BID into an activated tBID (Gross et al., 1999). Activated tBID binds and homoligomerizes BAK or BAX to induce the release of cytochrome c during ligand mediated apoptotic cell death (Korsmeyer et al., 2000; Wei et al., 2000; Wei et al., 2001). Hence, proteolytic activation of BID acts as an intermediate to recruit both mitochondrial and receptor pathways during the development of apoptosis.

Interestingly, death receptor independent activation of caspase 8 by DNA damaging agents has been reported (Engels et al., 2000; Wesselborg et al., 1999; Wieder et al., 2001). These DNA damaging agents were able to efficiently induced cell death by cleaving and activating pro-caspases including caspase 8 even when death receptors were inhibited (Pirnia et al., 2002; Wieder et al., 2001). This suggests that apoptotic cell death initiated by DNA damaging agents in p53 wild-type cells can recruit individual members of both the intrinsic and extrinsic pathways. Thus, these two pathways are not mutually exclusive and may even cooperate to bring about the timely demise of cells harboring extensive DNA damage.

1.3E p53-independent apoptotic cell death

Since p53 is a critical anti-tumor factor, it's not suppressing that this protein is commonly mutated in most cancer cells. It should be noted that, p53 belongs to family of three other proteins: p53, p63, and p73 (Kaghad et al., 1997; Murray-Zmijewski et al., 2006; Yang et al., 1998). This family shares more than 60% amino acid homology in their DNA binding domain, which makes any member of this family competent to activate downstream targets in the absence of wild-type p53 (Yang and McKeon, 2000). Both p73 and p63 have been shown to induce apoptosis in the absence of wild-type and when p53 is present, to co-operate with p53 induced apoptotic cell death (Flores et al., 2002; Agami et al., 1999).

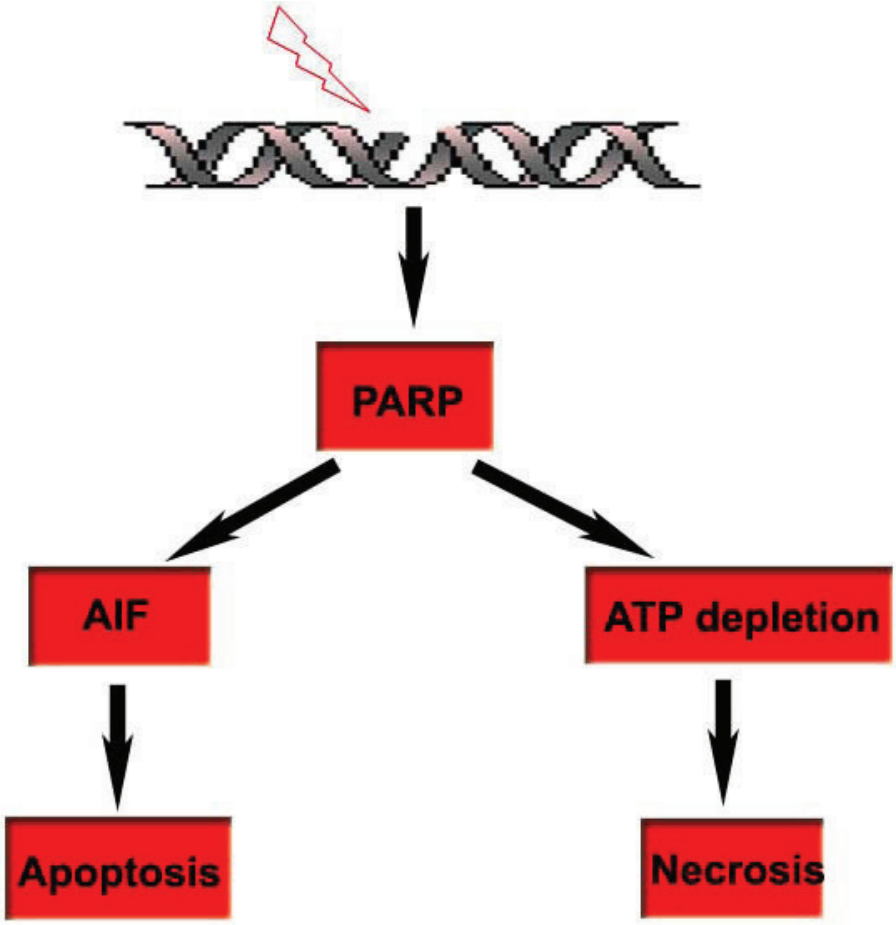


Figure 8. PARP-1, nuclear protein is activated by DNA damage. Activated PARP-1 can initiate two morphologically distinct cell deaths.

Poly(ADP-ribose)polymerase-1 (PARP-1) has also been reported to mediate caspase independent apoptotic cell death in tumor cells. PARP-1 is an abundant nuclear protein activated by single ssDNA, which stimulates its catalytic activity (Menissier-de Murcia et al., 1989). When activated, PARP-1 converts NAD^+ (nicotinamide adenine dinucleotide) a glycolytic intermediate to poly(ADP-ribose) or PAR(Benjamin and Gill, 1980).

It has been suggested that the ability of PARP-1 to convert NAD into PAR results in the activation of a signal transduction pathway that eventually results in the release of apoptosis

inducing factor (AIF) and endonuclease G from the mitochondria membrane (Andrabi et al., 2006; Yu et al., 2006). AIF and endo-G translocate into the nucleus and fragment DNA in a manner similar to apoptotic cell death (Cipriani et al., 2005; Joza et al., 2001; Li et al., 2001; Susin et al., 1999). The end result of the signal pathway is the induction of a form of cell death which shows some of the characteristics of apoptosis, such DNA fragmentation, but has no caspase activation and occurs in the absence of active p53 (Susin et al., 2000).

1.3F Necrosis

The catalytic activity of PARP-1, which converts NAD^+ to PAR, has been implicated in initiating two morphologically distinct forms of cell death (Figure 8). Necrosis, a form of programmed cell death, is characterized by electron-lucent cytoplasm, swelling of cellular organelles and loss of membrane integrity (Zong et al., 2004). Recently, it has been proposed that, necrosis, like apoptosis occurs by well orchestrated signaling mechanism that requires the activation of specific molecular markers (Zong, 2006).

Through the production of poly(ADP-ribose) polymers, PARP-1 utilizes most of the cellular NAD^+ pool which is an essential intermediate of the glycolytic pathway (Bouchard et al., 2003; Chiarugi, 2005). To maintain the energetic balance required for metabolism, the cell metabolizes available ATP in an attempt to replenish the cellular pool of NAD^+ (Zong et al., 2004). A futile cycle is generated which falls short of increasing cellular NAD^+ and eventually leads cell death by bioenergetic failure (Chiarugi, 2005).

CHAPTER 2

Experimental Methods

2.1 Reagents

MC was supplied by D.M. Vyas (Bristol-Myers Squibb). DMC was synthesized in our laboratory as previously described (Palom et al., 2002; Kinoshita et al., 1971). Nuclease P1 (Penicillium citrinum, EC 3.1.30.1), Etoposide, MTT reagents, MG115, Nocadozole, and doxycycline were purchased from Sigma, Allentown, PA. RPMI 1640, McCoy's 5A, and Penicillin-Streptomycin were purchased from Mediatech, Herndon, VA. Fetal Bovine Serum (FBS) and gentamycin (G418) were purchased from Gemini Bio-Products, West Sacramento, CA. Hygromycin B was purchased from Calbiochem La Jolla, CA. The MultiCaspase Detection kit was purchased from Guava Technologies, Hayward, CA. Phosphodiesterase I (snake venom diesterase, *crotalus adanonteous* venom, EC 3.1.4.1) and alkaline phosphatase (*Escherichia coli*, EC 3.1.3.1) were obtained from Worthington Biochemical Corp., Freehold, NJ. Sep-Pak C-18 cartridges were purchased from Waters Corp., Milford, MA.

2.2 Cell lines

The isogenic colon cancer cell lines, DLD-1 and DA-2, were generous gifts from Bert Vogelstein (Johns Hopkins School of Medicine), whose group used a two-step procedure to establish a tetracycline-off system for controlled wild-type p53 expression in DA-2 cells (Yu et al., 1999). DLD-1 cells do not express wild-type p53 and DA-2 cells maintained in the presence of doxycycline express a trace amount of wild-type p53. DLD-1 cells were grown in McCoys 5A media containing 10% FBS and penicillin (50 units/ml) –streptomycin (50µg/ml) solution, while DA-2 cells were maintained in an additional 0.4mg/ml G418, 20ng/ml doxycycline, and 0.25mg/ml Hygromycin B.

MCF-7 human breast cancer cells (wild-type p53), obtained from American Type Culture Collection, were grown in RPMI medium supplemented with 10% fetal bovine serum and penicillin (50 units/ml) –streptomycin (50μg/ml) solution.

2.3 Drug Treatments and plasmid

Cells were treated with MC or DMC (dissolved in 30% methanol) at the concentrations and times indicated in the figure legends. Where indicated, the topoisomerase poison etoposide (dissolved in dimethyl sulfoxide) was used to compare cytotoxicity at the indicated concentrations and time points. The $pCS3^{+}$-6Myc plasmid expressing Myc-Chk1 protein was kindly provided by You-Wei Zhang (Salk Institute, La Jolla, CA).

2.4 Isolation of nuclear DNA from drug-treated cells

MCF-7 human breast cancer cells were seeded at 80% confluence overnight prior to drug treatment at 37^{o}C in 5 % CO_2. Approximately 2.7 x 10^7 cells were treated with 10 μM MC or 10 μM DMC for 24 hours. At the end of the incubation period, cells were harvested, washed 2x in ice-cold PBS, and spun down after each wash at x 300g. Nuclear DNA was isolated using a Qiagen blood and cell culture DNA maxi kit following the manufacturer's instructions (Qiagen, Valencia, CA). The isolated DNA was suspended in TE buffer overnight and subjected to further analysis as described.

2.5 ***Enzymatic digestion of DNA from drug-treated cells and isolation of the adduct fraction by Sep-Pak chromatography***

The lyophilized DNA, typically 20-30 A_{260} units, obtained from 10^8 cells was digested to the nucleoside level by the following protocol: Nuclease P1 (1.0 unit/A_{260} unit of DNA) was added to the DNA in dilute aqueous acetic acid at pH 5.0 (2.5 A_{260} units/mL), followed by incubation for 4 h at 37 ^{o}C. The pH was adjusted to 8.2 by addition of 0.5 M Tris, and $MgCl_2$

was added to a concentration of 1.0 mM. Addition of snake venom diesterase (2.25 units/A_{260} unit of DNA) and 2 hours incubation at 37 ^{o}C were followed by the addition of alkaline phosphatase (1.6 units/A_{260} unit of DNA) and incubation overnight at 37 ^{o}C. The digest was then heated at 90 ^{o}C for 1 hour to hydrolyze guanine-*N7* adducts. The solution was concentrated to 1 mL and applied to a Sep-Pak C-18 cartridge ("Classic Short Body", Waters), previously washed consecutively with 10 mL acetonitrile, 10 mL H_2O and 2 mL of 10 mM ammonium acetate. The unmodified nucleosides were eluted with 120 mL H_2O, then 10 mL 5% methanol, followed by quantitative elution of the adducts with 60% methanol–water (20 mL). This last fraction was lyophilized, and submitted to analysis by mass spectroscopy, described below.

2.6 Mass Spectrometry

LC-ESI MS/MS. An Applied Biosystems ABI-5000 tandem mass spectrometer (Concord, ONT, Canada) was interfaced directly with an Agilent Model 1100 liquid chromatograph (Santa Clara, CA) equipped with a degasser, autosampler, column oven and diode array detector. The column used in this work was Phenomenex Luna 3 μ, C8 (Tomasz and Palom, 1997), 100 Å, 150 x 2mm. The LC column was maintained at 35 ^{o}C. Mobile phase conditions: mobile phase A, 95/5 water/acetonitrile, containing 0.1% formic acid, 5 mM ammonium formate; mobile phase B, 5/95 water/ acetonitrile, containing the same modifiers. Elution program: 100 % A for 15 minutes, 0-30% B in 30 minutes, 30 % B for 15 minutes. The flow rate was 0.2 mL/min. The diode array detector recorded UV spectra from 190 to 400 nm.

The ABI Turbo V ion source electrospray inlet consists of an ABI TurboIonSpray probe though which the LC effluent is released as a nebularized spray. A high potential on the probe ionizes the spray droplets. Heated dry nitrogen gas-heated impinging of the spray speeds evaporation of the droplets before they reach the entrance to the mass spectrometer. A modest

flow of dry nitrogen gas out of the entrance of the mass spectrometer, referred to as curtain gas (CUR), minimizes chemical background noise in the instrument.

The mass spectrometer recorded data throughout each LC run in multiple reaction monitoring (MRM) mode. The analytes eluted between 5 and 15 min. Curtain gas (CUR) was 15 mL/min. Nebularizer gas (GS1) was 50 mL/min, heater gas (GS2) 40 mL/min, ionspray potential (IS) 5000 V. Temperature of the GS2 gas, referred to as source temperature (TEM) was 650 ^{o}C. The MS inlet heater plate (ihe) was ON and at its fixed value of 100 ^{o}C. Pressure of the nitrogen gas in the collision cell (CAD) was 6.0 and the CAD entrance potential was 10 V. The parent ions and their fragment pairs were monitored sequentially throughout the course of the run, dwelling on each parent-fragment pair for 50 msecconds before moving onto the next. The cycle was repeated at approximately every 1.5 seconds. For each adduct, the parent ion and its two fragment ions are listed in Table 1, along with the corresponding experimental variables.

2.7 Quantitative analysis

Separate calibration curves of each adduct in the 6-adduct standard mixture were constructed by 2- to 100-fold range dilutions of the standard adduct mixture. The undiluted mixture contained 5 nmol/1.0 mL of each adduct. Two fragment ions were monitored for a particular adduct, resulting in two calibration curves, one for each MRM fragment pairs. The adduct samples were analyzed in duplicate injections and their quantities were calculated separately from each of the two different fragment calibration curves. The four values thus obtained for each adduct were averaged and standard errors were calculated. DNA adduct frequencies of the samples were calculated from the formula:

DNA adduct frequency (mol adduct/mol DNA-nucleotide) =

(picomol adduct/μmol DNA digested) x10^{-6}

where "μmol DNA digested" was calculated from the A_{260} values of the DNA samples before digestion, using 6,500/$mol^{-1}cm^{-1}$ as the molar extinction coefficient E_{260} of mammalian DNA (Bizanek et al., 1993).

2.8 Quantitative reverse transcription-PCR

RNA was isolated using the Qiagen RNeasy mini kit. 5μg of RNA was used for cDNA synthesis using the high capacity cDNA archive kit (Applied Biosystems). The primer-probes for *p21, chk1, puma, bax, gadd45 and mdm2* (Applied Biosystems, Celera Discovery Systems Assays on Demand) and *actin* (Applied Biosystems Pre-developed Assay Reagents) were utilized for Taqman PCR using the Applied Biosystems 7500 sequence detection system (Perkin Elmer) as follows: one cycle, 2 minutes (50°C); one cycle, 10 minutes (94°C); and 40 cycles, 15 seconds (94°C) and 1 minute (60°C).

2.9 Protein Extract Preparation

Nuclear and cytoplasmic extractions were prepared using a variation of the Dignam Protocol (Dignam et al., 1983) as described in (Abbas et al., 2002). For whole cell extraction, briefly, at the end of the incubation period, cells were harvested, washed 2x in ice-cold PBS, and spun down after each wash at x300g. Cell pellets were dissolved in RIPA buffer (0.1% SDS, 0.5% deoxycholate, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 50mM tris pH 8.0, 1mM PMSF, 1μg/ml aprotonin, 1μg/ml leupeptin and 1% Phosphatase inhibitor cocktail) and incubated on ice for 10 minutes with periodic shaking. Pellets were then centrifuged at x9,300 g for 15 minutes. The supernatants were collected and kept at -80^{o}C for future analysis

2.10 Histone Extraction

Cells were harvested by centrifugation at 4^{o}C at 2000 rpm for 5 minutes. The pellets were washed once in phosphate buffered saline (PBS), and lysed in 4ml extraction buffer (10% Triton

X-100 (v/v), 50mM phenyl-methylsulfonyl fluoride, 2% sodium azide (w/v) in PBS). Pellets were then resuspended in 0.2N hydrochloric acid overnight at 4°C. Extracts were centrifuged again at 4°C at 2000 rpm for 10 minutes and the supernatant was collected and stored at -20°C. Twenty microgram protein aliquots were boiled and separated by 10% SDS-PAGE.

2.11 Western blot analysis

Protein samples were size-fractionated by electrophoresis in 10% SDS denaturing poly-acrylamide gels and electrotransferred to nitrocellulose membranes (Amersham). For ubiquitinated products, samples were separated on a 10% and 8% (bottom and top respectively) denaturing gel prior to transfer. The resulting blots were incubated with the following primary antibodies: p53 specific monoclonal antibodies [1:1:1 mixture of 421, 240, and 1801 antibodies) as described (Bargonetti et al., 1992; Bargonetti et al., 1993)], anti-Chk1 (Santa Cruz Biotech., cat. no. sc-7898), anti-ubiquitin (Dako, cat. no. Z0458), anti-proteasome (Biomol, anti-β5 cat. no. PW8895), anti-myc (Santa Cruz Biotech., cat. no. sc-40), anti-gamma histone 2AX (H2AX) (Upstate Cell Signaling), anti-dimethylated histone 3 lysine 9 (H3K9) (Upstate Cell Signaling), anti-poly(ADP-ribose) polymerase (PARP) antibody (Pharmingen), anti-poly(ADP-ribose) (PAR) (Alexis Biochemical), anti-procaspase-8 or -3 antibodies (Oncogene Research), anti-caspase-2 (Millipore), anti-ATR (Fisher Scientific), and the polyclonal anti-actin antibody (Sigma). The membranes were then incubated in anti-mouse or anti-rabbit secondary antibodies (Sigma) and the signals were visualized by chemiluminescence. Densitometry analysis was performed using NIH image J and values were normalized to actin.

2.12 MTT Assay

Sensitivity following treatment with DNA damaging agents was determined for each cell line using the tetrazolium dye-based microtitration assay. Cells were seeded at 1.25×10^5 cells and

allowed to attach overnight. Cells were then treated with mitomycins or etoposide and incubated for the indicated times. Following incubation, reconstituted 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/ml MTT powder dissolved in balanced salt solution without phenol red) was added to the cells in an amount equal to 10% of the culture medium volume, and incubated at 37°C in 5% CO_2 for an additional 2 hours. The cells were harvested and centrifuged at 1850 rpm for 5 minutes at room temperature, and the resulting pellets were resuspended in 0.04N hydrochloric acid diluted in isopropanol. Samples were incubated for 5 minutes at room temperature, and then centrifuged at 14,000 rpm for 2 minutes. Supernatant absorbance at 550nm was read. Data is represented as percent population of viable cells in each sample relative to the untreated sample.

2.13 MultiCaspase Assay

Following treatment, at least $5.0x10^4$ of DLD-1 and D-A2 cells were harvested, washed, and stained as indicated by the manufacturer's protocol. Stained samples were immediately analyzed for multiple caspase activity in the Guava Personal Cell Analysis System™ (Guava Technologies).

2.14 Immunofluorescence microscopy

Immunostaining for poly(ADP-ribosyl)ated or phosphorylated H2AX proteins was performed on cells plated on glass coverslips. After drug treatment, cells were fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were permeabilized with PBS containing 0.2% Triton X-100 and 1% FBS for 5 minutes at -20°C and washed three times in 1% FBS-PBS. Cells were incubated with polyclonal anti-poly(ADP-ribose) antibody (BD Pharmingen – 1:200) or monoclonal anti-gamma H2AX (Upstate Cell Signaling – 1:250) in 1% FBS-PBS at room temperature for 1 hour. Following incubation with primary antibody, cover

slips were rinsed three times with 1% FBS-PBS solution and incubated with either secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Santa Cruz – 1:200) or FITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch – 1:200) in 1% FBS-PBS. Cells were rinsed three more times with 1% FBS-PBS. Cover slips were mounted onto slides with 4', 6'-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Images were collected with a Nikon fluorescence microscope.

2.15 Confocal microscopy

After drug treatment, cells were fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were permeabilized with PBS containing 0.2% triton x-100 and 1% FBS for 5 minutes at -20^{o}C and washed 3x in 1% FBS-PBS. Cover slips were immediately mounted onto slides with 4', 6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.). Nuclear morphology was visualized using spinning disk confocal microscopy or immunofluorescence microscope. All confocal images were captured using 100x objective and immunofluorescence microscopy at 60x. NIH image J was used to determine nuclear area as follows: the captured images were open using the image J software. A circle was drawn around each nuclear in a field and quantified. The following formula was used to calculate the nuclear size: size (μm) = area (given in pixel) / cm/pixel. The final value was then converted to μm/pixel.

2.16 RNA interference and transfections

For siRNA experiments, cells were seeded at 60% confluence in media without penicillin–streptomycin and allowed to attach overnight. Cells were transfected with 25 nM of each of the specified siRNA (all from Dharmacon) for 24 hours using Lipofectamine 2000 (Invitrogen). At the end of the incubation period fresh media without penicillin–streptomycin

was added to cells for 2 hours, after which cells were either left untreated or treated with 10 μM MC or 10 μM DMC for an additional 24 hours. Protein extracts were obtained from cells for Western blot analysis. For MTT analysis, after siRNA transfections, cells were incubated for an additional 2 hours with MTT solution and analyzed for proliferation.

For over-expression experiments, cells were plated at 90% confluency and transfected with 4 μg of each indicated plasmid for 24 hours using Lipofectamine 2000. Cells were then either left untreated or treated with 10 μM MC or 10 μM DMC for an additional 24 hours followed by protein extraction or MTT analysis for proliferation.

2.17 Proteasome activity assay

Cells were washed 2x in PBS and lysed in buffer A (50 mM Tris-HCL pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, 1mM DTT, and 10% glycerol). Extracts were homogenized on ice and spun down at 4^{o}C for 15 minutes at 15,000 g. Protein concentrations of extracts were determined (Bradford assay) and adjusted using Buffer A. 30 μg of proteins were analyzed on a non-denaturing gradient PAGE gel (5, 4, and 3% from bottom up) with Rhinohide polyacrylamide strengthener (Molecular Probes). Bromophenol blue was added to the samples, loaded and run at 4^{o}C for 3 hours at 150 volts. Gels were then covered with 0.4 mM of the proteasome substrate, Suc-Leu-Leu-Val-Tyr-AMC (Bachem) diluted in Buffer B (buffer A modified to contain 1 mM ATP) and kept at 37^{o}C for 30 minutes on a rocker. Proteasome activity bands were visualized when gels were exposed to UV (360nm). Images were collected using a digital camera.

2.18 Microarray

DLD-1 cells were seeded at 80% confluence overnight prior to drug treatment at 37^{o}C in 5 % CO_2. Cells were treated with 10 μM MC or 10 μM DMC for 24 hours. At the end of the incubation period, cells were harvested, washed 2x in ice-cold PBS, and spun down after each

wash at x 300g. Protein extracts were made, labeled using Cy3 or Cy5 dye (Amersham Biosciences), and incubated on array slide as outlined in Sigma Panorama Antibody Microarray-Cell Signaling Technical Bulletin (CSAA1). Array slides were scanned using ScanArray G_x Microarray Analysis System (PerkinElmer, Waltham, MA). Microarray images were analyzed using ScanArray Express, Microarray Analysis System software, version 4.0.0.0004 (PerkinElmer LAS, Inc., Waltham, MA).

2.19 Colony Forming Assay (Clonogenic Assay)

DLD-1 and DA-2+DOX cells were seeded at either 1 x 10^4 or 5 x 10^4 cells overnight prior to drug treatment at 37^{o}C in 5 % CO_2. Cells were treated with concentration of drugs indicated in figure, for 1 hour. At the end of the incubation period, cells were washed 2x in Hank's Balanced Salt Solution (GIBCO, NY). After washing, fresh media was added and plates were incubated for a period of 11 days (media was changed every third day during the duration of the experiment). After the incubation period, plates were rinsed 2x with 1x PBS, incubated at room temperature with Methylen Blue Solution (Fluka, Allentown, PA) for 30 minutes. Plates were then washed again with dH_20 until blue stain was washed off, and allowed to dry overnight at room temperature. Images were collected using a digital camera.

CHAPTER 3

Rapid down-regulation of Chk1 by stereoisomeric DNA adducts is associated with mitomycin cytotoxicity

3.1 INTRODUCTION

The p53 protein is an important tumor suppressor and is frequently mutated in cancer cells (Hollstein et al., 1991; Hollstein et al., 1999). DNA damage activates and stabilizes wild-type p53, which results in increased transcription of multiple p53 target genes involved in cell cycle arrest or apoptotic cell death (Harris and Levine, 2005; Levine et al., 2006). It has been estimated that over 50% of all cancers harbor a mutation in the p53 gene that interfere with the ability of the protein to effectively induce cell death (Hollstein et al., 1991). Loss of p53 function has been associated with increased resistance to chemotherapeutic agents (Johnstone et al., 2002). In addition, loss of p53 or its downstream target, p21, disrupts the G_1/S checkpoint in response to DNA damage (Deng et al., 1995; Waldman et al., 1995). Lack of a G_1/S checkpoint causes cells to depend entirely on their intra-S and G_2/M checkpoints to ensure genomic integrity (Russell et al., 1995).

Within each cell is an elaborate DNA damage response system capable of detecting potentially deleterious changes to the genetic material. The absence of this critical intrinsic safeguard mechanism has been shown to facilitate unregulated growth and tumor formation. The ATR-Chk1 pathway regulates chromosome fidelity at the G_2/M transition and is especially important to cells lacking a functional p53 checkpoint pathway (Enders, 2008; Osborn et al., 2002). The phosphatidylinositol 3-kinase-related kinase ATR, a DNA damage and replication stress response protein, is part of a complex network of checkpoint proteins which are activated in response to deleterious lesions that affect replication fork progression (Enders, 2008; Zou and Elledge, 2003). In response to replication stress, ATR phosphorylates Chk1 on two critical residues, Ser-317 and Ser-345 (Zhang et al., 2005; Zhao and Piwnica-Worms, 2001). When activated, Chk1 delays entry into mitosis by phosphorylating and inactivating Cdc25A and

Cdc25C, two phosphatases required for cell cycle progression (Zhang et al., 2006). In the absence of Chk1, cells with DNA damage continue through mitosis culminating in cell death by mitotic catastrophe due to the lack of G_2/M checkpoint (Castedo et al., 2004; Syljuasen et al., 2004; Huang et al., 2005). Disruption of the Chk1 G_2/M checkpoint kinase is a provocative death target, especially for cells with compromised p53 since these cells lack an efficient G_1/S checkpoint (Kawabe, 2004; Dixon and Norbury, 2002; Mukhopadhyay et al., 2005; Didier et al., 2008).

Mitomycin C (MC), a bioreductive DNA alkylating agent, is a well known antitumor, antibiotic and chemotherapeutic agent (Kennedy et al., 1980; Tomasz et al., 1981; Rockwell et al., 1982). Within the intracellular compartment, MC is metabolized by reductive enzymes to generate reactive DNA alkylating species and oxygen radicals through redox cycling (Tomasz and Lipman, 1981). Activated MC alkylates guanine at the N^2-position to form DNA monoadducts and DNA intrastrand and interstrand cross-links adducts (Tomasz and Palom, 1997). 10-Decarbamoyl mitomycin C (DMC), a derivative of MC, has also been shown to bind DNA forming a similar, but not identical array of DNA adducts (Palom et al., 2002; Paz et al., 2008). We recently demonstrated that equimolar concentrations of DMC produce more DNA adducts in human cells than MC and most of the adducts have altered stereochemistry (Paz et al., 2008). Specifically, the chirality of the mitosene linkage to guanine-N^2 of DNA of MC is opposite of that of DMC (mitosene-1-α vs mitosene-1-β) (Figure 1) The critical cytotoxic lesion produced by chemotherapeutic DNA damaging agents such as mitomycin C, has been proposed to be the interstrand cross-link adducts (Iyer and Szybalski, 1963; Dronkert and Kanaar, 2001). These interstrand cross-link DNA modifications inhibit strand separation during replication and transcription which in turn activates ATR and ATM checkpoint pathways (Osborn et al., 2002).

Although both MC and DMC produce these cross-link DNA adducts, interestingly, DMC more rapidly induces death of cells than MC (Boamah et al., 2007; Abbas et al., 2002).

In this study, we report that the rapid Chk1 protein depletion following DMC treatment correlates with the differential response of human cells to the two mitomycins (MC and DMC). Our data indicate that DMC is a better modifier of DNA than MC, generating increased DNA adduct frequency, especially the mitosene-1-β guanine adducts (Palom et al., 2002; Paz et al., 2008). Here, we also report that the DNA damage observed with DMC treatment corresponds to depletion of Chk1 through a proteasome mediated pathway. We see that the cell morphology is vastly different for cells treated with MC than for cells treated with DMC. Importantly, gene silencing of Chk1 by siRNA increased the cytotoxicity of MC DNA-adducts in the absence of wild-type p53. However the reintroduction of Chk1 followed by treatment with DMC barely rescued cell survival. Therefore, while Chk1 depletion increases the killing capacity of the N^2-guanine adducts, other factors are likely required to cooperate with reduced Chk1 for the induction of DMC provoked death.

3.2 RESULTS

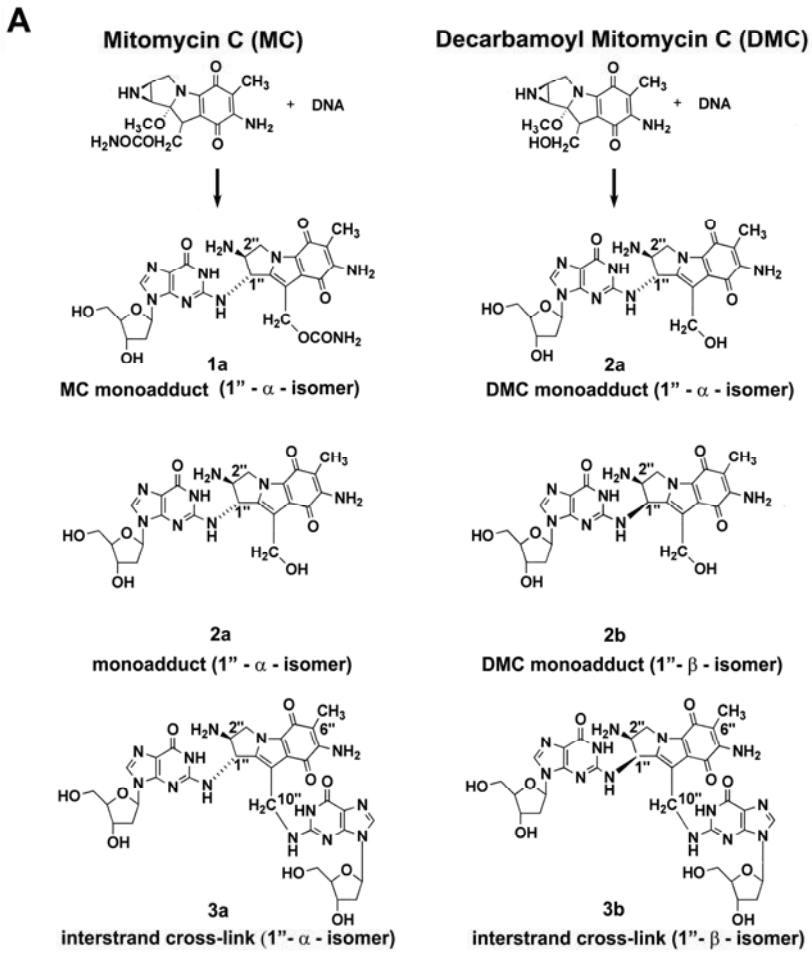
3.2 A DMC generates more mitosene 1-β DNA adducts than MC and causes nuclear shrinkage in the presence or absence of wild-type p53.

We previously reported that DMC has increased cytotoxicity compared to MC in the presence or absence of wild-type p53 with 24 hours of drug treatment (Boamah et al., 2007; Abbas et al., 2002). To further examine the differential response to these two mitomycins, we investigated the frequency of DNA adduct formation in the wild-type p53 breast cancer cell line, MCF-7 (Paz et al., 2008). It was observed that after 24 hours of treatment with MC or DMC, the

overall adduct frequency formed by DMC was more than that observed with MC, although both drugs produced a similar spectrum of adducts. Strikingly, the predominant stereochemistry of the 1”-mitosene linkage of the DMC adducts to DNA was 1”-β (2b, 3b), opposite to the predominant 1”-α stereochemistry of the MC adducts (1a, 2a, 3a) (Fig. 1). Interestingly, a dramatic reduction in nuclear size of MCF-7 cells treated with DMC for 24 hours was observed but no such change resulted in the MC treated population (Fig. 2*A*, *MCF-7*).

To assess if the nuclear shrinkage following DMC treatment of MCF-7 cells was cell type specific, we compared the morphology of drug treated MCF-7 and isogenic colon cancer cells with or without wild-type p53 at multiple timepoints (DA-2 and DLD-1, respectively) (Figs. 2*A,* 2*B, and* 2*C*). Following 24 hours of DMC treatment, both DLD-1 and DA-2 cells showed similar nuclear shrinkage following DMC treatment as observed in MCF-7 (Fig. 2*A*). After 24 hours of MC treatment, only a small population of cells showed reduction in the nuclear size (Fig. 2*A*). Overall, DMC treatment caused a reduction of nuclear size in all cell lines tested, which was not observed with MC treatment (Fig. 2*A*). This change in nuclear morphology correlated with the increased DMC cytotoxicity we reported previously as observed over prolong drug treatment (Fig 2*B*).

Additionally, we observed a marked decrease in nuclear morphology of cells after 48 hours of DMC treatment (Fig. 2*B*). The nuclear morphology of MC treated cells, remained consistently larger. However, upon prolonged exposure to both MC and DMC, the nuclear morphology equally decreased in size (Fig. 2*C*)(it should be noted that data from the two timepoints, namely 48 hours and 72 hours, were collect from a single experiment). This decrease in nuclear morphology correlated very well with the cytotoxicity of the two mitomycins, suggesting an association between drug cytotoxicity and disruption of nuclear architecture.



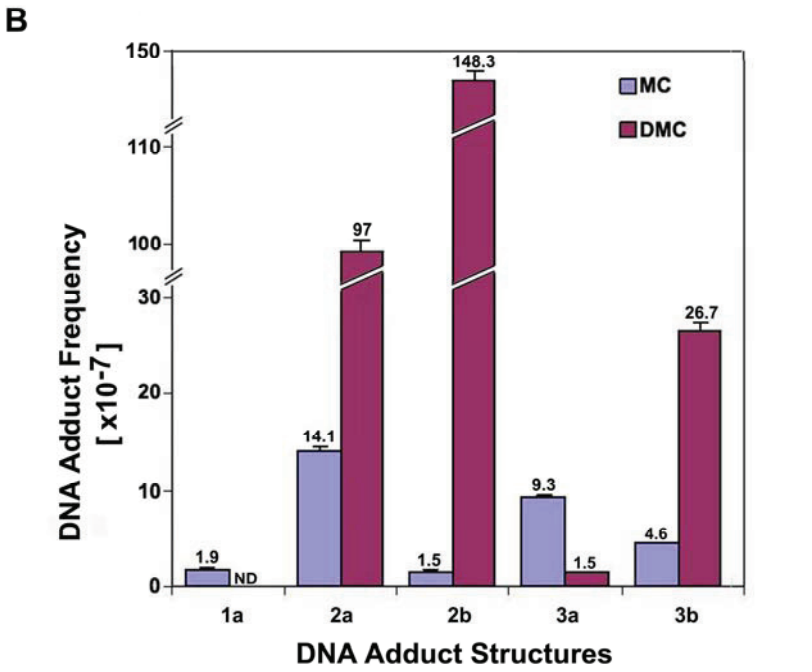


Figure 1. DMC generates more DNA adducts than MC. (A) Chemical structures of DNA adducts: 1a, 1"-α MC monoadduct; 2a, 1-"α DMC monoadduct; 2b, 1"-ß DMC monoadduct; 3a, 1"-α interstrand cross-link; 3b, 1"-ß interstrand cross-link.
(B) MCF-7 cells were left untreated or treated for 24 hours with 10 µM MC or 10 µM DMC. After treatments, DNA was isolated from the MCF-7 cells and digested to nucleosides. Data represent 3 independent injections of DNA adducts from the same experiment. Standard errors are represented by bars at the top of each column.

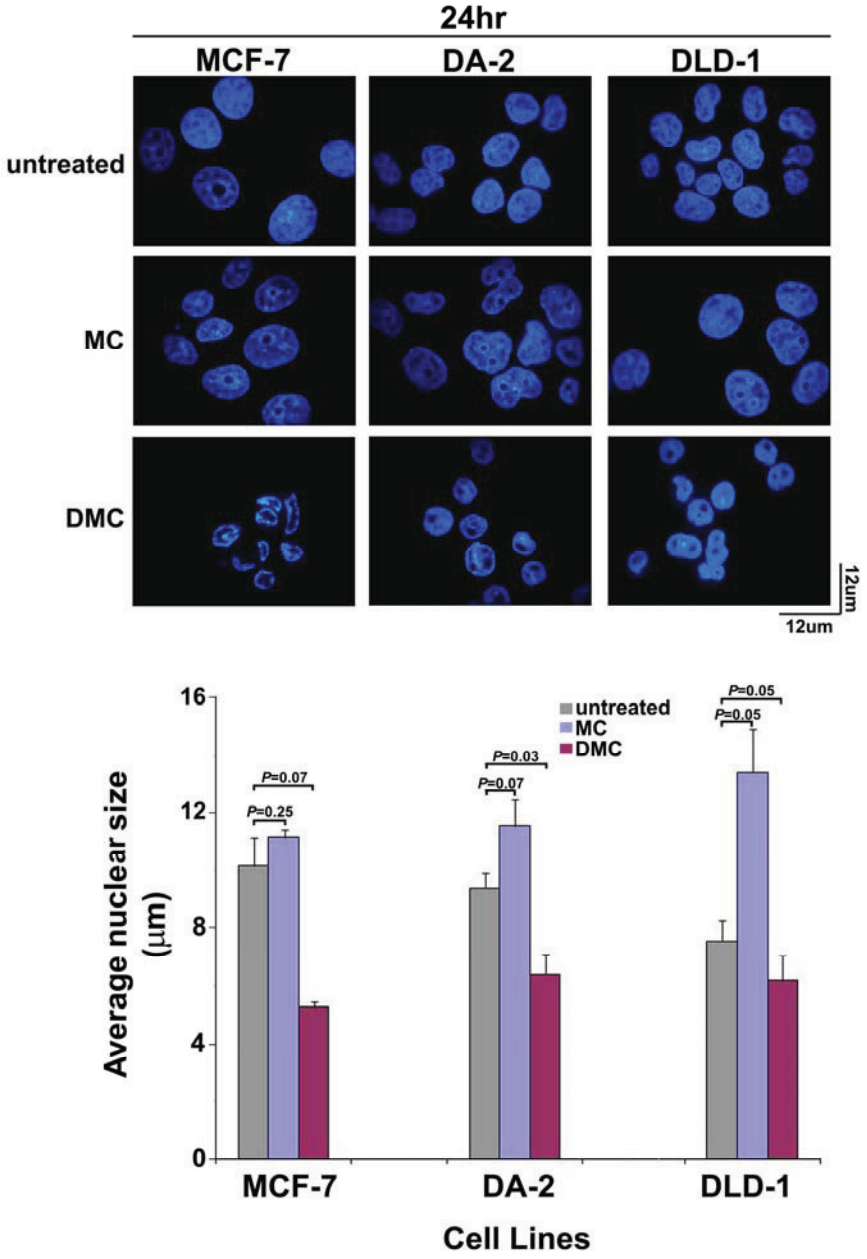


Figure 2A. DMC cytotoxicity is associated with decrease in nuclear size. MCF-7, DA-2, and DLD-1 cells were left untreated or treated for 24 hours with 10 μM MC or 10 μM DMC.

Representative regions of DAPI stained slide with nuclei from 3 independent experiments are depicted (top panel). Nuclei size was determined after staining cells grown on a cover slip with DAPI (lower panel). Average values obtained from 2 independent experiments are calculated. For each independent experiment the number of nuclei measured for all treatment condition (N) = MCF-7: 29; DA-2: 32; DLD-1: 21. Error bars denote s.e.m. Significance determined using a one-tailed paired *t*-test. Images were collected on a confocal microscope. Nuclear size was determined using Image J software.

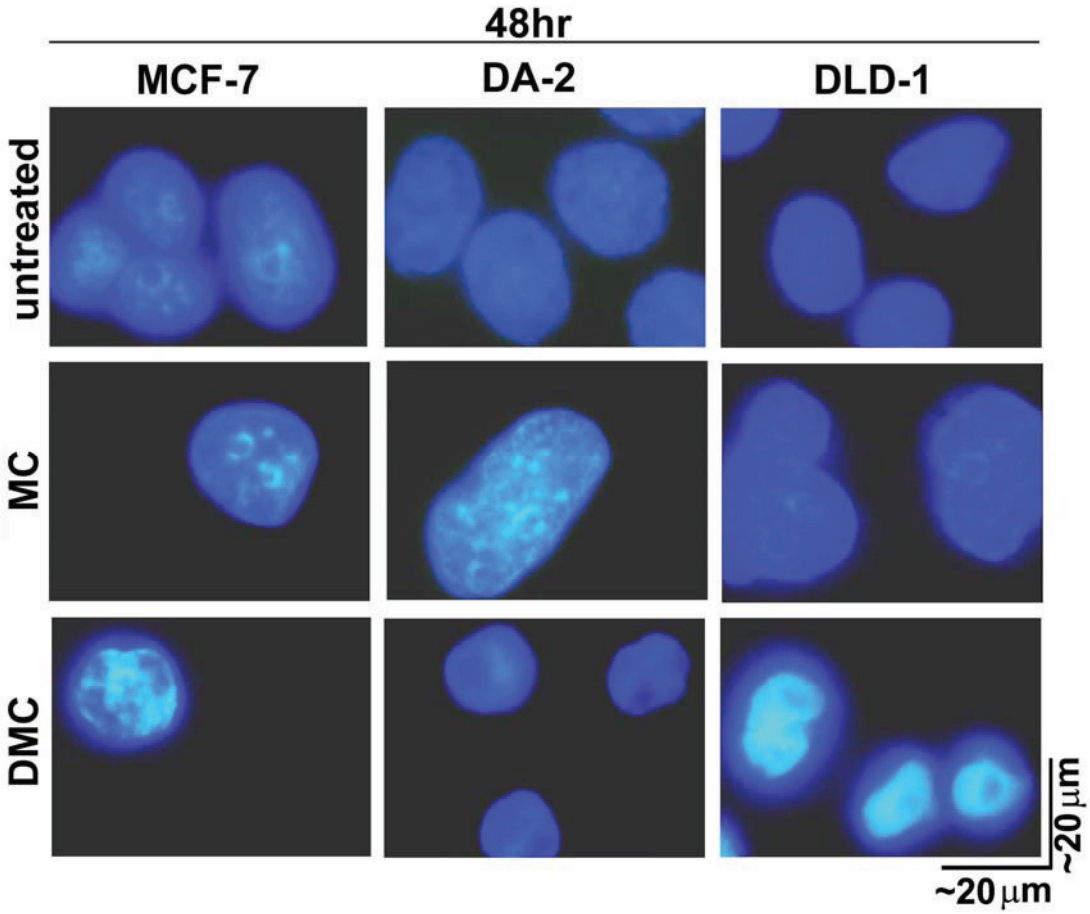


Figure 2B. DMC treatment caused a significant decrease in nuclear size. MCF-7, DA-2, and DLD-1 cells were left untreated or treated for 48 hours with 10 μM MC or 10 μM DMC.
Representative region of DAPI stained slide with nuclei are depicted. Image scale bar value was approximated due to errors encountered during image capturing. For this reason, average nuclear size values could not be calculated.

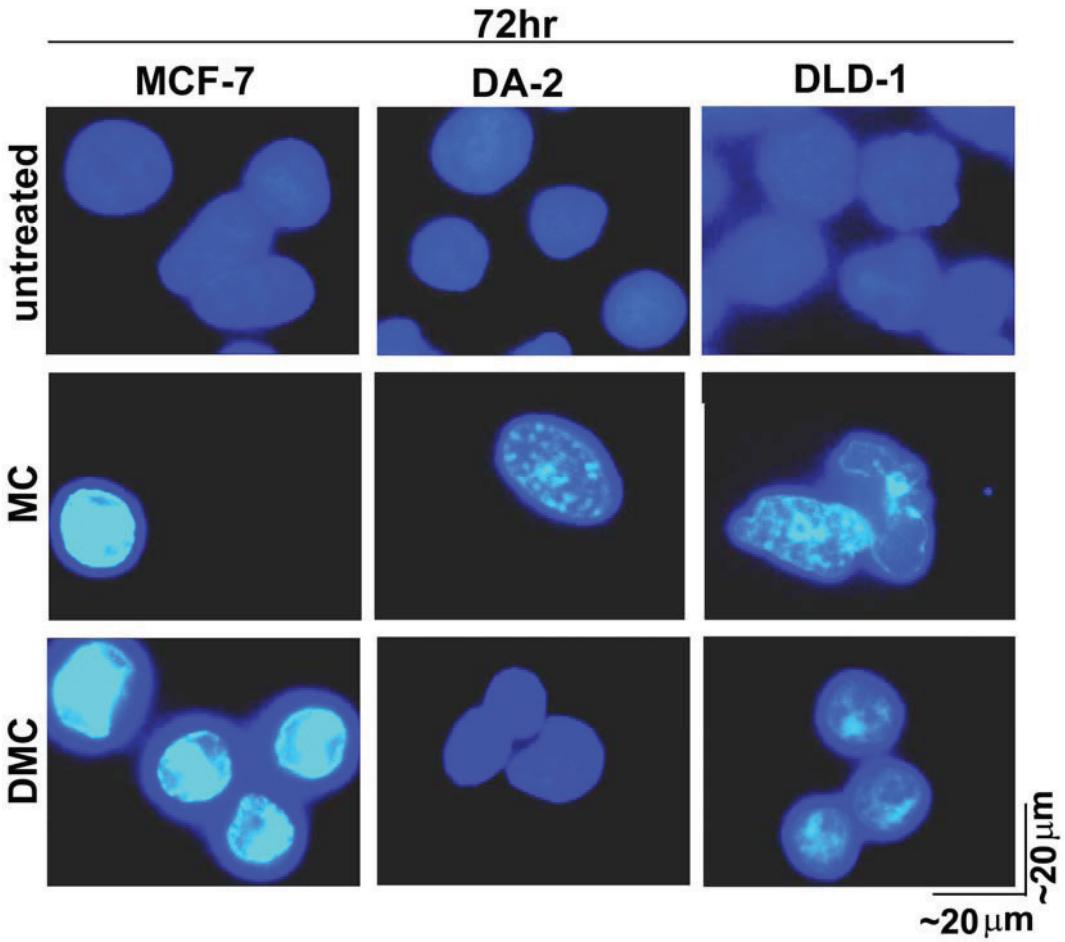


Figure 2C. Surviving cells after prolonged exposure to MC or DMC show variable nuclear size. MCF-7, DA-2, and DLD-1 cells were left untreated or treated for 72 hours with 10 μM MC or 10 μM DMC. Image scale bar value was approximated due to errors encountered during image capturing. For this reason, average nuclear size values could not be calculated.

3.2B Depletion of Chk1 by siRNA increased MC cytotoxicity in the absence of wild-type p53.

DMC treatment results in the depletion of Chk1 protein 24 hours post treatment while MC treatment does not (Boamah et al., 2007). Since we observed differential regulation of Chk1 during MC- and DMC- induced cellular cytotoxicity, we hypothesized that the Chk1 protein might play a role in the differential cytotoxicity observed for the two drugs. To address this hypothesis, we depleted the levels of endogenous Chk1 using siRNA and observed the outcome of drug treatment (Fig. 3*A see lanes 7-9*). In the absence of Chk1 siRNA, down-regulation of Chk1 protein was only seen in DMC treated cells (Fig. 3*A see lanes 3 and 6*). In the presence of Chk1 siRNA, we observed dramatic reduction in Chk1 protein and therefore we were able to investigate if this increased the cytotoxicity of MC (Fig. 3*A see lanes 7-9*).

We observed a significant decrease in cell proliferation in MC treated samples when Chk1 was depleted by Chk1 siRNA (Fig. 3*B*). Interestingly, we observed a statically significant change in cellular viability of MC treated cells when siRNA to Chk1 was added as indicated by a P-value of 0.001 and also with DMC treatment after siRNA mediated gene silencing of Chk1 with a P-value of 0.008 (Fig. 3*B*).

Chk1 protein is essential for the maintenance of genomic integrity and, Chk1 mouse knockdown is embryonic lethal (Enders, 2008). Perhaps, the lack of spontaneous cell death after siRNA mediated depletion of Chk1 in DLD-1 cells was because we examined viability in a short period. We suspect that prolonging propagation will greatly affect the viability of these Chk1 depleted cells, even in the absence of any DNA damaging agents.

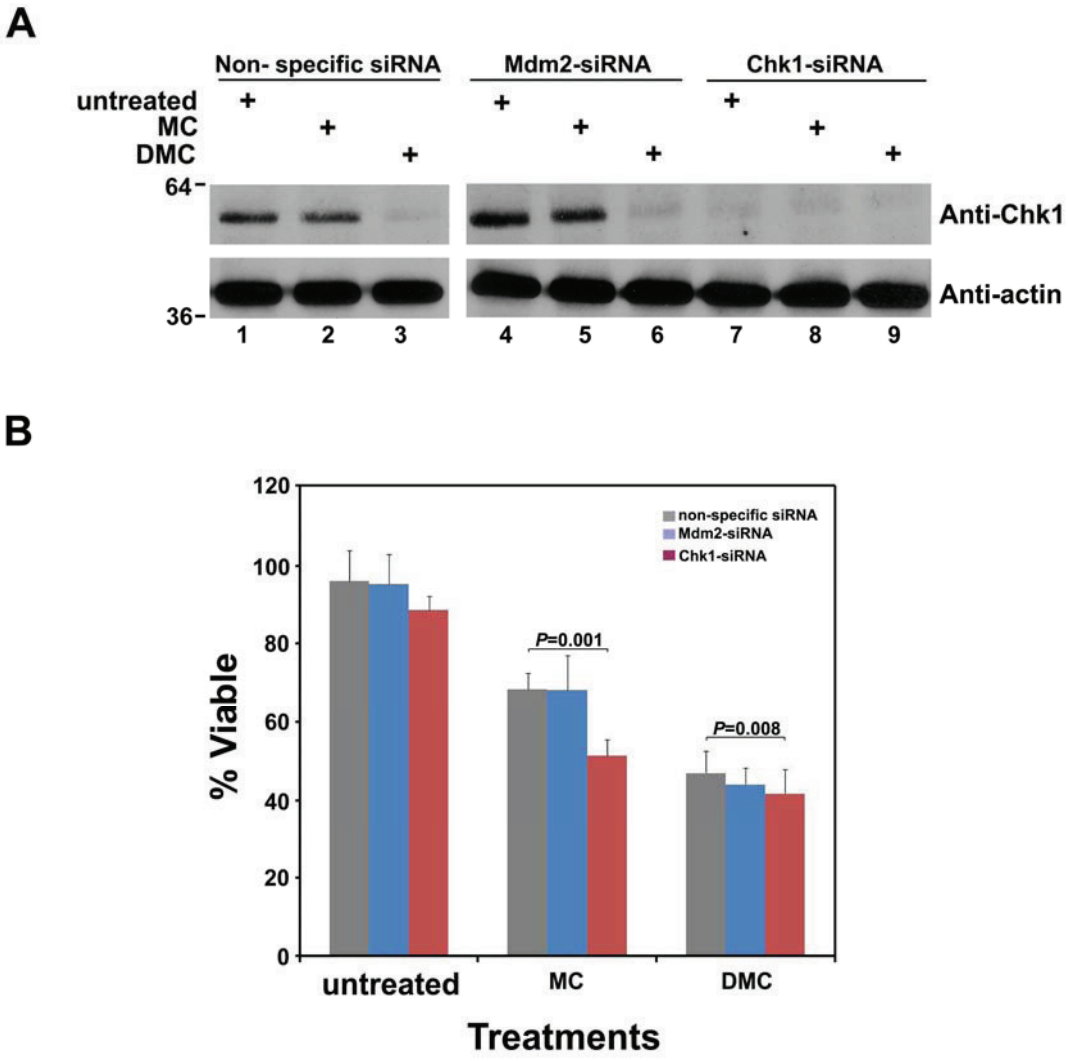


Figure 3. Chk1 down-regulation increased MC cytotoxicity in the absence of wild-type p53. (A) Cells were left untransfected or transfected with 25nM non-specific siRNA, 25nM Mdm2 siRNA, and 25nM Chk1 siRNA for 24 hours. After the transfection incubation period, cells were either left untreated, or treated with 10µM MC or 10µM DMC for an additional 24 hours. Whole cell extracts were obtained from these cells and analyzed for Chk1 expression. Actin was used as a loading control. (B) To determine the percent proliferation, cells were treated as indicated above. MTT solution was added to each sample and incubated for an additional 2 hours. Values were obtained from 3 independent experiments. Significance determined using a two-tailed paired *t*-test.

3.2C Chk1 depletion following DMC treatment can be rescued by inhibition of the proteasome.

To determine if Chk1 down-regulation was mediated through the ubiquitin proteasome pathway, we exposed DLD-1 cells to proteosome inhibitors. Total ubiquitinated protein products increased when cells were treated with MG115 (Z-LLnVal) (Fig. 4*A*, *compare lanes 1 and 2*) or with MG132 (Z-LLLal, data not shown). As expected DMC, but not MC, treatment caused the depletion of Chk1 protein (Fig. 4*A*, *lanes 3 and* 5). However, unexpectedly, the total ubiquitinated proteins also decreased. Importantly Chk1 levels and total ubiquitinated proteins were recovered in the presence of MG115 (Fig. 4*A*, compare *lanes 5 and 6*).

To further confirm that the recovery of Chk1 was due to inhibition of proteasome activity and not due to a change in transcript levels, we analyzed the levels of *chk1* transcripts under similar conditions. While DMC alone caused a 50% reduction in *chk1* transcription in both the DA-2 and DLD-1 cells, MC treatment resulted in increase transcript levels (Fig. 4*B*). Paradoxically, this increase in transcript levels did not result in an increase in Chk1 protein levels. MG115 did not upregulate *chk1* transcripts in MC and DMC treated sample. The recovery of Chk1 upon successful inhibition of the proteasome pathway implicates the proteasome as the primary mechanism in DMC mediated Chk1 degradation (Figs 4*A*, *lanes 5 and 6*).

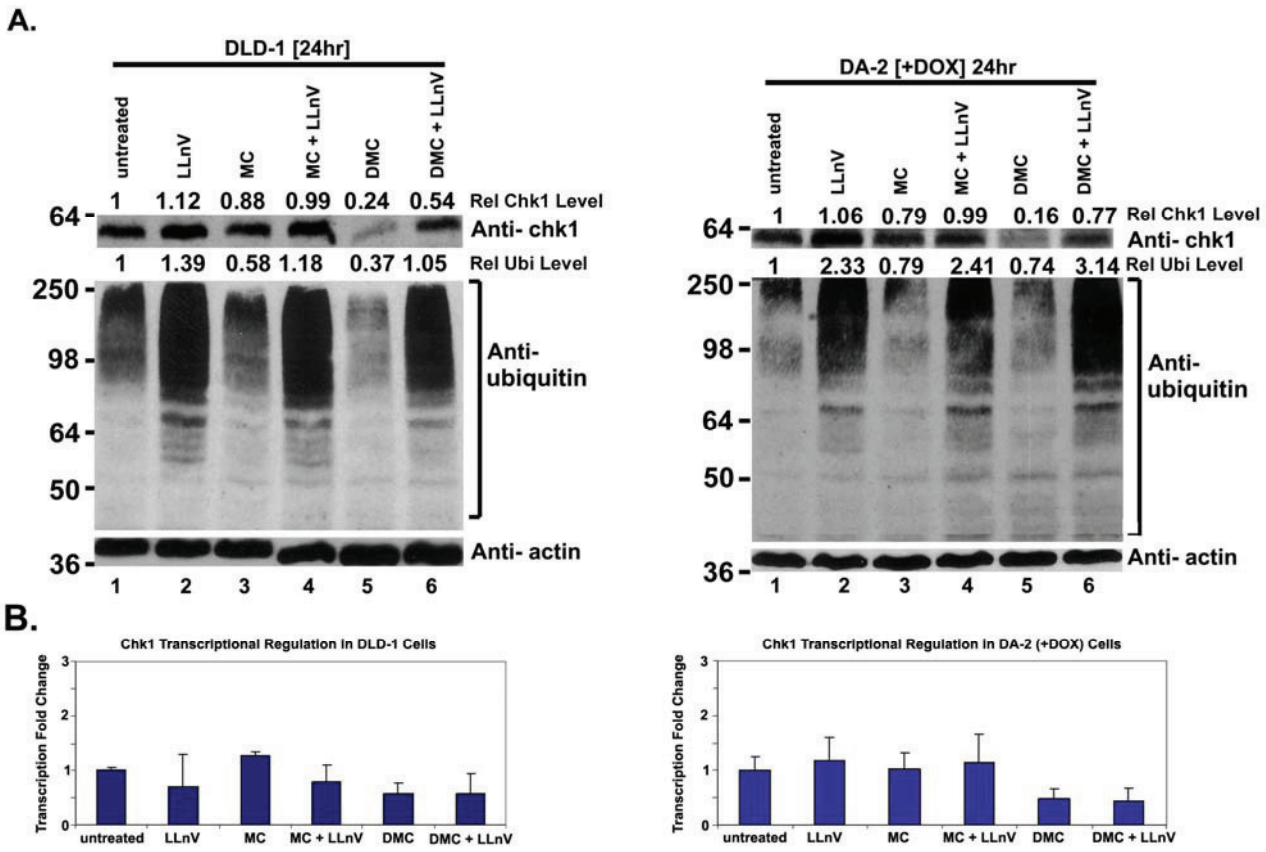


Figure 4. DMC cytotoxicity activates a proteasome dependent depletion of Chk1. (A) Whole cell extracts obtained from DLD-1 (left panel) and DA2+DOX (right panel) cells were left untreated (lane 1), treated with 4μM LLnV (lane 2), 10μM MC or 10μM DMC (lanes 3 and 5), 10μM MC or 10μM DMC with 4μM LLnV (lanes 4 and 6) were analyzed by Western blotting for Chk1 protein (top panels) and accumulation of ubiquitinated proteins (bottom panels). Actin was used as a loading control. Numbers at the top of each sample lane represent the relative Chk1 or Ubiquitin protein levels, normalized to actin. (B) Quantitative real-time PCR (RT-PCR) analysis of *Chk1* mRNA from DLD-1 (left panel) and DA2+DOX (right panel) cells left untreated, treated with 4μM LLnV, 10μM MC or 10μM DMC, 10μM MC or 10μM DMC with 4μM LLnV. Results were normalized to untreated samples and *actin* values.

3.2D DMC DNA adducts activate signaling, without increasing proteasome activity.

Increased degradation of total ubiquitinated protein suggested that perhaps DMC DNA adducts signaled for an increase in proteasome activity. We investigated this hypothesis after we observed increased down-regulation of Chk1 in addition to a substantial decrease in total ubiquitinated products after treatment with DMC (Fig 4, *lane 5*). We used an in gel assay to detect in cell lysates the cleavage of the fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC, by the proteasome core particle (CP) with both regulatory particles (RP_2CP), core particle with one regulatory particle (RP_1CP), and the core particle only (CP). The inhibitor, MG115 decreased proteasome activity in DLD-1 cells (without wild-type p53) (Fig. 5*, left panel, lane 2*). However, MC and DMC treatment did not increase proteasome activity compared to untreated (Fig. 5, *compare lanes 3 and 4 to lane 1*). The increased degradation of ubiquitinated substrates in DMC treated cells was not due to a change in proteasome levels since DMC-treated and untreated cells showed similar proteasome levels (Fig 5, *compare left panel to right panel*). The observed decrease in ubiquitinated products in the absence of an increase in proteasome activity suggested that DMC DNA adducts might increase signaling through increased damage and by a block to the DNA replication or transcription machinery.

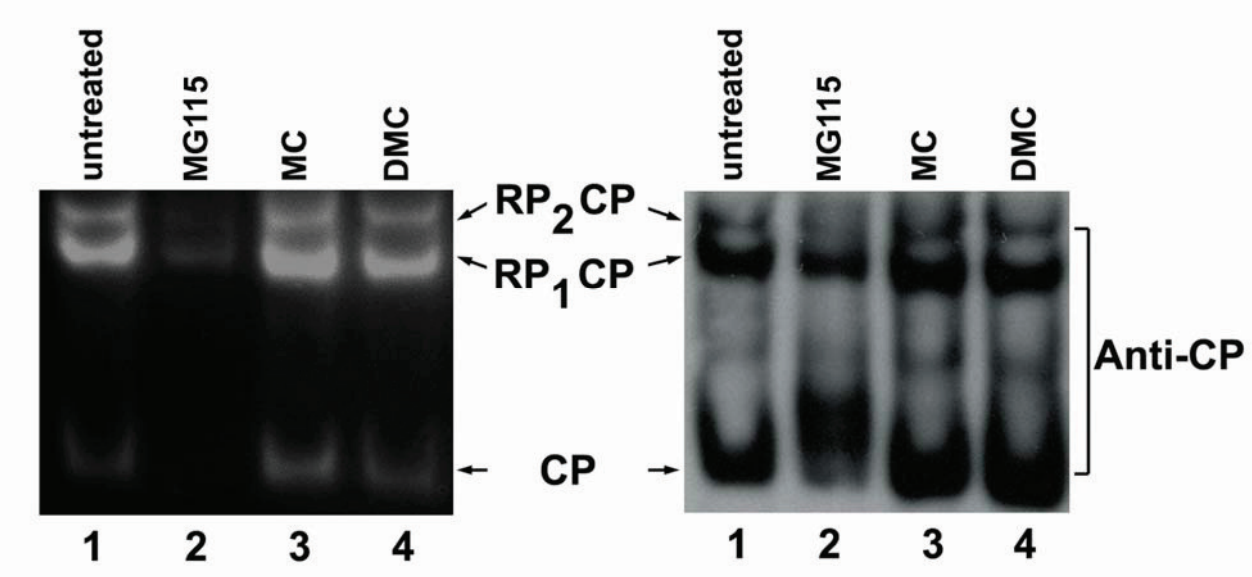


Figure 5. DMC cytotoxicity activates a proteasome dependent depletion of targets without increasing proteasome activity. DLD-1 cells were left untreated (lane 1), treated with 4μM LLnV (lane 2), 10μM MC (lane 3), or 10μM DMC (lane 3). Extracts were obtained from cells and analyzed on a native polyacrylamide gel for proteasome activity (left panel) and proteasome subunits (right panel).

3.2E Over-expression of Chk1 slightly attenuates DMC induced cell death in DLD-1 cells.

To address the potential of stabilized Chk1 to attenuate DMC cytotoxicity, we over-expressed a Myc tagged Chk1 protein, which was successfully induced under all treatment conditions (Fig. 6). Interestingly, only the endogenous Chk1 protein was selectively down-regulated by DMC while the exogenous Myc-Chk1 protein was not (Fig. 6, *lane 6*). Treatment with MC did not dramatically influence either endogenous or exogenous Chk1 (Fig. 6, *compare lanes 2 and 4*). We then examined the role of stabilized Myc-Chk1 in mitomycin induced cellular cytotoxicity (Fig. 6, *graph*). When exogenous Chk1 was introduced, viability was slightly increased for both MC and DMC. Under conditions where the vector alone was transfected into

cells, both MC and DMC showed significant reduction in cellular proliferation, with DMC showing increased cytotoxicity relative MC (Fig. 6*, graph*).

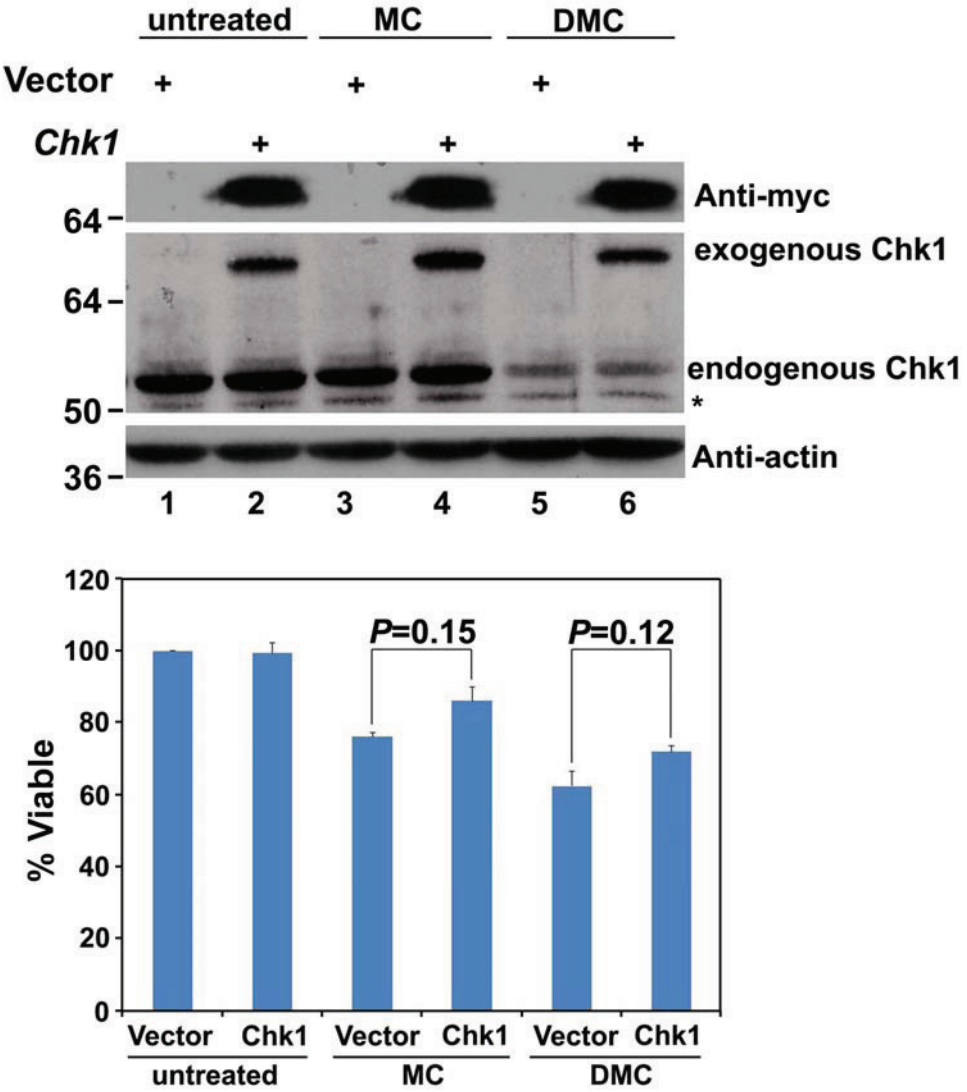


Figure 6. Over-expression of Chk1 slightly decreases DMC cytotoxicity. For over-expression, DLD-1 cells were transfected with 4µg of either empty vector or *Myc-Chk1* for 24 hours. After the transfection incubation period, cells were either left untreated, or treated with 10µM MC or 10µM DMC for an additional 24 hours. Whole cell extracts were obtained from these cells and analyzed for Chk1 expression. Actin was used as a loading control. To evaluate the proliferation of cells over-expressing *Myc-Chk1* after treatment with MC or DMC, DLD-1 cells were treated as indicated above. MTT solution was added to each sample and cells were incubated for an additional 2 hours. Values were obtained from 3 independent experiments. (*) Denotes non-specific bands. The pCS3^{+}-6Myc plasmid expressing Myc-Chk1 protein was kindly provided by You-Wei Zhang (Salk Institute, La Jolla, CA).

3.2F. Increased DMC DNA adducts interfere with certain transcriptional activities resulting in the down-regulation of multiple targets in DA-2 cell lines.

In the absence of any changes in proteasome activity upon treatment with MC or DMC, we investigated the impact of increased DNA adduct production on transcription of p53 target genes. We measured the ability of p53 to induce transcription upon treatment with MC or DMC to determine if increased DNA adducts could block p53 mediated activated transcription. We analyzed the transcriptional activity of wild-type p53 in DA-2 (it should be note that DA-2+DOX cells express low levels of wild-type p53) cells grown in media containing doxycycline. We observed a reduction in nuclear size in both MCF-7 and DA-2 after treatment with DMC, but not with MC (Figs. 2*A* and 2*B*). Both MC and DMC treatment stabilized p53 as determined by Western blot analysis. However, MC showed higher induced p53 protein levels compared to DMC (Figs. 7). The stabilized p53 after 24 hrs of treatment with MC robustly activated p53 transcription of the target genes *Mdm2* and *p21*. Interestingly, 24 hrs of DMC treatment, which stabilized p53, did not result in the transactivation of *Mdm2* and gave only minimal activation of *p21* (Figs. 7). This suggests that the increased frequency of DNA adducts, perhaps of the mitosene-1-β-DNA stereochemistry, produced by DMC interfered with the ability of the basal transcription machinery to produce transcripts. We have previously noted that early time points (3 and 6 hrs) after MC and DMC treatment are able to activate transcription of the p53 target genes (Abbas et al., 2002). Therefore we predict that this 24 hour treatment causes a block to the transcription because of increased amounts of DNA-adducts formed.

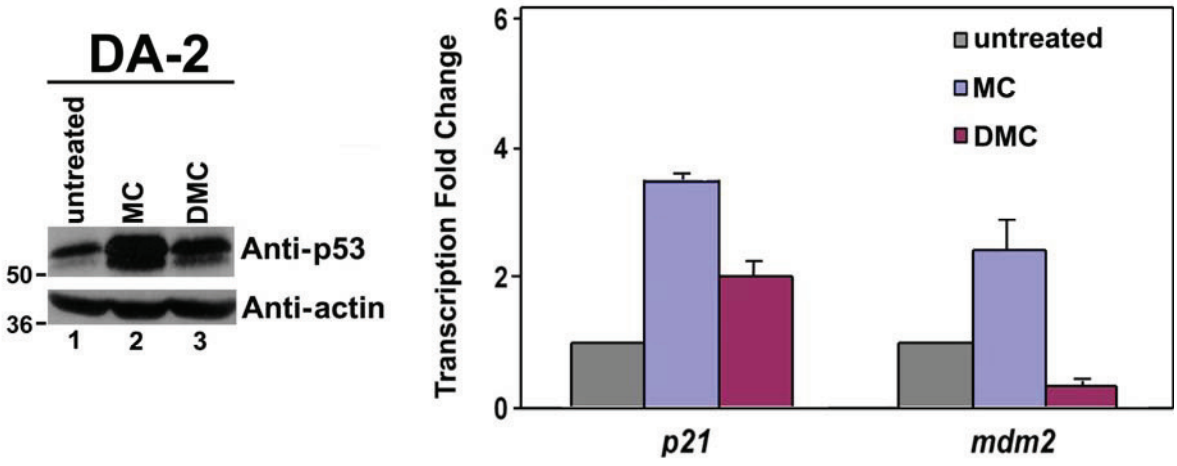


Figure 7. DMC increased DNA adducts disrupt transcription
DA-2 cells were left untreated (lane 1), or treated for 24 hours with 10µM MC (lane 2), or 10µM DMC (lane 3). After treatments, whole cell extracts were obtained from cells and analyzed for p53 protein stability. Actin was used as a loading control. For quantitative PCR analysis, cells were left untreated or treated for 24 hours with 10µM MC or 10µM DMC. Transcriptional activity of p53 target genes, *p21* and *mdm2* mRNA, were analyzed by quantitative real-time PCR (RT-PCR). Results were normalized to untreated samples and *actin* values.

3.3 DISCUSSION

Down-regulation of Chk1 has been described as an alternative pathway for inducing cell death when the more common apoptotic pathways have been sabotaged in cancer cells (Dixon and Norbury, 2002; Carrassa et al., 2004). We have investigated how increased and altered production of DNA adducts can result in Chk1 down-regulation and increase cell death especially in the absence of a p53 checkpoint (Boamah et al., 2007). We observed that treatment with DMC produces more mitosene-1-β-DNA adducts than MC (Palom et al., 2002; Paz et al., 2008). The cytotoxicity of these drugs significantly differ (Palom et al., 2002), especially in the absence of wild-type p53 activity (Abbas et al., 2002). In view of the close structural similarity between MC and DMC, the differences in their biological effects in human tumor cell cultures

were unexpected. In this report, we further characterize these effects and propose to correlate them with the known structural differences of their DNA adducts.

Interstrand cross-link (ICL) DNA adducts are the clinically relevant cytotoxic lesions produced upon exposure to MC and DMC. The intrinsic ability of the cellular machinery to recognize and excise ICL lesions from the genome and activate cell death when genomic integrity is beyond repair underlies the sensitivity of various cell lines to DNA cross-linking (Dronkert and Kanaar, 2001). The increased production of mitosene-1-β-DNA adducts following DMC treatment might account for the increased cytotoxicity observed with this derivative compound. The nature of the mitosene-1-β interstrand cross-links and how they contribute to cytotoxicity is just beginning to emerge and will provide useful tool for dissecting how DNA structures signal to alternative cell death pathways.

DMC treatment of cells also caused a reduction in nuclear size which is consistent with mitotic catastrophe (Carrassa et al., 2004). Nuclear shrinkage during DMC induced cell death was independent of wild-type p53. While nuclear condensation is a hallmark of apoptotic cell death, we have documented that caspase-3 and -9 activation, and PARP cleavage are limited in DMC treated cells lacking p53 (Boamah et al., 2002). Our data therefore suggests an association with cell death by DMC to be by mitotic catastrophe. It should be noted that, micronuclei formation has been reported as a characteristic of cells undergoing DNA damaged induced mitotic catastrophe (Carrassa et al., 2004; Chan et al., 1999). The possibility of cytokinesis in the absence of complete DNA replication due to increased cross-links and loss of Chk1 dependent checkpoint, could account for the observed micronuclei. While we do not have direct evidence for this, our confocal microscopy images of DAPI stained nuclear (Fig 2) show brightly stained condensed nuclear architecture when the cells were treated with DMC for 24 hours.

It is known that ICLs disrupt replication and transcription activities (Dronkert and Kanaar, 2001). ICLs are mostly cytotoxic to cells in the S phase which are actively replicating because these aberrant DNA structures inhibit elongation by stalling the polymerase. The stalled polymerase activates DNA damage response factors and checkpoint regulators (Osborn et al., 2002). The ATR- Chk1 pathway operates at the G_2/M transition and it is mostly activated in response to replication arrest to prevent inappropriate firing of late origins, collapse of the replication fork, and premature entry into mitosis (Enders, 2008). The ability of Chk1 to arrest the cell has been shown to prolong cell survival after DNA damage (Hirose et al., 2001; Wang et al., 2002). It is shown here that increased production of mitosene-1-β-DNA adducts by DMC correlates with a signaling mechanism that results in the down-regulation of Chk1. In the absence of Chk1 the DNA-damaged cells continue through the G_2/M checkpoint eventually causing death by mitotic catastrophe. Recently, it was reported that the ATR/Chk1 was highly specific to replication blockers as opposed to the alternative ATM/Chk2 regulatory pathway (Cho et al., 2005; Myers et al., 2009). This suggests that the primary pathway activated by replication blockers to induce recovery from DNA damage and prolong cell survival is through the ATR/Chk1 pathway. The regulation of Chk1 we have observed correlates well with the differential cytotoxicity of MC and DMC at early timepoints. Previously, we observed that prolonged exposure to MC could also result in Chk1 down-regulation and loss of cellular viability, this is may be due to increased DNA adduct formation with more time (Boamah et al., 2007). Consistent with our current data, Sugiyama et al reported that disruption of the G_2 checkpoint using UCN-01, a Chk1 inhibitor, potentiated the cytotoxicity of MC and this effect was independent of the wild-type p53 (Sugiyama et al., 2000).

In this report, we observed that at 24 hours of exposure to MC when Chk1 was down-regulated by siRNA, MC induced similar cytotoxicity to that observed with DMC. Interestingly, upon depletion of Chk1 using siRNA, the viability of these cells was similar as observed with Mdm2-siRNA and non-specific siRNA. While the loss of Chk1 has been shown to be lethal, the lack of significant cell death after depletion of Chk1 in our samples maybe because we maintained these cells for only 48 hours. Perhaps, extensive culturing beyond this 48 hour time point will recapitulate the expected cell death outcome observed upon depletion of Chk1.

In contrast, over-expressing Chk1 caused only a slight decrease in the cytotoxicity of the two mitomycins at 24 hours post treatment. We cannot exclude the possibility that lack of upstream or downstream factors that impinge on Chk1 activity are down-regulated by DMC DNA adduct signaling. Down-regulation of these factors might render exogenous Chk1 insufficient to dramatically affect the cellular outcome after over-expression. However, since we observed a slight increase in survival after Chk1 over-expression, we believe that the over-expressed Chk1 contributes to increased survival but requires additional factors.

The regulation of Chk1 has been implicated in the response of cells to replication blockers. Phosphorylation of Chk1 at Ser345 as opposed to Ser317 by ATR, preferentially destabilizes Chk1 by marking the protein for ubiquitin mediated proteolysis (Zhang et al., 2005). We observed that when the proteasome activity was inhibited by MG115, Chk1 protein levels were recovered even when cells were treated with DMC. Our data supports the hypothesis that DMC down-regulates Chk1 by causing increased ubiquitination and subsequent degradation by the proteasome. Importantly, we saw that this mechanism of Chk1 down-regulation happened in the absence of increased proteasome activation. Although we did not observe any increase in proteasome activity in DMC treated samples, we observed a decrease in total ubiquitinated

protein products upon treatment with DMC. This supports the above hypothesis that DMC DNA adducts signal for altered protein post-translational modifications that cause rapid degradation of the tagged polypeptides. It is substantiated by our microarray data that showed that DMC treatment caused a dramatic reduction in the levels of many proteins on the Sigma Panorama Cell Signaling array.

When we examined the p53 transcription activity, we observed that MC caused much higher levels of activated p53 target genes than DMC. This may be due to the fact that DMC DNA adducts negatively affect promoter activation because the transcription machinery will stall, perhaps due to increased and variably shaped DNA adducts. This suggests that even in the presence of wild-type p53 protein, DMC might activate cell death by a p53 independent pathway.

In conclusion, we observe that DMC induces p53-independent cell death in cancer cells, mediated by degradation of Chk1. We propose that Chk1 degradation is correlated with the predominant formation of mitosene-1-β guanine adducts of DMC, in contrast to the mitosene-1-α adducts formed with MC (Fig 8). This alteration of the chirality of the drug linkage to DNA undoubtedly changes the alignment of DMC adducts, resulting in a different DNA damage signaling mechanism. The alignment of the MC mitosene-1-α monoadduct and of the 1-α cross-link in oligonucleotide duplexes have been well characterized (Norman et al., 1990; Tomasz et al., 1987; Sastry et al., 1995). The alignment of the 1-β adducts of DMC have not been studied yet. The mitomycins represent the first case in which DNA linkage chirality is proposed to modulate the biological response to DNA damage.

Depletion of Chk1 was recently reported to activate an ATR-caspase-2 apoptotic cell death pathway that is independent of wild-type p53 (Sidi et al., 2008). This mode of p53-

independent apoptotic cell death does not activate the canonical intrinsic and extrinsic cell death markers. Our analysis of DMC-induced cell death failed to indicate activation of any of the multiple critical apoptotic cell death markers, however, we observed an increase in caspase activity in the absence of wild-type p53 (Boamah et al., 2007). It is possible that the observed increase in caspase activity originates from caspase-2 activation, since none of the more common pro-apoptotic caspases were depleted in our analysis (Boamah et al., 2007). We are currently investigating the role of caspase-2 in DMC induced cell death in cell lines with mutations in the p53 pathway. Interestingly, hypoxia, which has been shown to protect cancer cells, was also reported to activate the ATR/Chk1 pathway to increased cell survival and protection against ROS mediated DNA damage (Hammond et al., 2002; Hammond et al., 2003). DMC, which has been shown to have increased DNA adduct formation under hypoxic conditions, can possibly have therapeutic use due to the ability of its adducts to signal for a rapid Chk1 degradation, thereby counteracting the cancer-protective effects of hypoxia.

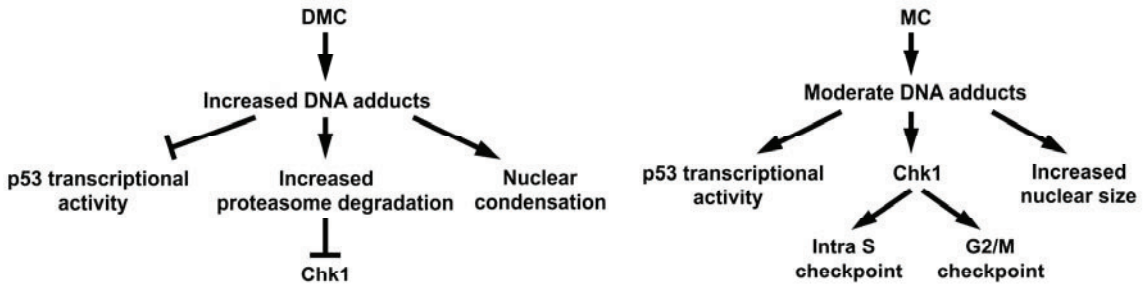


Figure 8. Regulation of molecular targets by DMC and MC DNA adducts. DMC treatment generates increased DNA adducts while MC treatment produces moderate DNA adducts. The increased DNA adducts produced by DMC interferes with p53 transcriptional activity and signal for the rapid depletion of endogenous Chk1 which may disrupt the G_2 /M cell cycle checkpoint and lead to nuclear condensation. MC DNA adducts stabilize Chk1 which may prolong the Intra S and G_2 checkpoints, increase nuclear size and activate p53 transcriptional activity.

CHAPTER 4

MC and DMC DNA adducts signal to different molecular targets to induce p53-dependent and p53-independent cell death

4.1 INTRODUCTION

The *p53* gene is the most commonly mutated gene in human tumors (Nigro et al., 1989b); drug induced pathways that can lead to cell death in the absence of p53 are important since mutations in the p53 gene are common in diverse types of human cancer (Hollstein et al., 1991). We previously reported that both MC and DMC stabilize p53 protein and activated cell death in ML-1 cells (Abbas et al., 2002). Interestingly, while MC and DMC are approximately equitoxic to ML-1 cells, DMC is more cytotoxic than MC to the K562 cell line which lacks p53, suggesting that DMC provokes an alternate and p53-independent cell death pathway (Abbas et al., 2002). The increased cytotoxicity of DMC in cells lacking a functional p53 pathway suggests DMC may activate a pathway which results in cell death, and may have chemotherapeutic potential by triggering a p53-independent cell death pathway.

DNA alkylating agents, have been reported to induce Poly(ADP)ribose polymerase (PARP) activity during cellular cytotoxicity (Zong et al., 2004). PARP is an abundant, highly conserved nuclear protein involved in maintaining genomic integrity (Bouchard et al., 2003; Smith, 2001; Chiarugi, 2002). Single and double strand breaks has been reported to induce PARP catalytic and DNA binding properties (Koh et al., 2005; Jeggo, 1998). Activated PARP consumes NAD+ to form polymers of poly(ADP)ribose (PAR), which is used to postranslationally modify itself and a select group of histones and nuclear proteins involved in the maintenance of chromatin architecture and DNA repair (Smith, 2001; Tong et al., 2001). In cells with functional PARP activity, the degree of PARP induction by genotoxic agents has been suggested to determine the fate of the cell (Ivana Scovassi and Diederich, 2004). In the presence of low levels of DNA damage, PARP acts a survival factor by inducing detection and repair of the damage region. In contrast, damages that are beyond repair induce PARP hyperactivation

which has been reported to induce both a p53 independent apoptotic and non apoptotic cell death (Bouchard et al., 2003).

While a number of reports concerning cellular signaling by MC-DNA adducts exist, studies on molecular signaling by DMC-DNA adducts are extremely limited. Since we observed increased cytotoxicity with DMC compared to MC, especially in cells lacking wild-type, we investigated the molecular targets induced by DMC-DNA adducts. Previously, we determined that MC and DMC activate the p53 pathway and cause increased transcription of p53 downstream target genes (Abbas et al., 2002; Abbas et al., 2004; Arva et al., 2005). MC has been shown to activate caspases-8 and -3 (Engels et al., 2000; Park et al., 2000; Vit et al., 2001), however, the caspases activated by DMC have not been reported. Very little is known about how DMC activates molecular targets during cell death, aside from its ability to activate the p53 pathway and its increased cytotoxicity as compared to MC for cells lacking a functional p53 pathway (Abbas et al., 2002).

In this study, using isogenic p53 cell lines, DA2+DOX and DLD-1, we compared molecular targets activated by DMC in the presence and absence of wild-type p53 relative to MC induced target activation. We observed that both MC and DMC activated procaspases-8 and -3 at an early timepoint, and induced PARP activity and Chk1 down-regulation after prolonged treatment. Interestingly, the down-regulation of Chk1 coincided with MC and DMC induced cellular cytotoxicity in cells without wild-type p53

4.2 RESULTS

4.2A DMC is more cytotoxic than MC to isogenic colon cancer cells lacking wild-type p53 and equally cytotoxic to cells expressing wild-type p53.

In order to further substantiate the increased effectiveness of DMC in promoting cell death in cell lines lacking wild-type p53, we used etoposide, MC, and DMC to induce DNA damage in the isogenic colon cancer cell lines DLD-1 (expressing no wild-type p53) and its derivative DA-2, which has low level expression of wild-type p53 in the presence of doxycycline (+DOX) (Fig. 1B). MTT analysis detects changes in cytotoxicity in response to drug treatment with a high degree of precision for detecting living, but not dead cells (Mosmann, 1983). In fact, when compared to the clonogenic assay, the MTT assay shows excellent reproducibility (Shimoyama et al., 1989).

MTT assay results showed that 24 hours DMC, but not MC or etoposide, treatment substantially decreased live cells in both DLD-1 (no p53) and DA-2 cells (+DOX: low p53) (Fig. 3A); at 48 hours of treatment, DMC was more cytotoxic than MC to DLD-1 cells but the two drugs were equally cytotoxic to DA-2 +DOX cells. At 24 hours and 48 hours a greater difference of cytotoxicity of DMC and MC occurred in the DLD-1 isogenic colon cancer cells without wild-type p53 (Fig. 1A and for p53 levels see Fig. 1B, lanes 1-3). However by 72 hours etoposide and MC were almost as cytotoxic to DLD-1 cells. When low wild-type p53 expression was present in DA-2 +DOX cells, DMC only demonstrated more cytotoxicity than MC at the 24 hour time point (Fig. 1A, for p53 levels Fig. 1B, lanes 4-6). MC and DMC increased the amount of p53 protein in low expressing DA-2+DOX cells, likely due to DNA damage induced post-translational modifications of the p53 protein resulting in enhanced stability (Fig. 1B lanes 4-6).

In D-A2 (-DOX) cells, p53 levels were substantially higher in the untreated sample and no further increase was observed in the presence of MC or DMC (Fig. 1B, lanes 7-9).

To examine the relationship between decreased viability after mitomycin drug treatment of DLD-1 and DA-2+DOX cells and the induction of cell death pathways we analyzed apoptotic markers in cell extracts. During cell death the enzyme PARP is often cleaved, which has been associated with apoptosis (Kaufmann et al., 1993). We observed evidence of substantial PARP cleavage after MC and DMC treatment of DA-2+DOX cells (Fig. 1B lanes 5-6 and 8-9). The death of DLD-1 cells (no wild-type p53) after DMC treatment was therefore occurring in the absence of PARP cleavage (an apoptotic marker) as Western blot analysis showed barely detectable PARP cleavage in mitomycin treated DLD-1 cells (Fig. 1B, lanes 1-3). Importantly, only 40% of DMC treated cells were viable as determined by MTT analysis after 24 hours of drug treatment (Fig. 1A).

The depletion of procaspases-8 and -3 was also examined. DLD-1 cells treated with DMC showed a minor decrease in procaspase levels while barely detectable changes in the procaspases were seen in MC-treated samples (Fig. 1C lanes 1-3). In the presence of low level wild-type p53 expression MC treatment resulted in depletion of procaspases-8 and -3 while DMC treatment had a lesser effect (Fig. 1C, lanes 4-6). However, although procaspase depletion was evident after MC treatment, more cell death was seen in DMC treated cells than in MC treated cells at the same time point (Fig. 1A). The most significant procaspase depletion was detected in the presence of high levels of induced p53, when DA-2+DOX cells (Fig. 1C, lanes 7-9), and this was not influenced by mitomycin treatment.

Analysis of total caspase levels using the more sensitive Guava MultiCaspase assay showed a slight increase in caspase activity in DLD-1 cells after MC and DMC treatment (Fig.

1D). This was surprising because the procaspase depletion analysis showed a barely detectable change but the levels of caspase activation were lower in DLD-1 cells as compared with DA-2+DOX MC and DMC treated samples (Fig. 1D).

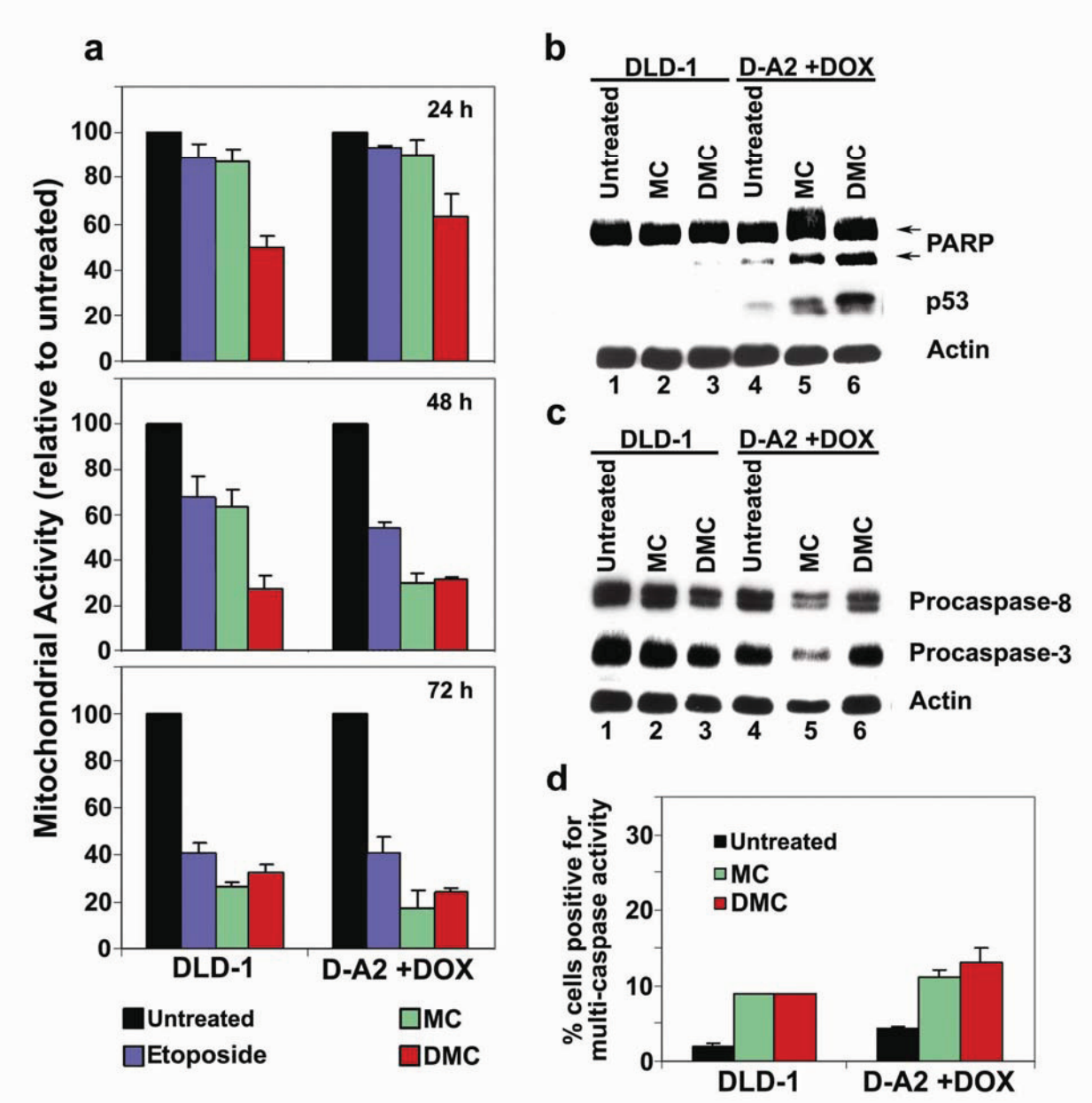


Figure 1. DMC-induced cytotoxicity in the absence of wild-type p53 is independent of PARP cleavage and caspase activation in isogenic cell lines. *A*, The cytotoxicity of the mitomycins in DLD-1 and D-A2 cells grown in the presence (+DOX) or absence (-DOX) of doxycycline was monitored using MTT assay. Cells were either left untreated or treated with 10uM MC or DMC for the indicated times. Data shows percentage of cells with mitochondrial activity relative to untreated samples and is representative of three independent experiments. Error bars indicate the standard deviation. *B*, Western blot analysis of DLD-1 and D-A2 cells grown in the presence (+DOX) or absence (-DOX) of doxycycline for 24 hours. Nuclear extracts from cells left untreated (*lanes 1, 4 and 7*) or treated with 10uM MC (*lanes 2, 5 and 8*) or DMC (*lanes 3, 6 and 9*) were monitored for PARP cleavage and p53 stability, using actin as a loading control. *C*, Caspase activation was monitored in DLD-1 and D-A2 cells grown in the presence (+DOX) or absence (–DOX) of doxycycline. Cytoplasmic extracts were prepared from cells left untreated or treated with 10uM MC or DMC for 24 h. Depletion of procaspases-8 and -3 in DLD-1 (*lanes 1-3*), D-A2 +DOX (*lanes 4-6*) and –DOX (*lanes 7-9*) cells was monitored by Western blot analysis, using actin as a loading control. *D*, Quantitative analysis of caspase activity in DLD-1 and D-A2 cells grown in the presence (+DOX) or absence (-DOX) of doxycycline was monitored by Guava MultiCaspase assay. Cells were either left untreated or treated with 10uM MC or DMC for 24 h. Bars are representative of percentage of cell population exhibiting caspase activity. Data is representative of three independent experiments. Error bars indicate the standard deviation.

4.2B MC and DMC differ in the activation of apoptotic target genes in isogenic cell lines.

We investigate the transcriptional activation of apoptotic genes induced by stabilized MC and DMC in DA-2+DOX cells (see Fig. 1B), and DLD-1 cells, which do not express wild-type p53 protein. We observed that after 24 hours of treatment, DMC caused down-regulation of selective apoptotic targets (*bax and puma*) while MC induced these targets about 2 fold, compared to untreated samples (Fig. 2A). Surprisingly, DMC robustly activated *gadd45*, compared to MC, although, the activation of this target is p53 independent, since in DLD-1 which do not express wild-type p53, similar induction was observed (Fig. 2A and B). In the absence of wild-type, the induction of p53 target genes were relatively low after treatment with MC and DMC (Fig. 2B)

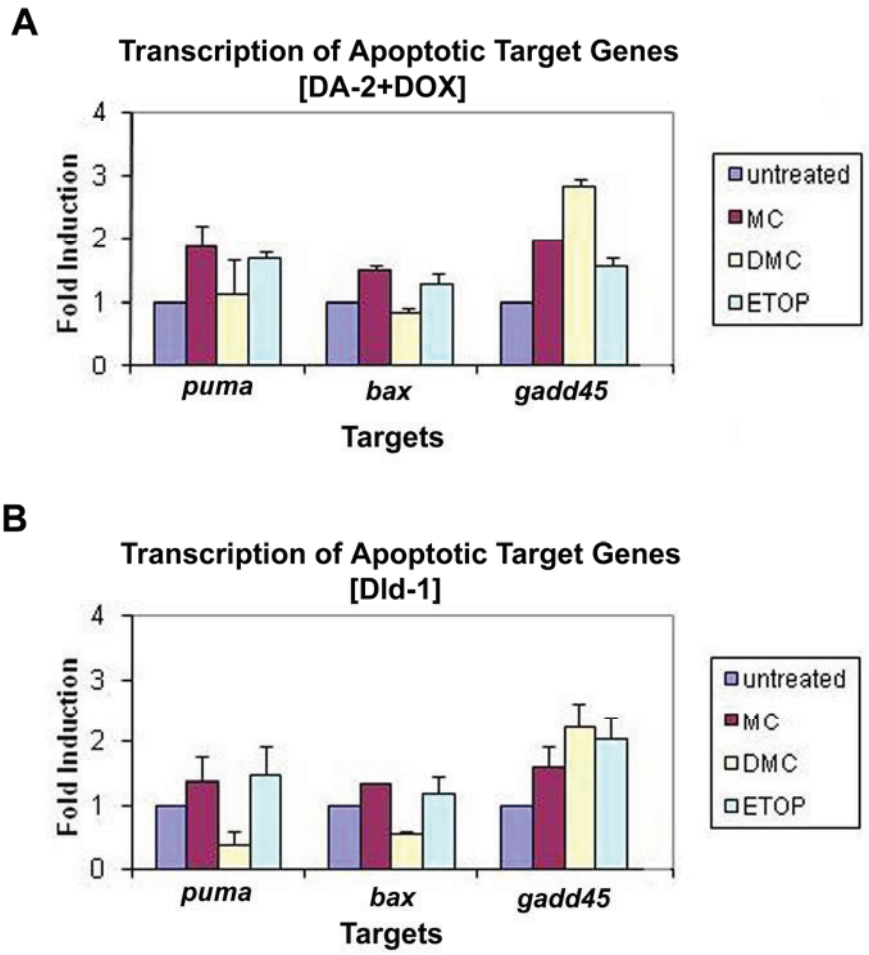


Figure 2. Apoptotic target gene activation by MC, compared to DMC, is robust in cells expressing wild-type p53.
(A) DA-2+DOX and (B) DLD-1 cells were left untreated (lane 1), or treated for 24 hours with 10μM MC, 10μM DMC, or 10μM etoposide for 24 hours Transcriptional activity of apoptotic target genes, *puma*, *bax*, and *gadd45* mRNA, were analyzed by quantitative real-time PCR (RT-PCR). Results were normalized to untreated samples and *actin* values.

4.2C Increased cytotoxicity of DMC in the absence of wild-type p53 is not the result of increased DNA double strand breaks.

To examine the degree of double strand breaks caused by the DNA damaging agents, we examined levels of phosphorylated histone 2AX (γH2AX) foci in cell nuclei and total γH2AX in cell extracts (Fig. 3A and Fig. 3B). Etoposide is well known for causing DNA double strand breaks. Importantly, in the absence of wild-type p53, DLD-1 cells showed less indication of

double strand breaks after treatment with DMC for 4 h than did the same cells treated with either MC or etoposide (Fig. 3A immunofluorescence and lanes 1-4 of the Western blot). In the absence of p53 all indicators of DNA double strand breaks remained greater in etoposide treated cells after 12 and 24 h of treatment but were the same in MC and DMC treated cells (Fig. 3A, lanes 5-10 and data not shown).

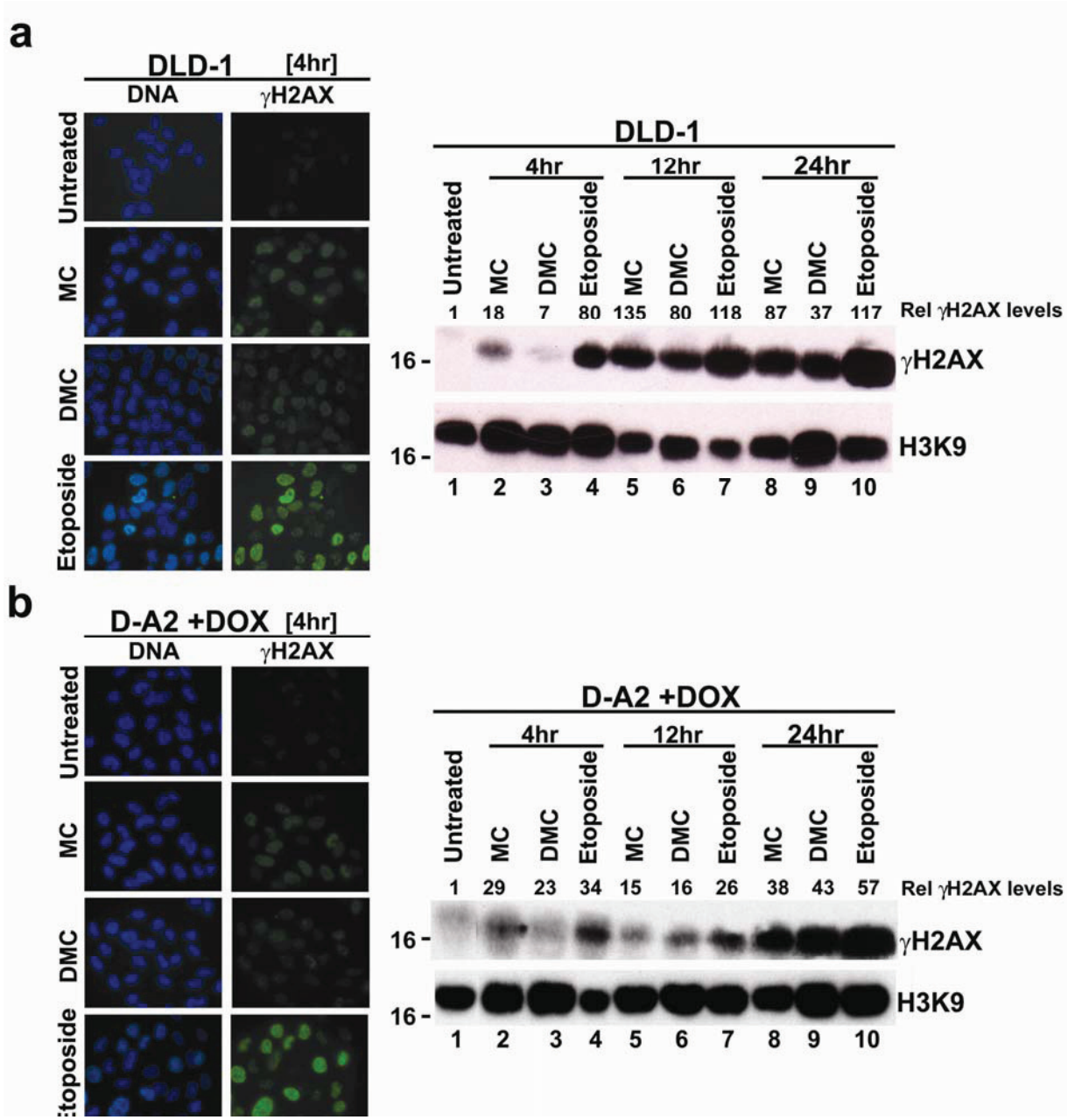


Figure 3. DMC-induced cell death in the absence of wild-type p53 cannot be attributed to increased DNA double strand breaks. ***A and B*****, Phosphorylated H2AX (γH2AX) was monitored in DLD-1 (A), and D-A2 (B) cells grown in the presence (+DOX) of doxycycline using fluorescence immunodetection and Western blot analysis. Cells were either left untreated or treated with 10uM MC or DMC for 4 h and stained with monoclonal antibody for detection of γH2AX (right panel). 4', 6-Diamidino-2-phenylindole (DAPI) was used to visualize the nuclei (left panel). Cell lysates from DLD-1 and D-A2 +DOX cells left untreated (*****lane 1*****) or treated with 10uM MC (*****lanes 2, 5 and 8*****), DMC (*****lanes 3, 6 and 9*****) or etoposide (*****lanes 4, 7 and 10*****) for the indicated time points were separated by SDS-PAGE and subjected to Western blotting using anti- γH2AX and anti-dimethylated H3K9 as a loading control. Numbers at the top of each sample lane represent the relative γH2AX protein levels, normalized to H3K9.**

4.2D DMC treatment signals for ADP-ribosylation of nuclear proteins in the absence of wild-type p53.

Increased PARP catalytic activity induced by DNA damage has previously been associated with non-apoptotic cell death (Halappanavar et al., 1999; Zong et al., 2004). We monitored PARP activity in DLD-1 and DA-2 cells by examining the levels of ADP-ribosylated proteins in cell extracts. Western blotting showed that DMC induced markedly higher levels of poly(ADP-ribosyl)ated proteins than MC when in the presence of low levels of p53 in DA-2+DOX cells (Fig. 4A, compare lanes 4-6). This striking increase in PARP activity was reproducibly seen by Western blotting and immunofluorescence in cell nuclei. In DLD-1 cells there was increased PARP activity after DMC treatment as compared to MC treatment but this was more profoundly detectable by immunofluorescence (Fig.4A, lanes 1-3 and Fig.4B).

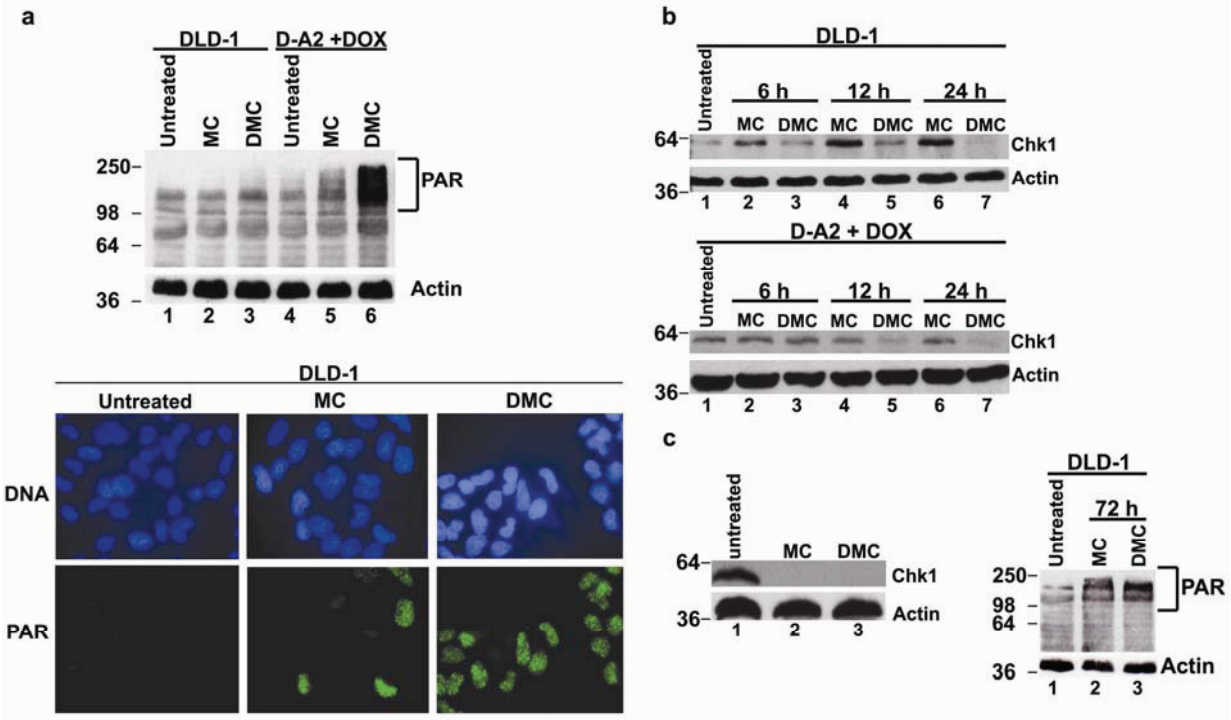


Figure 4. DMC, but not MC, induces robust PARP activity in cells with low, or no, wild-type p53. ***A*****, Western blot analysis for poly(ADP-ribose) (PAR) from DLD-1 and D-A2 cells grown in the presence (+DOX) or absence (–DOX) of doxycycline. Nuclear extracts were prepared from cells left untreated (*lanes 1, 4, and 7*) or treated with 10uM MC (*lanes 2, 5, and 8*) or DMC (*lanes 3, 6, and 9*) for 24 h and separated by SDS-PAGE. Actin was used as a loading control.** ***B*****, DMC significantly induces PARP activity in DLD-1 cells. Immunodetection of poly(ADP-ribosyl)ated proteins in the nuclei of DLD-1 cells was carried out using fluorescence microscopy. DLD-1 cells were either left untreated or treated with 10uM MC or DMC for 24 h and stained with polyclonal antibody for detection of poly(ADP- ribose) (PAR) polymers (lower panel). 4', 6-Diamidino-2-phenylindole (DAPI) was used to visualize the nuclei (upper panel).**

4.3 DISCUSSION

Here we report the activation of downstream targets by DMC in comparison to MC in isogenic cell lines. Activation of p53 targets results in the induction of apoptotic cell death. We observe that in DA2+DOX cells, expressing low levels of wild-type p53, DMC caused substantial cellular cytotoxicity in the presence or absence of wild-type p53 at early timepoint.

The difference in MC and DMC cytotoxicity gradually diminished after 48 hours of treatment, especially in DA2+DOX cells, indicating that prolong treatment with these two mitomycins can result in cell death by both drugs in the absence of wild-type p53. The disappearance of the cytotoxic difference between these two mitomycins upon prolong exposure may be attributed to MC being able to from more DNA adducts as DMC, especially the interstrand crosslink adduct.

We expanded our investigation of the differential cytotoxicity by examining the molecular targets activated by DMC in comparison to MC induced target activation. Cleavage of PARP has been reported as a marker of apoptotic cell death (Kaufmann, et al., 1993). Both MC and DMC induced substantial PARP cleavage in the DA2+DOX cells, which have wild-type p53 protein. MC induced PARP cleavage, however, did not result in cell death compared to DMC. Additionally, both drugs induced procaspase-8 and -3 activation. Although MC induced caspase activation caused PARP cleavage and stabilized p53 protein, we did not observe any substantial cytotoxicity in these cells caused by MC. It is not clear why caspase activation and PARP cleavage does not potentiate MC cytotoxicity in DA2+DOX cells. However, often these events are retractable and do not necessary result in cell death, as we observe with MC. In the absence of wild-type p53, both drugs barely activated caspases, although DMC induced substantial cellular cytotoxicity in these cells. Our data suggest that the novel cytotoxicity associated with DMC induced cell death in the absence of p53 can be attributed to activation caspase independent cell death targets.

Crosslink excision can result in the formation of double strand breaks, which has been shown to induce to γ-H2AX foci formation (Niedernhofer et al., 2004). We hypothesized that the increased cytotoxicity observed with DMC can may be attributed to its ability to form increased DNA adducts, which are later process to generate double strand breaks (DSB). We

observed that both MC and DMC generated similar γ-H2AX. Interestingly, the activation of γ-H2AX was induced at an early timepoint by both drugs in DLD-1 cells, which do not express wild-type p53 protein. Delayed γ-H2AX formation is observed in cells with wild-type p53. Upon induction by DNA damage, p53 protein induces cell cycle arrest, particularly at the G_1-S transition phase (Deng et al, 1995). Additionally, cross-link DNA adducts are detected and processed in the S-phase of the cell cycle (Dronkert and Kanaar, 2001). Perhaps, in cells expressing wild-type p53, the activation of p53 by MC and DMC induces a temporarily halt to the cell cycle progression from the G_1 phase into the S-phase, and thus delays the recognition and processing of DNA adducts into DSB products.

PARP activation, by DNA alkylating agents, has been reported to initiate p53-independent cell death (Zong et al., 2004). We investigated PARP activation in DA2+DOX and DLD-1 cells. We observed differences in PARP activity upon treatment with MC and DMC. This difference in PARP catalytic activity by these two mitomycins was also significantly robust when cells express wild-type p53. When activated by DNA damage, PARP has also been reported to ribosylate histones, which serves to loosen the compact nucleasomal structure by increasing the negative charge on histones and causing them to disassociate from the chromatin (Smith, 2001; Panzeter et al., 1993). In addition, other non-histone proteins such as DNA damage repair factors are ribosylated and recruited to the region of damage (Tong et al., 2001). Thus the ribosylation activity of PARP acts directly to produce efficient recognition and repair of toxic DNA lesions. How PARP activation results in cell death or DNA repair is unclear (Panzeter et al., 1993; Ivana Scovassi and Diederich, 2004). We observed that, DMC treatment robustly activated PARP catalytic activity compared to MC at an early timepoint. Interestingly, we observe that prolong exposure to both mitomycins caused similar levels of PARP activation.

Surprisingly, both drugs induced cellular cytotoxicity at the timepoint when the differences in PARP activity were similar. It is tempting to propose that the early induction of PARP catalytic activity by DMC may be an early cellular attempt to resolve strand breaks generated by DMC-induced DNA adducts and that over time, the sustained activity of PARP signals for cell death. Although we have no data to support this proposal, it will be interesting to investigate if the deletion of PARP can prevent cell death by DMC, especially at early timepoints.

It has been reported that, in cells without wild-type, inhibition of Chk1 activity sensitizes cells to chemotherapeutic agents (Russell et al., 1995). We investigated the association between cell death and Chk1 regulation after prolong exposure to MC and DMC. We observed that, at early timepoints, siRNA mediated down-regulation of Chk1 sensitizes cells to MC-DNA adducts (Boamah et al., submitted). We thus propose that, at timepoints where we observe MC cytotoxicity, we should see a corresponding decrease in Chk1 protein levels. We were particularly interested in cells without wild-type p53 because these cells have shown increased resistance to MC compared to cells expressing wild-type p53 (Abbas et al., 2002). When cells were treated with the mitomycins for 72 hours, we observed down-regulation Chk1 protein levels which interestingly corresponded to the cytotoxicity of both drugs at 72 hours. This data validates our previous report in which we observe that the limited cytotoxicity observed upon MC treatment can be associated with its inability to effectively down-regulate Chk1 protein.

In conclusion, we observed that treatment with MC and DMC differ with respect to the molecular targets activated during drug induced cellular cytotoxicity in cells expressing wild-type p53 at early timepoint (Figure 5). While we observed a significance differences between PARP and Chk1 regulation at early timepoint, it will be interesting to investigate if these two pathways converge to induce cellular cytotoxicity upon exposure to the mitomycins.

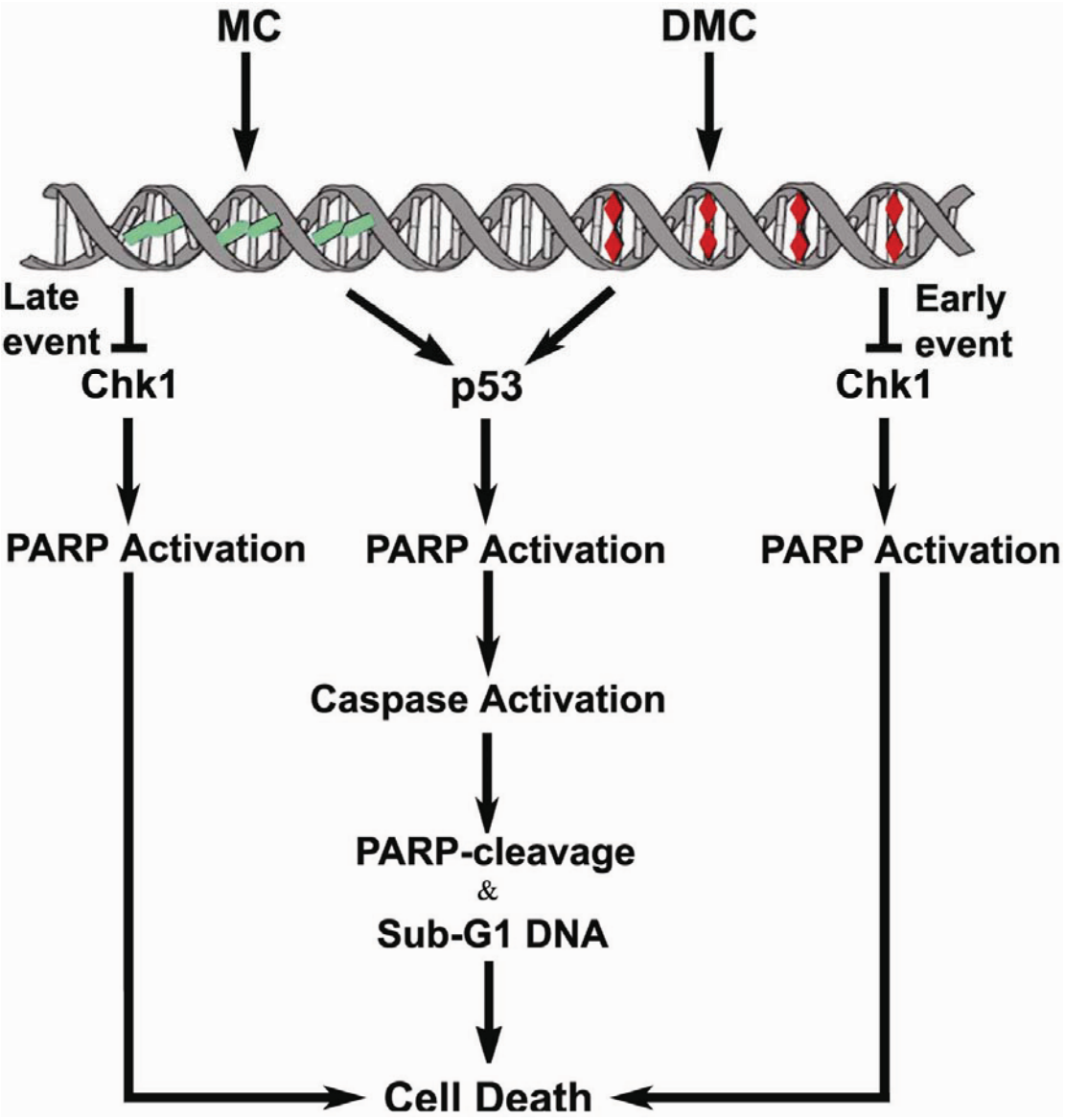


Figure 5. Schematic model depicting MC and DMC signal transduction pathways. Green and red objects represent the structurally different MC and DMC adducts, respectively. In cells with wild-type p53 many signals are activated with either MC- or DMC-DNA adducts, as indicated. In cells that lack wild-type p53 the programmed necrotic pathway marker of increased PARP activity is the only strongly evident marker in a caspase independent cell death pathway.

CONCLUSIONS

This thesis examined the molecular targets activated by DMC during drug induced cell death and compared them to MC-activated targets in the presence or absence of wild-type p53. Most importantly, the objective of this thesis was to investigate the p53-independent cell death properties of DMC. While the ability to induce death in cells expressing wild-type p53 is interesting, a more attractive alternative is to investigate the cytotoxic potential of chemotherapeutic drugs which are capable of initiating p53-independent cell death. This is because about 50% of all cancers have been shown to lack a functional p53 gene, while the rest harbor cellular alterations that disrupts the activity of the wild-type protein (Hollstein et al., 1991). In cells with compromised wild-type p53, most often, the biological activity of the protein can be induced by chemotherapeutic drugs. However, in cells that have mutations in the p53 gene, the function of the protein is permanently disrupted. Thus, having a chemotherapeutic drug that can initiate death in cells with compromised p53 or in cells which do not have wild-type p53, is an attractive way to target most, if not, all cancers.

While the p53 protein status is a critical determining factor in chemotherapeutic outcomes, recent reports have implicated Chk1, a G_2 checkpoint cell cycle regulator, as a mediator of chemotherapeutic sensitivity in the absence of wild-type p53 (Kawabe, 2004). Disruption of Chk1 protein activity potentiates the cytotoxicity of drugs which had limited cell death activity in the absence of wild-type p53.

In this thesis we observe that the ability to down-regulate Chk1, at an early time point, may mediate the novel p53-independent cytotoxic property of DMC compared to MC. While it is not clear exactly how DMC is able to down-regulate Chk1 and have

increased cytotoxicity compared to MC, we propose, based on our data, that this increased cytotoxicity can be attributed to the ability of the drug to generate increased DNA adducts, especially of the β-DNA adduct form.

CHAPTER 5

FUTURE STUDIES

5.1 Examine regulation of ATR and cell cycle proteins

ATR-Chk1 pathway regulates genome integrity at the G_2/M phase of the cell cycle (Enders, 2008). The phosphatidylinositol 3-kinase-related kinase, ATR, is activated in response to replication arrest to prevent inappropriate firing of late origins, collapse of the replication fork, and premature entry into mitosis (Osborn et al., 2002). Checkpoint 1 (Chk1) kinase, mediates these ATR dependent cellular events, halting the cell cycle to allow repair of damaged genetic material prior to mitosis (Zhang et al., 2006). Both MC and DMC have been shown to generate DNA cross-link products (Palom et al., 2002; Pan et al., 1993). These interstrand cross-link DNA structures activate the ATR-Chk1 pathway because they interfere with polymerase migration (Osborn et al., 2002).

In response to replication stress, ATR phosphorylates Chk1 on two critical residues, Ser317 and Ser345 (Bartek et al., 2004; Zhang et al., 2006). Phosphorylation of Chk1 at ser345 as opposed to ser317 by ATR, preferentially destabilizes Chk1 by marking the protein for ubiquitin mediated proteolysis (Zhang et al., 2005). Although we have shown that DMC induced Chk1 down-regulation is mediated through the ubiquitin pathway, we have yet to determine if Chk1 is actually ubiquitinated or phosphorylated by mitomycin-DNA adducts. To address this, we will examine Chk1 phosphorylation in MC and DMC treated samples in the presence or absence of LLnV using Western blot analysis. Our expectation will be that, while both MC and DMC should activate ser345 and Ser317 phosphorylation, there should be a greater pool of Ser345 in the combined treatment of LLnV/DMC treated samples compared to MC alone. The expectation is that MC alone will increase Ser317 phosphorylation, and thus explain why Chk1 is not destabilized under this treatment condition. In addition, we have to determine if indeed

Chk1 is ubiquitinated, by directly immunoprecipitating Chk1 and probing for the presence of ubiquitinated products, or vice verse, using anti-ubiquitin antibody for immunoprecipitating. However, in the case of DMC treated samples, this will be in the presence of LLnV, since Chk1 is protected from degradation only after the proteasome is inhibited.

It will be interesting to also determine ATR protein activation after treatment with MC and DMC. Since we observe that Chk1 is not destabilized after MC treatment, it is possible that ATR will also be regulated in a similar manner, perhaps to sustain active Chk1 levels in MC treated samples. Our protein array data suggests that DMC treatment caused down-regulation of multiple targets (Figs. 13-15). In DMC treated samples, it will not be surprising if ATR is down-regulated. However, since Chk1 down-regulation is associated with its ATR-dependent phosphorylation (as seen in preliminary data in Fig. 11), at early timepoints, we should observe ATR protein activation in these samples. Thus the absence of ATR in DMC treated samples, should influence the regulation of exogenous Myc-chk1, since an active ATR is required for its activation. The down-regulation of ATR after treatment with DMC will partially explain the cellular outcome observed after the reintroduction of Myc-chk1.

Using DA-2 +DOX and DLD-1 cell lines, we can monitor the expression levels of ATR at early time-point after mitomycin treatment using Western blot analysis. Based on the data obtained, we will further investigate how inhibition (using UCN-01 or Go6976) of ATR activity will specifically affect MC induced cellular cytotoxicity. Additionally, to confirm the observed cellular outcome after disruption of ATR activity, we will use

siRNA knockdown technique to fully examine the role of ATR in mitomycin induced cell death.

It is also possible that, lack of downstream factors, due to rapid down-regulation after treatment with DMC, accounts for the observed minimal survival after over-expression of Chk1. When activated, Chk1 delays entry into mitosis by phosphorylating and inactivating Cdc25A and Cdc25C, two phosphatases required for cell cycle progression (Chen et al., 2003; Zhang et al., 2005). DMC may activate a signaling mechanism that may bypass the usual Chk1 mediated pathway, and thus cause a rapid down-regulation of Cdc25A and Cdc25C phosphatases. If this is the case, then the reintroduction of Myc-chk1 will not affect drug induced cytotoxicity. However, the down-regulation of these phosphatases is unlikely, because, if these downstream targets are absent, then it is expected that cell cycle will be inhibited, since these targets are essential for the cell cycle progression. DMC treatment down-regulated Chk1 and the nuclear morphology of these cells is indicative of mitotic catastrophe. To induce mitotic catastrophe, cells have to be cycling which requires that Cdc25A and Cdc25C phosphatases are present to ensure G_2/M transition (Kovelman and Russell, 1996). It is likely that the lack of activity from the Myc-chk1 is due to lack of upstream regulators. It will be interesting to investigate the stability of these cell cycle phosphatases after treatment with the mitomycins.

Since Chk1 is not depleted following treatment of cells with or without wild-type p53 by MC, we will also investigate the cellular activity of Chk1 protein after treatment with MC. It will be interesting to know if the Chk1 in MC treated cells is indeed active.

We will also examine the phosphorylation status of the cell cycle phosphatases, as a measure of Chk1 activity specifically in MC treated samples.

5.2 H3ser10 phosphorylaion

We investigated pH3ser10 levels of DLD- and DA-2 + DOX cells treated with MC or DMC. We addressed this question to determine if in the absence of Chk1, as observed after DMC treatment, cells are still actively replicating. This will test the hypothesis that DMC treated cells are inducing cell death through mitotic catastrophe due to lack of Chk1, while MC treated cells with Chk1 prevents entry into mitosis and cell death.

Mitosis requires Aurora-B kinase dependent increase in pH3ser10 levels which results in chromosome compaction (Crosio et al., 2002; Li et al., 2006). We analyzed the cell cycle profile and pH3ser10 levels using FACs analysis (Figures 1 and 2) as well as the protein levels of pH3ser10 (Figure 3). MC treatment caused a significant change in the cell cycle profile compared to DMC (Figure 1; compare MC to DMC treatment). Treatment with both mitomycins caused a dramatic decrease in pH3ser10 protein levels relative to untreated (Figure 3; compare lanes 2 and 3 for both cell lines). The percentage of cells positive for pH3ser10 also decreased in our FACs analysis relative to the untreated (Figure 2). Hydorxyurea which inhibits DNA synthesis and causes the accumulation of cells specifically in the G_1 phase of the cell cycle, was used as a negative control (Figures 2 and 3). For our positive control, we used nocodazole, a drug known to inhibit microtuble polymerization. Nocodazole treatment causes G_2 cell cycle arrest and increase in pH3ser10 (Figures 2 and 3).

It is interesting that DMC treated cells did not show an increase in the levels of pH3ser10. Based on the nuclear morphology and loss of Chk1 protein, DMC treated cells should exhibit active mitosis. More importantly, it should be noted that PARP-1-mediated poly(ADP-ribosyl)ation disrupts the kinase activity of Aurora-B, causing a decrease in pH3ser10 levels after exposure to DNA damaging agents (Monaco et al., 2005). We observed a dramatic increase in poly(ADP-ribosyl)ation upon treatment with DMC, compared to MC (Figure 4; compare lanes 3 and 5). Based on the data by Monaco et al, it is possible that the lack of pH3ser10 after treatment with DMC can be attributed to robust induction of PARP-1 activity. This increase in PARP-1 activity may cause a decrease in Aurora-B protein kinase activity. In the absence of a functional Aurora-B kinase, cells continue into mitosis causing defects in chromosome condensation, segregation, and cellular divisions (Hauf et al., 2003). It is possible that the observed defects in nuclear morphology observed after DMC treatment can be attributed to increase production of DNA adducts coupled with loss of Aurora-B kinase activity. It will be interesting to investigate the regulation of Aurora-B with respect to mitomycin induced cell death.

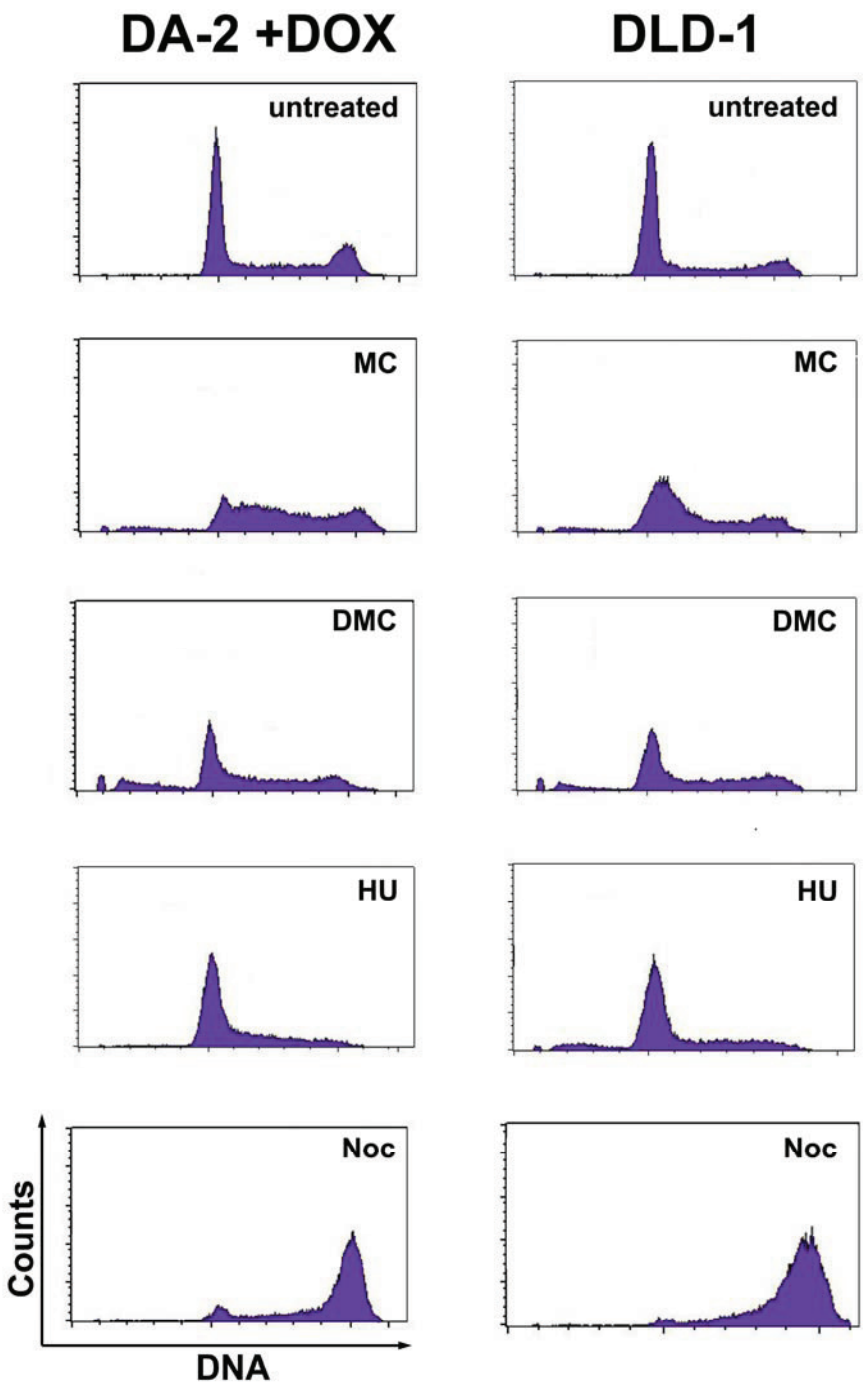


Figure 1. MC treatment dramatically changes cell cycle profile compared to DMC. Cells were left untreated or treated with 10μM MC or 10μM DMC for 24 hrs, stained with propidium iodide, and analyzed for cell cycle profile using FACs.

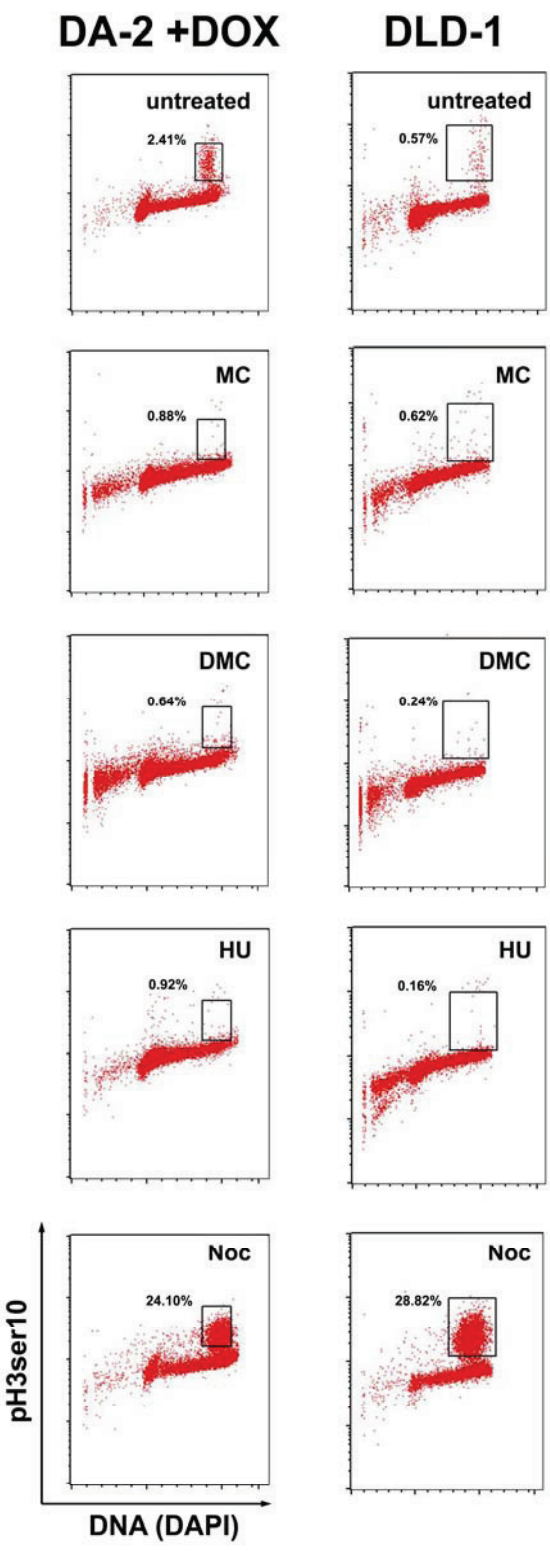


Figure 2. DMC treatment failed to increase cells positive for the mitotic marker, pH3ser10
Phosphorylated H3 (pH3ser10) was monitored in DA-2+DOX and DLD-1 cells using FACs analysis. Cells were either left untreated or treated with 10uM MC or DMC for 24 hours and stained with monoclonal antibody for detection of pH3ser10 and propidium iodide.

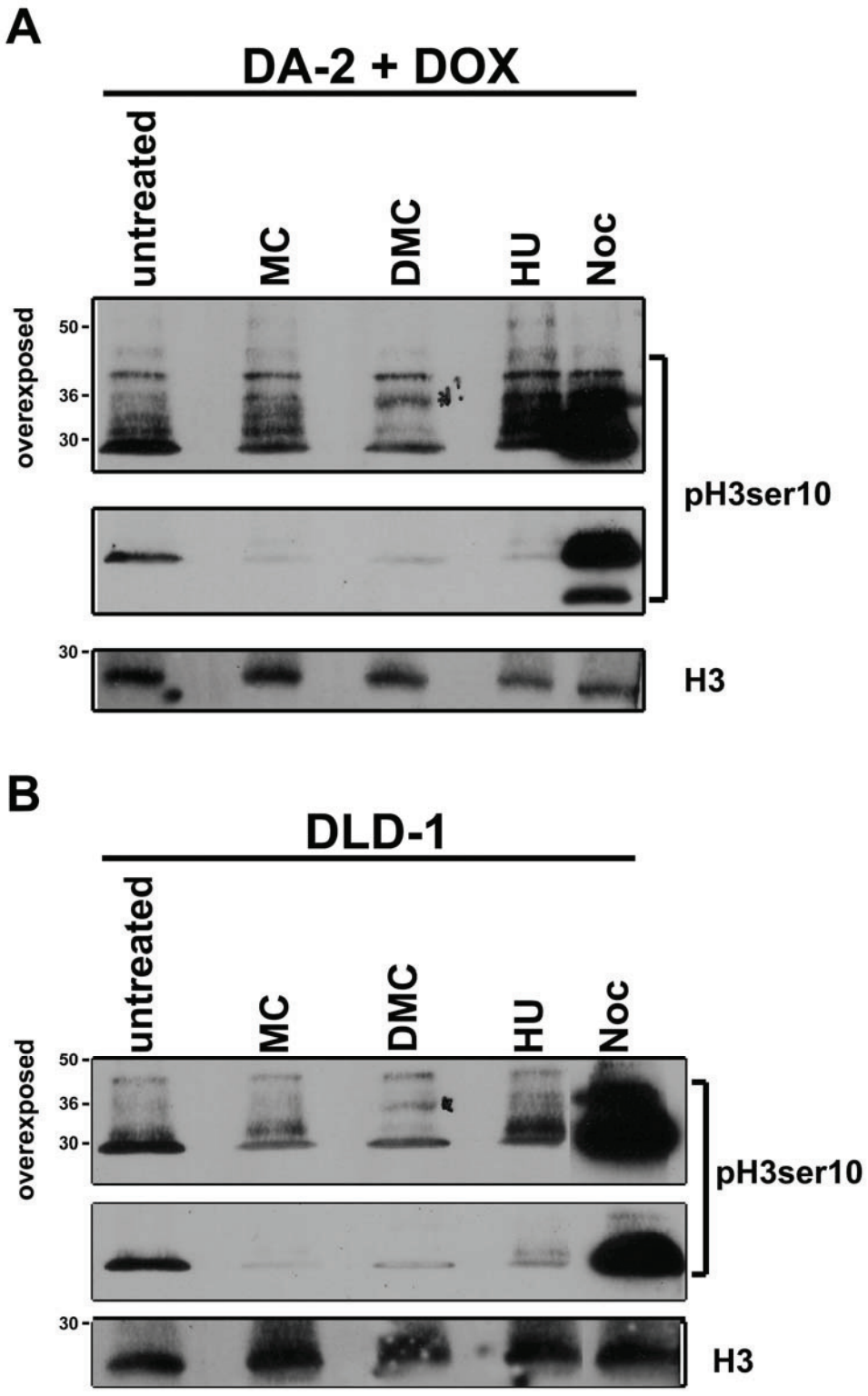


Figure 3. Both MC and DMC decrease pH3ser10 protein levels
Phosphorylated H3 (pH3ser10) was monitored in DLD-1 (A), and DA-2+DOX (B) cells using Western blot analysis. Actin was used a loading control.

We inhibited the activity of PARP-1 using DPQ (3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone) (Figure 4A; lanes 2, 4, and 6). This lack of activity is due to decrease in PARP-1, since protein levels remained the same in the presence or absence of DPQ (Figure 4B). It will be interesting to investigate if cells treated with DPQ/DMC will increase pH3ser10 levels. However, it should be noted that this experiment will not inhibit the cytotoxicity of DMC since we have already shown that inhibition with DPQ does not attenuate DMC cytotoxicity (Figure 4C).

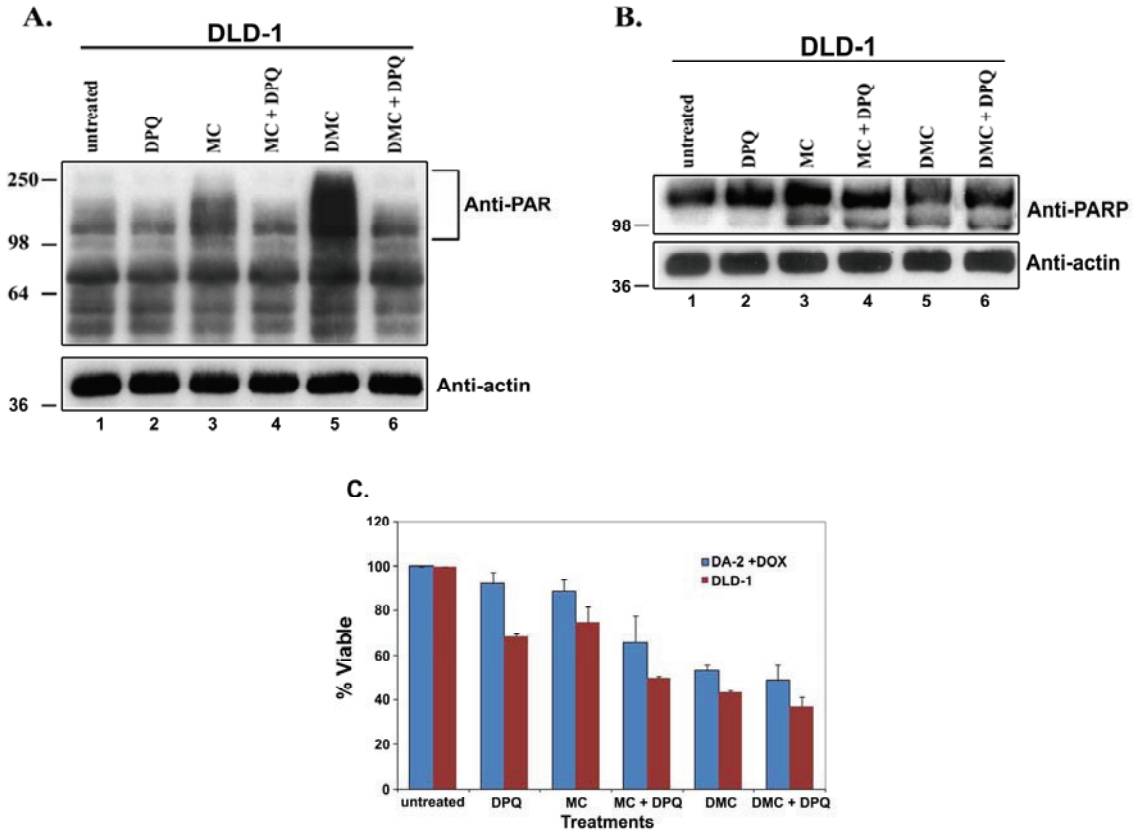


Figure 4. PARP-1 inhibition does not attenuate DMC induced cellular cytotoxicity.
(A) Poly(ADP-ribose) and PARP-1 (B) protein levels were monitored in DLD-1 cells treated with 10uM MC or DMC in the presence or absence of 3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ) using Western blot analysis. Actin was used a loading control. (C) The cytotoxicity of the mitomycins in DLD-1 cells treated with 10uM MC or DMC in the presence or absence of 3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ) was measured using MTT assay. Cells were either left untreated or treated with 10uM MC or DMC 24 hours. Data shows percentage of cells with mitochondrial activity relative to untreated samples and is representative of three independent experiments. Error bars indicate the standard deviation.

5.3 Investigating inhibition of cellular proliferation using the clonogenic assay.

We measured the viability of cells treated with MC or DMC using the MTT assay. Our data, using the MTT assay, indicated differential cytotoxicity at early timepoints by MC and DMC (Boamah et al., 2007; Abbas et al., 2002). The observed cellular outcome at early timepoints by MC and DMC is comparable to previously reported difference in proliferation using the clonogenic assay (Palom et al., 2002). The MTT assay, while a robust assay for measuring cytotoxicity, does not indicate if the surviving cells are capable of division or DNA repair. Thus, we investigated the clonogenic potential of DA-2 and DLD-1 cells treated with MC and DMC.

Cells were plated at different densities and treated with increasing concentration of the mitomycins for only one hour and then the drugs were washed out. DLD -1 cells, seeded at 1 x 10^4 cells, treated with MC showed a decrease in clonogenic ability at lower drug concentration (0.5μM) compared to DMC at a similar drug concentration (Fig. 5 and Fig. 6). This difference in cellular outcome decreased with increasing drug concentrations. Interestingly, at higher drug concentrations, we observed that both MC and DMC induced similar inhibition. When DLD-1 cells were seeded at lower density, both MC and DMC treatments showed similar clonogenic inhibition at all the different concentrations, suggesting that the initial number of cells seeded can influence the outcome of drug test in this cell lines (Fig. 6).

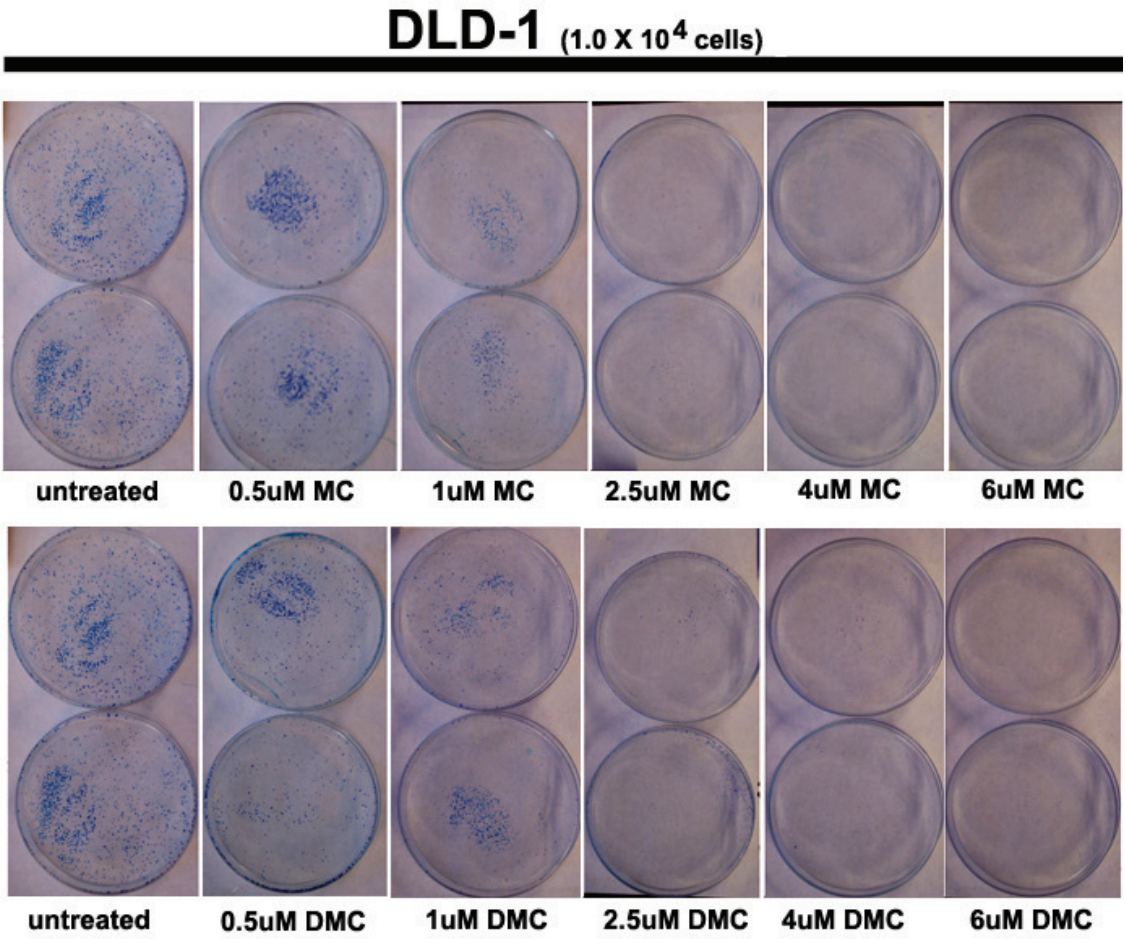


Figure 5. Both MC and DMC inhibit cellular proliferation in the absence of wild-type p53.
DLD-1 cells were seeded at 1 x 10^4 cells and allowed to attach overnight. Cells were then either left untreated or treated with 10 µM MC or 10 µM DMC for 1 hour, followed by incubation in drug free fresh media for the duration of the experiment. For each condition, samples were seeded in duplicates. Top panel shows MC treated samples, while bottom panel shows DMC treated samples.

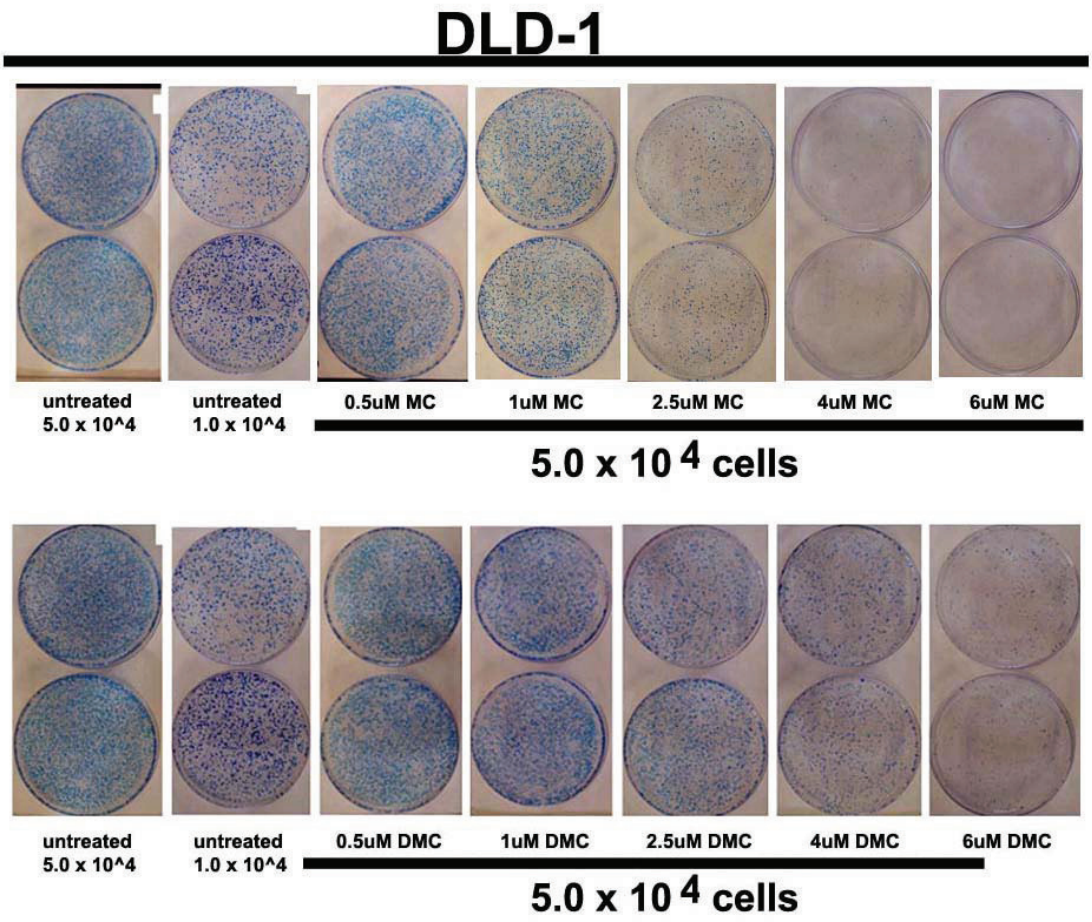


Figure 6. MC inhibits cellular proliferation at lower concentrations compared to DMC in cells without wild-type p53. DLD-1 cells were seeded at 1 x 10^4 or 5 x 10^4 cells and allowed to attach overnight. Cells were then either left untreated or treated with 10 µM MC or 10 µM DMC for 1 hour, followed by incubation in drug free fresh media for the duration of the experiment. For each condition, samples were seeded in duplicates. Top panel shows MC treated samples, while bottom panel shows DMC treated samples.

We observed in DA-2 cells, at both cell densities (5x 10^4 and 1 x 10^4cells), that MC shows increased clonogenic inhibition at early concentrations compared to DMC (Fig. 7 and Fig. 8). We observed that at the lower end of the dose response curve (0.5μM) of drug treatment, the inhibitory properties of MC and DMC differ in DA2+DOX cells, with MC being more cytotoxic.

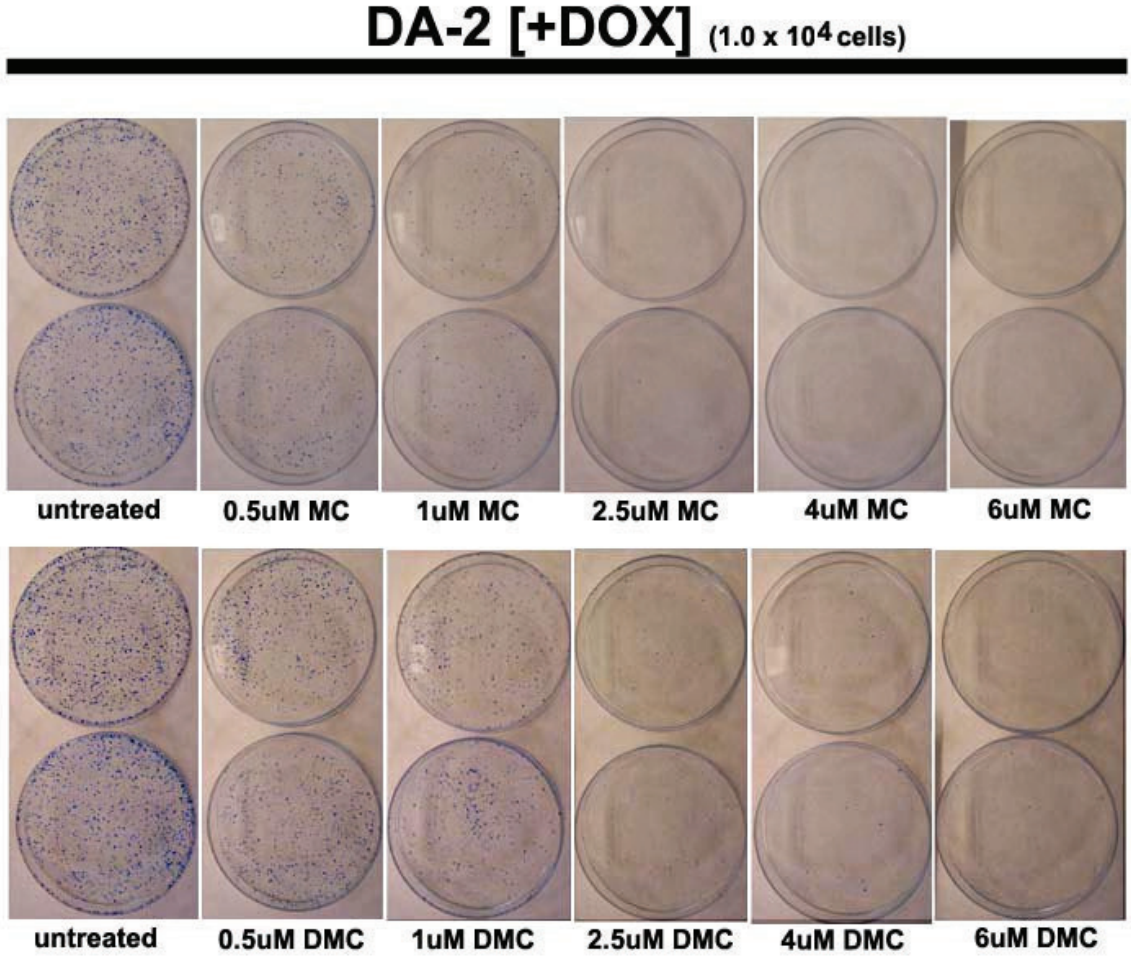


Figure 7. Treatment with MC causes a decrease in colony formation at lower drug concentrations compared to DMC in cells with wild-type p53.
DA2+DOX cells were seeded at 1 x 10^4 cells and allowed to attach overnight. Cells were then either left untreated or treated with 10 μM MC or 10 μM DMC for 1 hour, followed by incubation in drug free fresh media for the duration of the experiment. For each condition, samples were seeded in duplicates. Top panel shows MC treated samples, while bottom panel shows DMC treated samples.

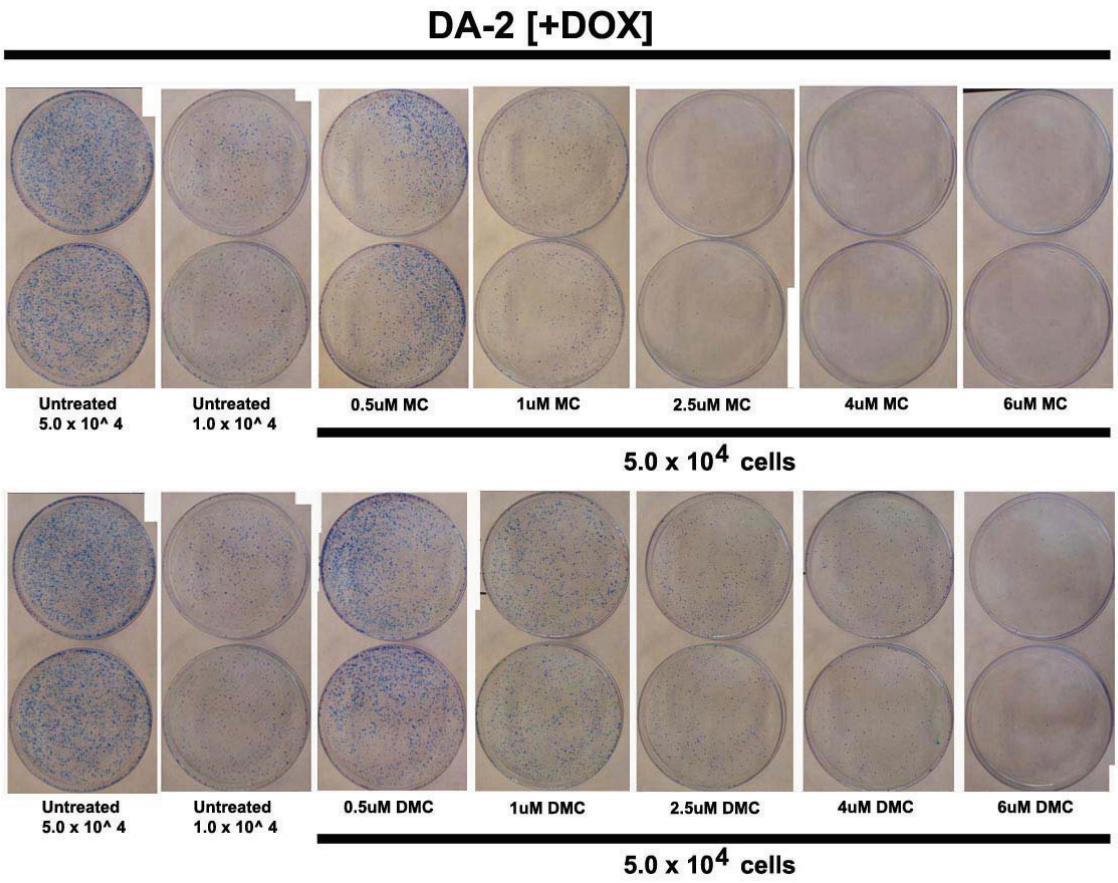


Figure 8. MC inhibits colony formation compared to DMC in cells with wild-type p53.
DA2+DOX cells were seeded at 1 x 10^4 or 5 x 10^4 cells and allowed to attach overnight. Cells were then either left untreated or treated with 10 µM MC or 10 µM DMC for 1 hour, followed by incubation in drug free fresh media for the duration of the experiment. For each condition, samples were seeded in duplicates. Top panel shows MC treated samples, while bottom panel shows DMC treated samples.

Both MC and DMC inhibited cellular proliferations, although MC was more effective at lower concentrations compared to DMC. Our data supports previously published work in which a similar assay showed that MC and DMC have similar cytotoxicity in EMT-6 mouse mammary cells and Chinese hamster ovary cell lines under

aerobic conditions (Rockwell et al., 1995). It's interesting that, under hypoxic conditions using the clonogenic assay, measurement of the cytotoxic differences between MC and DMC is similar to our reported MTT assay data (Palom et al., 2002). Using the MTT assay as a measure of cytotoxicity, we observe that MC and DMC differ in cytotoxicity, to DA-2 and DLD-1 cells, when treated for 24 hours without washing out drugs. However, this difference is not striking using the one hour washout clonogenic assay as measure of cellular proliferation. Together, our data supports previous reports that both MC and DMC can interfere with the clonogenic ability of cells, although at high drug concentrations. Interestingly, when DA-2 and DLD-1 cells were treated with 10μM MC or 10μM DMC for 24 hours, and assayed for clonogenic potentials, both drugs had no surviving colonies (data not shown). Perhaps, investigating the cytotoxicity of MC and DMC at densities different from what is reported here, we can reproduce what we observe with the MTT assay. Alternatively, longer treatment time might be required for DMC adduct formation.

5.4 Examine the role of caspase-2 in mitomycin induced cell death

We detected, in DA-2 and DLD-1 cells, an increase in caspase activity using the Guava multicaspase activity assay. Most intriguing was the observation that DLD-1 cells, which do not express wild-type p53, showed a significant increase in caspase activity upon treatment with MC or DMC (See chapter 4, Fig 1 and Chapter 5, Fig. 9). Furthermore, we examined the disappearance of specific procaspases that mediate apoptotic cell death. While we observed a significant decrease in procaspase protein levels which support our observed increase in caspase activity in DA-2 cells,

interestingly, in DLD-1 cells the examined procaspases were not activated. However, it should be noted that this analysis did not include caspase-2.

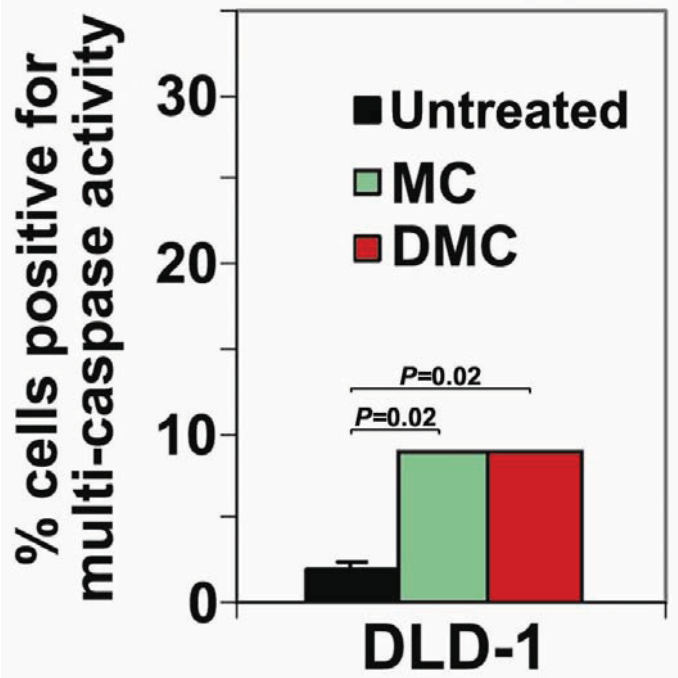


Figure 9. Caspase activity is increased by MC and DMC in the absence of wild type p53.
Reproduced from Chapter 4 Fig. 1D with slight modifications. Error bars are shown for each treatment conditions. Significance determined using a one-tailed paired *t*-test. A significant threshold value of 0.05 is used.

It was recently reported that, IR-induced DNA damage results in p53-independent apoptotic cell death through the activation of caspase-2 (Sidi et al., 2008). This mode of apoptotic cell death was shown to bypass activation of the canonical intrinsic and extrinsic cell death pathway effectors. Our analysis of DMC induced p53-independent cell death shows barely detectable levels of activation of most of the critical markers associated with intrinsic and extrinsic apoptotic cell death pathways (Boamah et al., 2007).

Additionally, Chk1 depletion was observed to signal for caspase-2 mediated apoptotic cell death in the absence of wild-type p53 (Sidi et al., 2008). We also observed a decrease in Chk1 protein by DMC in the absence of wild-type p53, while siRNA mediated Chk1 depletion increases the cytotoxic efficiency of MC. Taken together, our data suggest that DMC induced cell death may activate caspase-2 and bypass the mitochondrial and receptor cell death pathway.

Oligomerization of procaspase-2 has been shown to cause autocleavage and production of active cleaved caspase-2 (Schweizer et al, 2003). Additionally, caspase-2 is activated by a large multimeric PIDDosome complex containing PIDD (p53-induced protein with a death domain), RAIDD (receptor interacting protein-associated ICH-1/CED-3 homologus protein with a death domain), and caspase-2 (Tinel and Tschopp, 2004). The PIDDosome complex member, PIDD which is a p53 downstream target gene, suggest that caspase-2 activation may require active p53 during drug induced apoptotic cell death. However, it was also recently reported that caspase-2 can be activated by an undetermined mechanism independent of the PIDDososme complex (Manzl et al., 2009). This indicates that caspase-2 activation does not require a functional p53 protein or other caspase family members. Although we did not observe activation of our examined procaspases in the absence of wild-type during DMC induced cell death, it is possible that the observed caspase activity upon treatment with DMC can be attributed to caspase-2. Thus, we investigated the role of procaspase-2 in mitomycin induced cellular cytotoxicity.

We investigated caspase-2 processing after treat with MC and DMC in DLD-1 and DA-2 cells. Caspase-2 exits as a 50 kDa pro-form, which can be autoprocessed or

cleaved to produce fragments with approximate size of 32 kDa and 19 kDa (Manzl et al., 2009; O'Reilly et al., 2002). We observed that in DLD-1 cells (top panel), treatment with DMC caused the reduction of cleaved caspase-2 below untreated level at both time points analyzed (Fig.10; lanes 3 and 5). However, in DA-2 cells (bottom panel), DMC caused cleavage and activation of procaspase-2, but after 24 hours post treatment, the cleaved fragment was reduced (Fig.10; compare lanes 3 and 5).

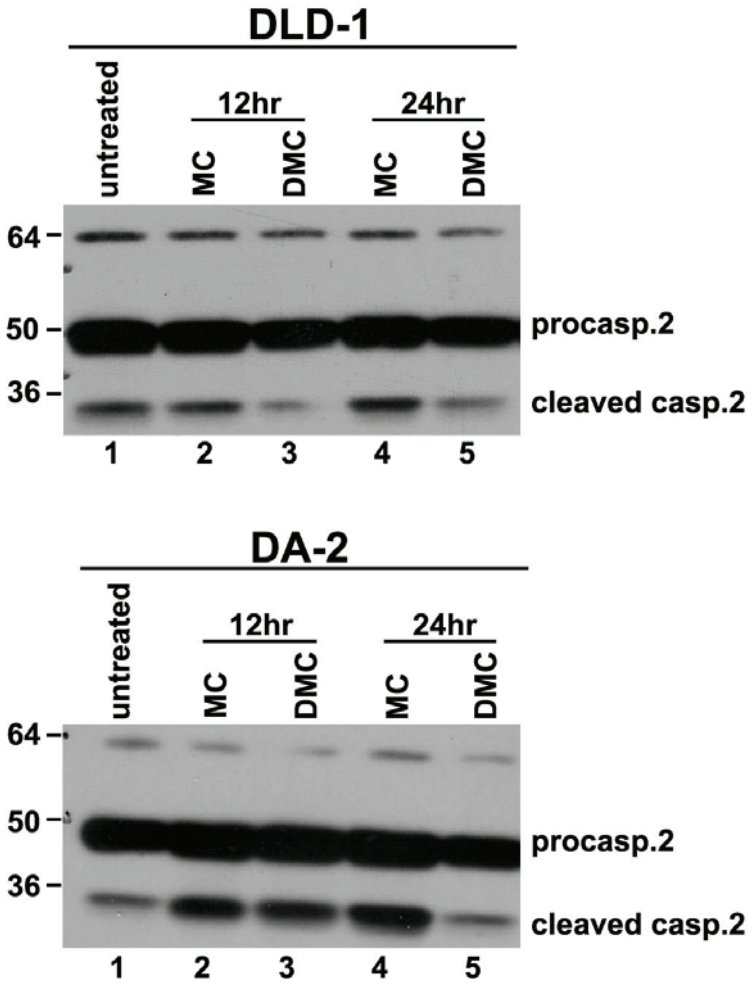


Figure 10. DMC treatment depletes cleaved caspase-2 in the presence or absence wild-type p53.
DA2+DOX cells either left untreated or treated with 10 µM MC or 10 µM DMC for the indicated timepoints. Extracts were obtained from cells and analyzed for caspase-2 activation.

It is unclear how this fragment is reduced; however, preliminary data suggest a proteasome mediated down-regulation (data not shown).

Treatment with MC caused cleavage of caspase-2 in both DLD-1 and DA-2 cells (Fig.10). Interestingly, the cleaved fragment was stabilized even after 24 hours of treatment with MC (Fig.10; see lane 4 for both top and bottom panels).

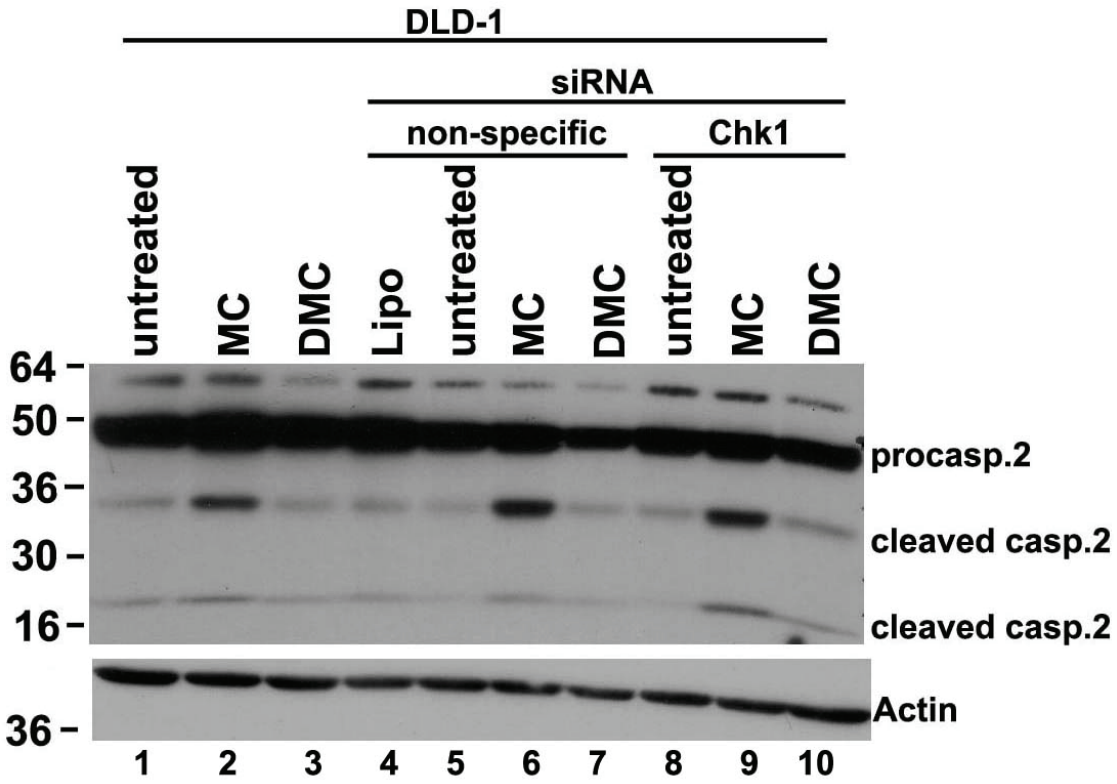


Figure 11. MC treatment stabilizes cleaved caspase-2.
DLD-1 cells were either left untreated or treated with non-specific or Chk1 siRNA for 24 hours, followed by treatment with 10 µM MC or 10 µM DMC for an additional 24 hours. Extracts were obtained from cells and analyzed for caspase-2 activation. Actin was used as loading control.

Caspase-2 phosphorylation and catalytic activity of has been implicated in the maintenance of a G_2/M DNA damage checkpoint (Shi et al., 2009). Since we observe a

decrease in cellular viability in the absence of wild-type p53 in MC treated samples upon depletion of Chk1, we investigate the cleavage of caspase-2 under similar conditions (Fig. 11). It is possible that the disappearance of the 32 kDa cleaved fragment in DMC treated samples is associated with the disruption of a G_2/M DNA damage checkpoint, which might results in cell death by mitotic catastrophe. To our surprise, even under conditions where Chk1 depletion caused a statistically significant reduction of cellular viability, the levels of cleaved caspase-2 remained similar (Fig. 11).

To further explore the role of caspase-2 in mitomycin induced cellular cytotoxicity, we investigated its localization in the presence or absence of DNA damage. We observed that, treatment with MC and DMC caused a marked increase in the levels of cytoplasmic cleaved caspase-2 (Fig. 12., see the 32 kDa fragment in each lane). Interestingly, the levels of this caspase-2 fragment increased with prolong treatment of both MC and DMC in cytoplasmic fractions, while the procaspase-2 form remained similar under all treatment timepoints. The 19 kDa caspase-2 fragment localized mostly to the cytoplasmic compartment after treatment with DMC compared to MC (Fig. 12., compare lanes 3 and 4 to lanes 5 and 6). Upon prolong exposure to the mitomycins, we observed a significant depletion of the 19 kDa fragment in MC treated samples, while the protein remained localized in the cytoplasmic fractions in DMC treated extracts (Fig. 12., compare lanes 7 and 8 to lanes 9 and 10).

ATR has been suggested to indirectly mediate caspase-2 activation in the absence of wild-type p53 through its downstream targets (Sidi et al., 2008). This ATR-caspase-2 regulation was proposed as an alternative pathway for the induction of p53-independent apoptotic cell death. We investigated ATR regulation after treatment with MC or DMC

(Fig. 12). ATR was mostly cytoplasmic, however, upon treatment with MC or DMC, a dramatic increase in ATR is observed in the cytoplasmic fractions at early timepoint (Fig. 3. compare lane 1 to lanes 3 and 5).

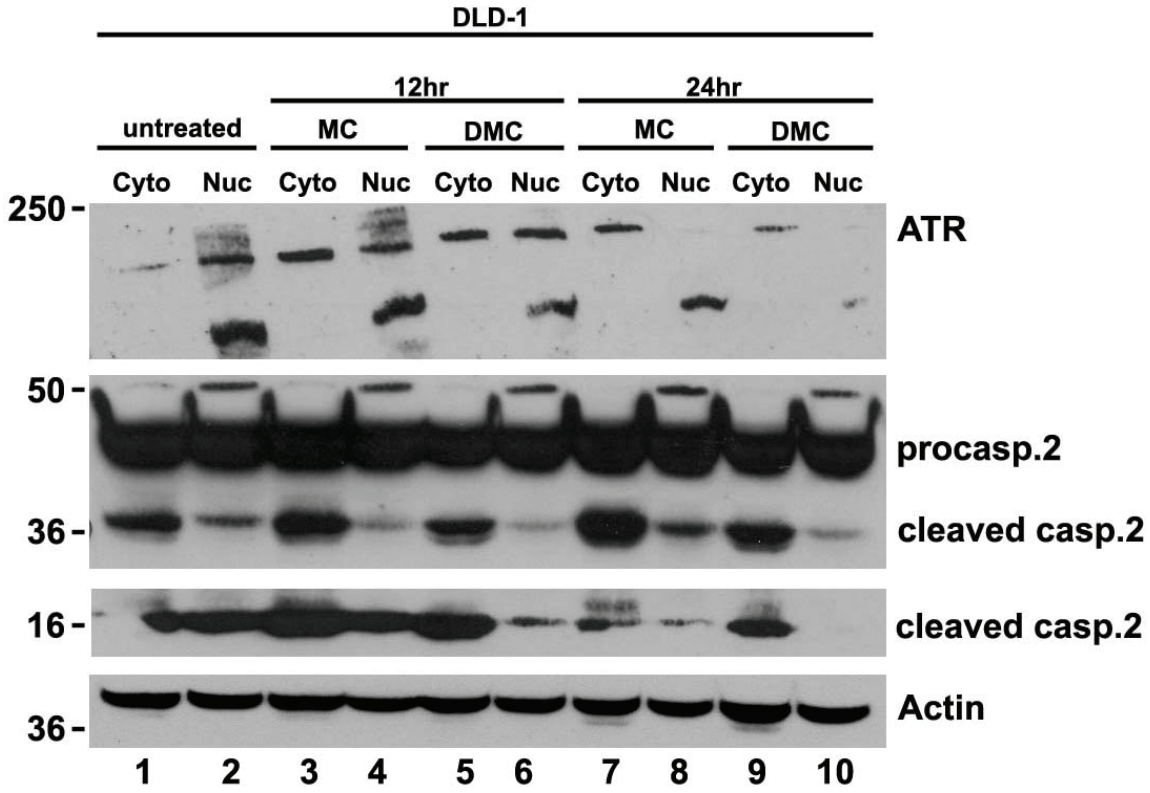


Figure 12. DMC treatment stabilizes cytoplasmic cleaved caspase-2.
DLD-1 cells were either left untreated or treated with 10 µM MC or 10 µM DMC for the indicated timepoints. Extracts were obtained from cells and analyzed for caspase-2 activation. The 16 kDa cleaved caspase-2 band was observed after prolong exposure. ATR antibody generates two unique bands, with the top band being primarily ATR. Actin was used as loading control.

Interestingly, nuclear ATR protein was depleted after 24 hours of treatment with MC and more notably with DMC (Fig. 12., compare lane 2 to lanes 8 and 10). The

cytoplasmic ATR was stabilized at this later timepoint in MC treated samples compared to DMC treatment (Fig. 3., see lanes 7 and 9).

While the exact mechanism of caspase-2 regulation in DNA damage induced cell death has not been fully delineated, our data suggest that caspase-2 processing might affect the cellular outcomes upon treatment with MC or DMC. We have yet to determine how inhibition of caspase-2, using specific inhibitors, will affect the cellular outcome upon treatment with MC or DMC. Treatment with z-VAD-fmk, a broad spectrum caspase inhibitor, has been reported to prevent processing of the 19 kDa fragment of caspase-2, not the 32 kDa fragment (O'Reilly et al., 2002). We observed an increase in the 19 kDa fragment of caspase-2 in DMC treated samples, but not after MC treatment. We have yet to determine the biological activity, if any, of this cleavage product upon treatment with DMC. Furthermore, z-VAD-fmk treatment does not disrupt the catalytic activity of caspase-2 (Garcia-Calvo et al., 1998). Using broad spectrum caspase inhibitors and caspase-2 specific inhibitors (z-VDVAD-fmk), will help to determine the role of caspase-2 in DMC induced cellular cytotoxicity. If indeed the 19 kDa fragment has a role in DMC induced cell death, then inhibition of the processing of this cleavage product should affect cell death induction in the absence of wild-type p53.

5.5 Determine which targets are differentially regulated by MC and DMC from microarray data.

We examined the regulation of 224 proteins, spotted on a Panorama Ab Microarray Cell Signaling slide as antibodies, using extracts obtained from DLD-1 cells treated with MC and DMC for 24 hours. The DLD-1 cells do not have wild-type p53 and shown a differential cellular viability and Chk1 regulation in response to treatment with

MC or DMC. Thus, these DLD-1 cells serve as a platform to examine, on a large scale, the p53-independent molecular targets that are differentially regulated upon exposure to MC or DMC. We analyzed protein status as opposed to mRNA because of the lack of direct correlation between mRNA and protein expression. Additionally, most of the proteins involved in cell death regulation or DNA damage response exist under normal cellular physiology as zymogens or in an inactive form. Upon exposure to DNA damaging agents, these proteins are usually either processed to an active intermediate or modified by the additions of groups, which induces their biological activity.

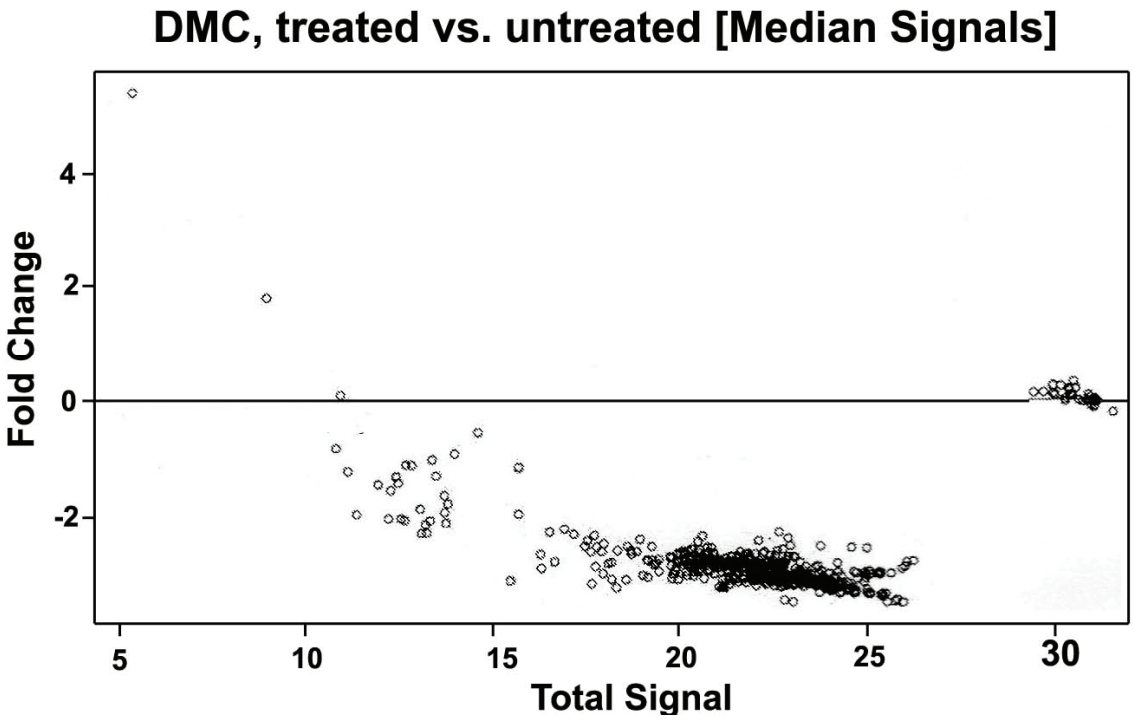


Figure 13.
DLD-1 cells were either left untreated or treated with 10 μM DMC for 24 hours. Extracts were obtained from cells and analyzed as indicated in the Sigma Panorama Antibody Microarray-Cell Signaling Technical Bulletin.

Our preliminary analysis indicates that, both MC and DMC downregulated all the protein targets spotted on the array slide. However, it should be noted that, while the DMC data was repeated twice (Fig. 13 and Fig. 14), the MC treated data was obtained from a single experiment (See Fig. 3). Western blot analysis of the extracts used for the array continuously showed differential regulation of Chk1 protein by MC and DMC (data not shown). Surprisingly, our array analysis showed downregulation of Chk1 by both MC and DMC. It is unclear if the quantitative data generated using the array was normalized correctly as this planned as the internal control.

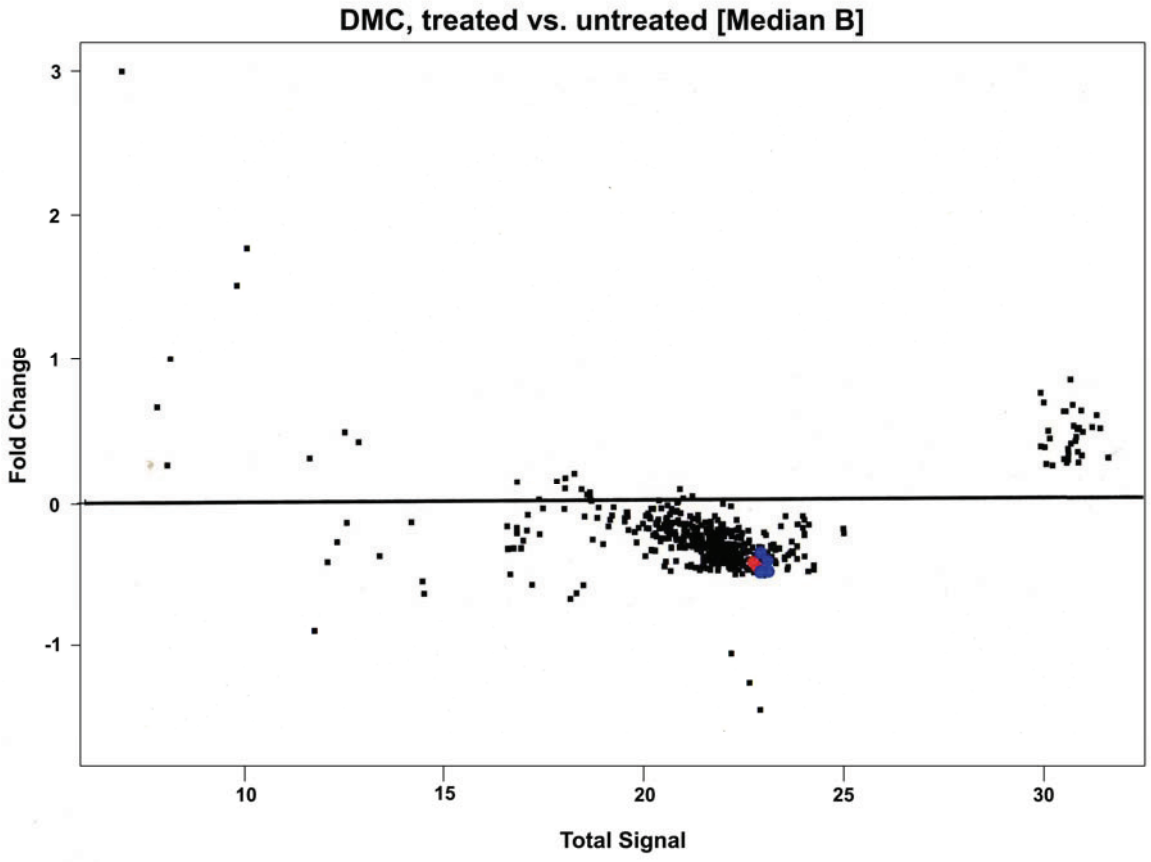


Figure 14.
DLD-1 cells were either left untreated or treated with 10 µM DMC for 24 hours. Extracts were obtained from cells and analyzed as indicated in the Sigma Panorama Antibody Microarray-Cell Signaling Technical Bulletin. Actin is blue and Chk1 is labeled red.

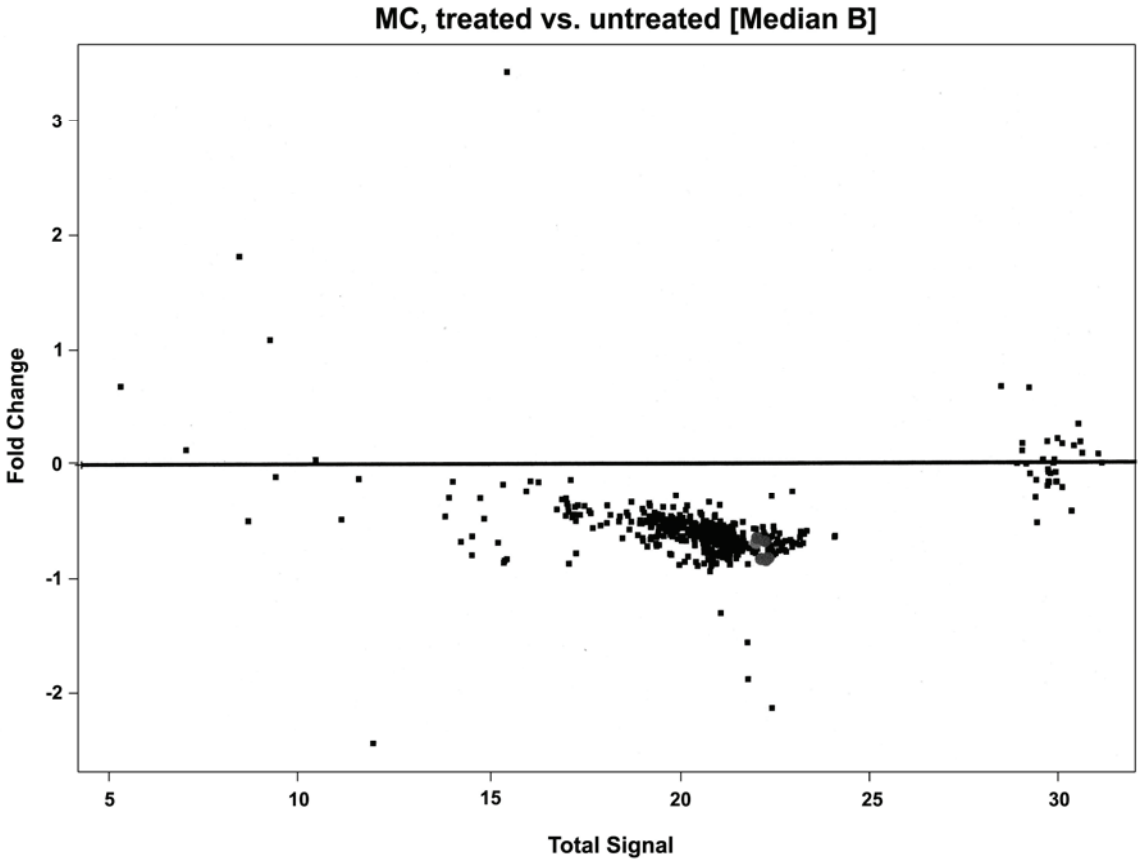


Figure 15.
DLD-1 cells were either left untreated or treated with 10 µM MC for 24 hours. Extracts were obtained from cells and analyzed as indicated in the Sigma Panorama Antibody Microarray-Cell Signaling Technical Bulletin. Chk1 protein is in color.

Cytoskeletal protein targets, such as actin and tubulin, spotted on the array slides as control, were recommended for normalization by the manufacturers. It is very disturbing that actin protein, which remains unchanged under most conditions when analyzed by Western blot, was downregulated, not only by DMC but MC as well. Although quantitative PCR analysis of actin mRNA shows downregulation of actin mRNA by DMC and not MC, all our Western blot data which exams differential regulation of target proteins by MC and DMC showed actin as stable when equal concentration of protein

was loaded on the gel. Accurate interpretation of Western blot data, require equal loading of sample. Clearly, further validation of each individual relevant protein target on the Panorama Ab Microarray Cell Signaling slide is required to conclusively support the array data. All the quantitative values obtained from our analysis are saved on Dr. Bargonetti's computer for future analysis.

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