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Mitomycin–DNA Adducts Induce p53-Dependent and p53-Independent Cell Death Pathways

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ABSTRACT 10-Decarbamoyl-mitomycin C (DMC), a mitomycin C (MC) derivative, generates an array of DNA monoadducts and interstrand cross-links stereoisomeric to those that are generated by MC. DMC was previously shown in our laboratory to exceed the cytotoxicity of MC in a human leukemia cell line that lacks a functional p53 pathway (K562). However, the molecular signal transduction pathway activated by DMC–DNA adducts has not been investigated. In this study, we have compared molecular targets associated with signaling pathways activated by DMC and MC in several human cancer cell lines. In cell lines lacking wild-type p53, DMC was reproducibly more cytotoxic than MC, but it generated barely detectable signal transduction markers associated with apoptotic death. Strikingly, DMC's increased cytotoxicity was not associated with an increase in DNA double-strand breaks but was associated with early poly(ADP-ribose) polymerase (PARP) activation and Chk1 kinase depletion. Alkylating agents can induce increased PARP activity associated with programmed necrosis, and the biological activity of DMC in p53-null cell lines fits this paradigm. In cell lines with a functional p53 pathway, both MC and DMC induced apoptosis. In the presence of p53, both MC and DMC activate procaspases; however, the spectrum of procaspases involved differs for the two drugs, as does induction of p73. These studies suggest that in the absence of p53, signaling to molecular targets in cell death can shift in response to different DNA adduct structures to induce non-apoptotic cell death.

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The genotoxic alkylating agent mitomycin C (MC) is a commonly used cancer drug previously demonstrated to generate a p53 response (*1–3*). Upon entering a cell, MC is enzymatically reduced into a highly reactive DNA-targeting electrophile (*4*). MC binds covalently to guanine residues to form monoadducts and intrastrand and interstrand cross-links (*5*). Although its reduction forms reactive oxygen species, the principal cause of MC cytotoxicity has been shown to be interstrand MC–DNA cross-links (*6*). Under the hypoxic conditions found in most solid tumors, greater cytotoxicity is achieved when cells are treated with the MC derivative 10-decarbamoyl-mitomycin C (DMC), which has had the carbamoyl group removed from the carbon at position 10 (*6, 7*). DMC produces high levels of 1′′-β-interstrand cross-links not formed by MC (see Figure 1 for flow chart of mitomycin adducts). Both MC and DMC signal to p53, causing an increase in p53 levels and p53-mediated cell death (*1*). Interestingly, while MC and DMC are equitoxic to ML-1 cells, DMC is more cytotoxic than MC to the K562 cell line, which lacks p53, suggesting that DMC provokes a strong p53-independent cell death pathway (*1*). Thus, DMC may have chemotherapeutic potential by triggering a p53-independent cell death pathway and may help us to determine the key features to target for inducing such a pathway. The *p53* gene is the most commonly mutated gene in human tumors (*8*); induction of cell death in the absence of p53 by molecular targeting is an important paradigm because mutations in the *p53* gene are common in diverse types of human cancer (*9*).

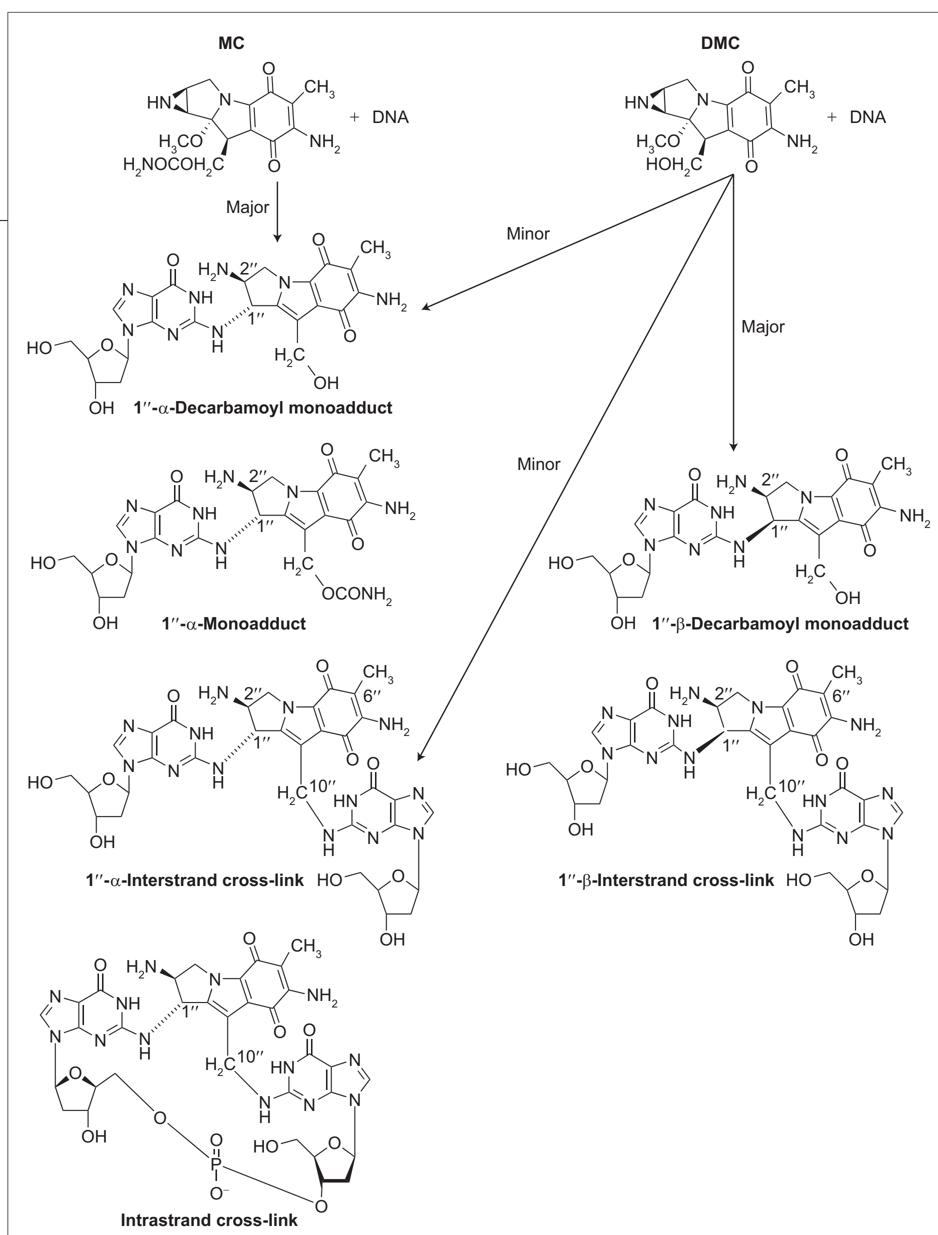

Figure 1. DNA adducts formed after exposure of mouse mammary tumor cells to MC and DMC. MC forms four different DNA adducts, shown in the left panel. DMC forms two DNA adducts that occur in response to MC (indicated by arrows), as well as two novel adducts, shown in the right panel.

While a number of reports concerning cellular signaling by MC–DNA adducts exist, studies on molecular signaling by DMC–DNA adducts are limited. Previously, we determined that MC and DMC activate the p53 pathway and cause increased transcription of p53 downstream target genes (*1*). MC has been shown to activate caspases-8 and -3 (*10, 11*); 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 with MC in cells lacking a functional p53 pathway (*1*). In this study, we compared molecular targets activated by DMC in the presence and absence of p53, and compared their activation by MC under the same conditions. This study was facilitated by the use of a well-documented colon cancer cell line (D-A2) in which doxycycline (DOX) concentration regulates wild-type p53 protein expression (*12*). This can be compared with the isogenic colon cancer line that lacks wild-type p53 expression (DLD-1) (*12*). We have systematically analyzed the outcomes of MC and DMC cellular treatments in the absence of wild-type p53 (using DLD-1 cells), and in the presence of wild-type p53 expression (using D-A2 cells plus DOX). Here we report that MC– and DMC–DNA adducts activate distinct molecular targets while causing similar levels of DNA double-strand breaks.

RESULTS AND DISCUSSION

MC and DMC Robustly Activate Procaspases in the Presence, but Not in the Absence, of p53. It is important to consider how chemotherapeutic DNA damage pathways function when activated in the presence and absence of wild-type p53, so chemotherapy can be designed for different molecular subtypes of cancers. The activation of a p53-independent cell death pathway in K562 cells after DMC treatment (*1*) led us to investigate the molecular mechanisms involved. We monitored caspase activation during DMC-mediated p53-independent cell death in K562 cells and other cell lines lacking wild-type p53 and compared the results with those obtained in the presence of wild-type p53 protein (Figure 2 and Figure 3). The activation of caspases during MC-induced apoptosis has been documented (*13, 14*); however, it has not been shown whether these caspases are activated in response to DMC or if caspase activation by the mitomycins requires the presence of p53 protein. A decrease in procaspase level occurs during caspase activation (*14*); therefore, we monitored depletion of procaspase-9, -8, and -3 as one indicator of caspase activation (Figure 2). In the absence of p53

in K562 cells, neither MC nor DMC caused detectable decreases in procaspase levels (Figure 2, panel a, lanes 1–4), although DMC was cytotoxic to these cells after 24 h of treatment, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) mitochondrial activity assay (*1*). Substantial caspase activation was detected in MC- and DMC-treated ML-1 (wild-type p53) cells. Reduced levels of procaspase-9 and -3 were detected with both drugs and a decrease in procaspase-8 resulted only after MC treatment (Figure 2, panel a, lanes 5–9).

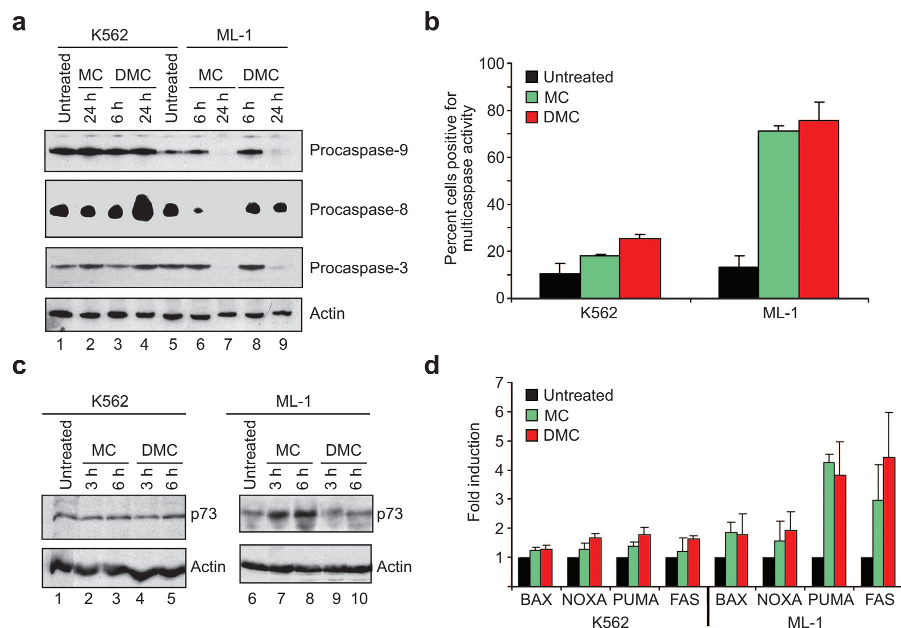

Figure 2. Procaspase activation, p73 increase, and apoptotic target gene activation in cells with and without wild-type p53. a) Depletion of procaspase-9, -8, and -3 in K562 (lanes 1–4) and ML-1 (lanes 5–9) cells treated with MC or DMC (5 μM) for 24 h was monitored by Western blotting. b) Quantitative analysis of caspase activity in K562 and ML-1 cells treated with MC or DMC (10 μM) for 24 h. Bars represent percentage of cell population exhibiting caspase activity. Data represent three independent experiments. Error bars indicate the standard deviation. c) p73 nuclear protein levels were monitored using protein extracts from K562 (lanes 1–5) and ML-1 (lanes 6–10) cells left untreated or treated with MC or DMC (5 μM) for 24 h. d) Quantitative real-time RT-PCR was used to analyze p53 apoptotic target gene transcription using messenger RNA isolated from K562 and ML-1 cells treated with MC or DMC (5 μM) for 12 h. Results indicate fold induction relative to the untreated sample. Data represent three independent experiments. Error bars indicate the standard deviation.

The activation of caspases by MC and DMC in the presence of p53 and the barely detectable activation in the absence of p53 were further validated using an assay for multiple-caspase activation (Figure 2, panel b). We measured multiple-caspase activity in K562 and ML-1 cells using the Guava Personal Cell Analysis System (sulforhodaminevalyl-alanyl-aspartyl-fluoromethylketone (SR–VAD–FMK)-based). This assay confirmed that K562 cells had extremely low levels of caspase activity in response to MC and DMC (with DMC inducing slightly higher levels), while robust caspase activation was observed in ML-1 cells treated with MC or DMC. Moreover, the addition of benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD)–FMK, a pan caspase inhibitor, did not protect K562 cells from DMC-induced cell death as assayed by MTT (data not shown). Taken together, these data suggest that cell death caused by DMC treatment of p53-null K562 cells is accompanied by only limited caspase activation, and consistent with this finding, caspase activation is not required for the DMC-mediated cell death pathway. Importantly, in wild-type p53 ML-1 cells, the DNA adducts produced by MC and DMC both resulted in robust activation of the cellular caspases (Figure 2, panels a and b).

Mitomycins Do Not Signal to p73 in the Absence of p53. Like p53 protein levels, p73 protein levels have been shown to be increased by DNA damaging agents such as doxorubicin, ionizing radiation, and cisplatin (*15, 16*). Studies have shown that p73 can induce numerous p53 target genes and can induce p53-independent apoptosis when overexpressed or activated by DNA damage (*17, 18*). We examined the ability of MC and DMC to signal for an increase in p73 protein levels in K562 and ML-1 cells. Neither MC nor DMC treatment increased the level of p73 in K562 cells (Figure 2, panel c, lanes 1–5). Significantly, no substantial activation of p53 target genes resulted from DMC treatment of K562 cells, as measured by quantitative reverse transcription PCR (RT-PCR) (Figure 2, panel d), further suggesting no activation of a p53 family member protein. Interestingly, in ML-1 cells, treatment with MC, but not with DMC, resulted in increased p73 protein levels (Figure 2, panel c, compare lanes 7 and 8 to lanes 9 and 10). However, RT-PCR demonstrated that down-

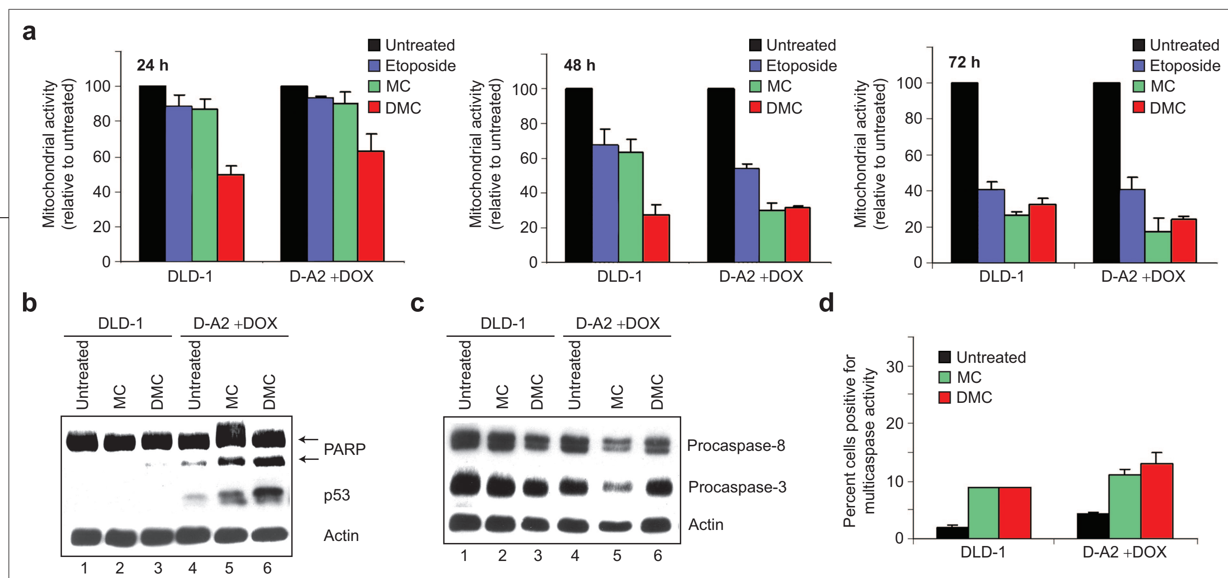

Figure 3. Drug-induced cytotoxicity, PARP cleavage, p53 level, depletion of procaspases, and level of caspase activity in DLD-1 and D-A2 (+DOX) cell lines. a) The cytotoxicity of MC and DMC (10 μM) in DLD-1 and D-A2 (+DOX) cells was monitored using MTT assay. Data show percentage of cells with mitochondrial activity relative to untreated samples and represent three independent experiments. Error bars indicate the standard deviation. b) Extracts from DLD-1 (lanes 1–3) and D-A2 (+DOX) (lanes 4–6) cells left untreated or treated with MC or DMC (10 μM) for 24 h were monitored for PARP cleavage and p53 levels. c) Depletion of procaspase-8 and -3 was monitored in DLD-1 (lanes 1–3) and D-A2 (+DOX) (lanes 4–6) cells under the same conditions as above. d) Quantitative analysis of caspase activity in DLD-1 and D-A2 (+DOX) cells treated with MC or DMC (10 μM) for 24 h. Bars represent percentage of cell population exhibiting caspase activity. Data represent three independent experiments. Error bars indicate the standard deviation.

stream target gene activation was similar after MC or DMC treatment of ML-1 cells (Figure 2, panel d), indicating that the p73 protein induced by MC did not influence transcription of the analyzed target genes. Therefore, the signaling to p73 can be facilitated by MC, but not by DMC, for the same cellular outcome after 24 h treatment of ML-1 cells.

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 line DLD-1 (expressing no wild-type p53) and its derivative D-A2, which has low-level expression of wild-type p53 in the presence of DOX (+DOX). We treated D-A2 cells with varying concentrations of DOX to regulate the amount of p53 protein expressed. The levels of p53 did not substantially increase with doses of DOX ranging from 20 to 5 ng mL^{-1}; therefore we used 20 ng mL^{-1} for low-level p53 expression. We were able to induce extremely high levels of p53 protein in the absence of DOX; however, these cells were apoptotic because of the overexpression of p53 (data not shown).

MTT analysis detects changes in cytotoxicity in response to drug treatment with a high degree of precision for detecting living but not dead cells (*19*), and when compared with the clonogenic assay, the MTT assay shows excellent agreement (*20*). MTT assay results showed that 24 h DMC, but not etoposide or MC, treatment substantially decreased viability in both DLD-1 (no wild-type p53) and D-A2 cells (DOX-regulated p53) (Figure 3, panel a; for p53 levels, see Figure 3, panel b). At 48 h of treatment, DMC was more cytotoxic than MC to DLD-1 cells, but the two drugs were equally cytotoxic to D-A2 (+DOX) cells. The strongest difference in the cytotoxicities of MC and DMC occurred in the DLD-1 cells (without wild-type p53) after 24 and 48 h of treatment. However, by 72 h etoposide and MC were also strongly cytotoxic to DLD-1 cells. When wild-type p53 expression was present in D-A2 cells (+DOX), DMC was more cytotoxic than MC at the 24 h time point but not at 48 and 72 h (Figure 3, panel a). Much faster kinetics for the induction of cell death were seen with DMC as compared with MC in either the presence or the absence of p53 when isogenic cell lines were compared.

We also examined the cytotoxicity of the mitomycins in two lung cancer cell lines: H460, which expresses wild-type p53, and p53-null H1299. With equimolar dosages of MC and DMC, we observed that DMC was more cytotoxic than MC to H1299 cells at 24 h, whereas both drugs were equally cytotoxic to H460 cells (data not shown). We previously showed that DMC was substantially more cytotoxic than MC to K562 cells, which lack functional p53 (*1*). Thus, DMC was substantially more cytotoxic than MC to each tested cell line lacking wild-type p53 protein.

During cell death, the enzyme PARP is often cleaved, which has been associated with apoptosis (*21*). Substantial PARP cleavage was observed after MC and DMC treatment of D-A2, but not DLD-1, cells (Figure 3,

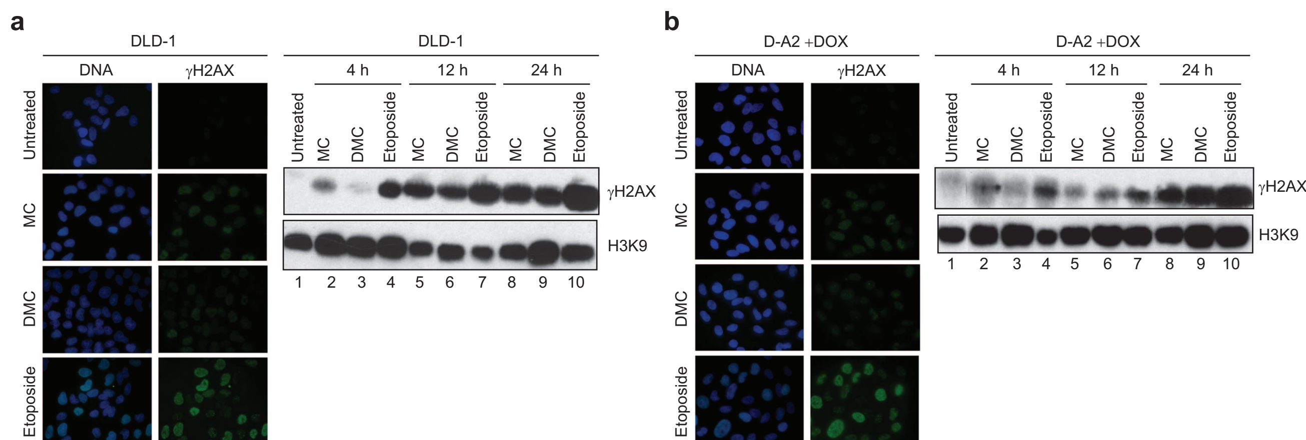

Figure 4. Phosphorylation of H2AX as a measure of double-strand breaks in cells treated with MC, DMC, or etoposide. Phosphorylated γH2AX was monitored in DLD-1 (a) and D-A2 (+DOX) (b) cells. Cells were left untreated or treated with MC, DMC, or etoposide (10 μM) for 4 h and stained with antibody for detection of γH2AX (right panel). DAPI was used to visualize the nuclei (left panel). Immunofluorescence results are typical of at least three independent experiments. Cell lysates from DLD-1 and D-A2 (DOX) cells left untreated (lane 1) or treated with 10 μM 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 monitored for γH2AX and dimethylated H3K9.

panel b, PARP panel). It is important to note that only 50% of DMC-treated DLD-1 cells were determined to be viable by MTT analysis after 24 h of drug treatment, where MC-treated cells did not lose viability (Figure 3, panel a). The death of DLD-1 cells (no wild-type p53) after DMC treatment was therefore occurring in the absence of PARP cleavage.

To examine other indicators of apoptosis, the depletion of procaspase-8 and -3 was examined. MC- and DMC-treated DLD-1 cells showed barely detectable changes in procaspases when normalized for loading by actin (Figure 3, panel c, lanes 1–3). In the presence of wild-type p53 expression, MC treatment resulted in depletion of procaspase-8 and -3, while DMC treatment had a lesser effect (Figure 3, panel c, 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 (Figure 3, panel a).

Analysis of total caspase levels using the sensitive Guava MultiCaspase assay showed a slight increase in caspase activity in DLD-1 cells after MC and DMC treatment but showed stronger activation in drug-treated D-A2 (+DOX) (low-level p53) samples (Figure 3, panel d). Importantly, PARP cleavage, procaspase depletion, and caspase activation indicate that DMC-induced cytotoxicity in the absence of p53 does not occur through a standard apoptotic cell death pathway. This suggests that in the absence of p53 activity, alternative cell death pathways lacking apoptotic markers can be rapidly activated by DMC and by 72 h can also be activated by MC, as a much slower response to the damage caused by the latter drug. Cells with p53 show indicators of apoptosis after both MC and DMC treatment, but the cell death could result from a combination of pathways.

Increased Cytotoxicity of DMC in the Absence of Wild-Type p53 Is Not the Result of Increased DNA Double-Strand Breaks. To examine whether the rapid cell death induced by DMC was due to increased double-strand breaks caused by the DNA damaging agent, we examined levels of phosphorylated histone 2AX (γH2AX) foci in cell nuclei and total γH2AX in cell extracts (Figure 4). Etoposide is well-known for causing DNA double-strand breaks (*22*). 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 MC or etoposide (Figure 4, panel a, 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 (Figure 4, panel a, lanes 5–10, and data not shown). The results were similar in wild-type p53-expressing cells (D-A2 +DOX, Figure 4, panel b). Interestingly, with

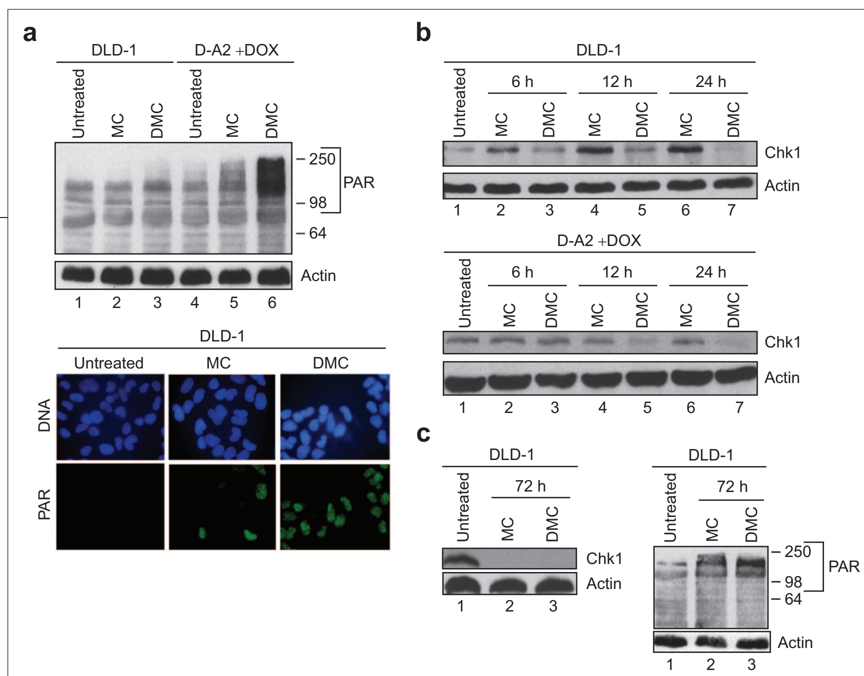

Figure 5. PARP activity and Chk1 protein levels regulated by MC and DMC in DLD-1 and D-A2 (+DOX) cell lines. a) DMC significantly induces robust PARP activity in DLD-1 and D-A2 (+DOX) cells at an early time point. Western blot of extracts obtained from DLD-1 and D-A2 (+DOX) cells left untreated (lanes 1 and 4) or treated with 10 μM MC (lanes 2 and 5) or DMC (lanes 3 and 6) for 24 h. Immunocytochemistry in DLD-1 cells detected nuclear PAR polymers (lower panel) in DMC-treated cells. DAPI was used to visualize the nuclei (upper panel). Immunofluorescence results are typical of three independent experiments. b) Chk1 protein levels are down-regulated by DMC and stabilized by MC at early time points. Whole cell extracts were prepared from DLD-1 and D-A2 (+DOX) cells left untreated (lane 1) or treated with 10 μM MC (lanes 2, 4, and 6) or DMC (lanes 3, 5, and 7) for 24 h. c) PARP activity and Chk1 protein are equally regulated by MC and DMC at later time points in cells without wild-type p53. Extracts from DLD-1 cells treated with MC or DMC (10 μM) for 72 h were analyzed for Chk1 protein and poly(ADP-ribosyl)ated proteins.

p53 expression, we reproducibly observed fewer double-strand breaks at the 12 h time point. This supports published work that p53 protein aids the DNA repair process (*23*) and, significantly, provides evidence that the increased cytotoxicity of DMC over MC is not a result of increased DNA double-strand breaks.

DMC Treatment Signals for Rapid ADP-Ribosylation of Nuclear Proteins and Chk1 Depletion. Increased PARP catalytic activity induced by DNA damage has previously been associated with non-apoptotic cell death induced by alkylating agents (*24, 25*). Activated PARP forms polymers of poly(ADP-ribose) (PAR), which are used to post-translationally modify select histones and nuclear proteins involved in the maintenance of chromatin architecture and DNA repair (*24, 26*). Because the mitomycins are alkylating agents, we monitored PARP activity in DLD-1 and D-A2 cells after drug treatment by examining the levels of ADP-ribosylated proteins in cell extracts. Western blotting showed that DMC induced higher levels of poly(ADP-ribosyl)ated proteins than MC when in the presence of p53 in D-A2 (+DOX) cells (Figure 5, panel a, compare lanes 4–6). This striking increase in PARP activity was reproducibly seen by Western blotting and immunofluorescence of cell nuclei(data not shown). In DLD-1 cells, there was increased PARP activity after DMC treatment as compared with MC treatment, which was more profoundly detectable by immunofluorescence (Figure 5, panel a, Western blot lanes 1–3 and immunofluorescence). The increased PARP activation in DMC-treated cells suggests that the DMC-treated cells invoke a more robust PARP-mediated repair pathway. Damage-repair factors are ADP-ribosylated and recruited to the regions of DNA damage (*27*). Further studies are needed to elucidate whether and how DMC–DNA adducts are able to differentially signal to the damage recognition factors.

Another target for p53-independent cell death is the depletion of Chk1, a kinase that is required for the DNA damage cell checkpoint (*28*). Chk1 depletion associates with death by mitotic catastrophe (*29, 30*) and is mediated by the ubiquitin proteolysis pathway (*31*). Therefore, we examined the ability of DMC and MC to carry out such depletion. In DLD-1 cells, the kinase Chk1 was depleted by 24 h of DMC treatment (Figure 5, panel b, DLD-1 panel, lane 7), while cells treated for 24 h with MC showed increased Chk1 expression (Figure 5, panel b, DLD-1 panel, lane 6). In D-A2 cells, the depletion of Chk1 by DMC was evident at an earlier time point of 12 h and remained at 24 h, while Chk1 levels were unaffected by MC treatment (Figure 5, panel b, D-A2 panel, lanes 1–7). These data suggest that DMC targets Chk1 for depletion by ubiquitin proteolysis, and it has been shown that Chk1 depletion is sufficient for the induction of cell death (*31*). The depletion of Chk1 combines with the activation of PARP for a rapidly induced p53-independent cell death that differs from what is induced by MC. Because both MC and DMC were equally cytotoxic to DLD-1 cells at 72 h (Figure 3, panel a), we examined the signaling to Chk1 and PARP at this time point to determine whether the two alkylating agents could eventually signal to the same pathway. At this equally cytotoxic time point, we found that both MC and DMC signaled for depletion of Chk1 and activation of PARP (Figure 5, panel c). This suggests that the non-apoptotic pathway uses these two targets to effect cell death.

Identification of a Caspase-Independent Cell Death Pathway. The data in DLD-1 cells (which lack wild-type p53) supported our results showing that the DMC-provoked cell death of p53-null K562 cells was not the result of apoptosis. There was no increase in sub-G1 DNA observed in DMC-treated DLD-1 cells (data not shown), suggesting an alternative cell death pathway that may be a convergence of pathways. DMC adducts have the ability to induce a rapid form of cell death that lacks strong apoptotic markers in the absence of p53 (see Figure 6), such as caspase activation, sub-G1 DNA, and PARP cleavage. Importantly, the activation of ADP-ribosylation of proteins by PARP and the depletion of Chk1 under such conditions suggests that the alternative DNA adducts signal for programmed necrosis, mitotic catastrophe, or both.

While our preferred hypothesis to explain the observed differences in cell death pathways is that they result from qualitative differences in the DNA adducts formed by MC and DMC, a potentially reasonable interpretation of the data is that the differences between the two drugs are due to different levels of DNA modification. Thompson and colleagues have described alkylating agents that mediate programmed necrosis in cancer cells in which apoptotic cellular programs are blocked. These investigators suggest necrotic cell death is an important component of cancer cell death (*24, 32*). It is clear that MC can provoke non-apoptotic cell death after an extended period of time. However, the specificity and rapid action of equimolar DMC provides evidence that it is a more potent drug for inducing p53-independent cell death.

It is interesting that MC and DMC signal differently in both the presence and absence of wild-type p53 protein, suggesting that some pathways can intersect while others remain distinct and some others are cell-type-specific. We have documented that DMC is more cytotoxic than MC to hypoxic EMT6 murine cancer cells (*6*), suggesting that DMC could be an effective treatment for oxygen-deprived solid tumors. With many cancers containing p53 pathway mutations, as well as mutations in apoptotic cellular programs, it is important to explore new drugs capable of killing cells with these deficiencies. Not only does DMC exhibit a similar ability to promote apoptosis in the presence of p53 as its source compound, MC, but it is clear from our studies in K562, H1299, and DLD-1 cells that it can effectively initiate non-apoptotic cell death in cell lines lacking wild-type p53. Cell death controlled by programmed necrosis and autophagy is actively being researched because these pathways are potential targets for chemotherapeutic drug discovery (*24, 32–34*). Alternative DNA adducts may specifically activate novel cell death pathways, which can be utilized in designing drugs that capitalize on non-apoptotic pathways.

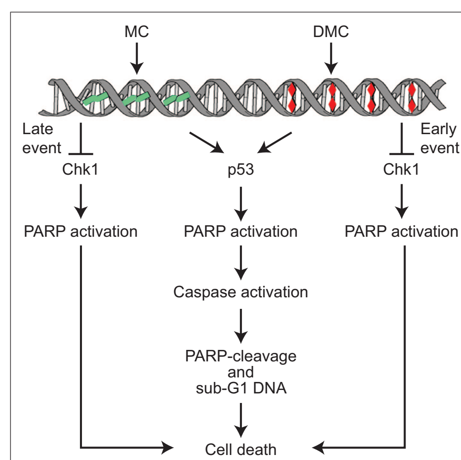

Figure 6. Schematic model depicting MC and DMC signal transduction pathways. Green and red objects represent structurally different MC and DMC adducts, respectively. In cells with wild-type p53, MC– and DMC–DNA adducts activated apoptotic cell death. In cells lacking wild-type p53, DMC rapidly activated non-apoptotic cell death, and MC slowly activated non-apoptotic cell death, with PARP activation and Chk1 depletion as markers.

METHODS

Reagents. MC was supplied by D. M. Vyas (Bristol-Myers Squibb). DMC was synthesized in our laboratory as previously described (*6*). Etoposide, MTT reagents, and DOX were purchased from Sigma. Roswell Park Memorial Institute (RPMI) media 1640, McCoy's 5A, and penicillin–streptomycin were purchased from Mediatech. Fetal bovine serum (FBS) and gentamycin (G418) were purchased from Gemini. Hygromycin B was purchased from Calbiochem. The MultiCaspase Detection kit was purchased from Guava Technologies.

Cell Culture. The myeloid leukemia cell line ML-1 with wild-type p53 (a gift from Michael Kastan, St. Jude Children's Hospital) and the erythromyeloid leukemia cell line K562 without p53 expression (ATCC) were grown in RPMI 1640 supplemented with 10% FBS (v/v) and 2500 units of pen–strep and maintained between 2.5×10^5 and 5×10^5 cells mL^{-1}. The isogenic colon cancer cell lines, DLD-1 and D-A2, were gifts from Bert Vogelstein (Johns Hopkins School of Medicine), who used a two-step procedure to establish a tetracycline-off system for controlled wild-type p53 expression in D-A2 cells (*12*). DLD-1 cells do not express wild-type p53 and D-A2 cells maintained in 20 ng mL^{-1} of DOX express low levels of wild-type p53. DLD-1 cells were grown in McCoy's 5A media containing 10% FBS (v/v) and 2500 units of pen–strep, while D-A2 cells required an additional 0.4 mg mL^{-1} G418, 20 ng mL^{-1} DOX, and 0.25 mg mL^{-1} hygromycin B. All cells were seeded overnight prior to drug treatment at 37 °C in 5% CO_2.

Drug Treatments. Cells were treated with MC or DMC (in 30% methanol (v/v)) at the concentrations and times indicated. Where indicated, the topoisomerase poison etoposide (in

DMSO) was used to compare cytotoxicity at the indicated concentrations and time points.

Protein Extraction. Nuclear extracts were prepared using a variation of the Dignam protocol (*35*), as described previously (*1*).

Histone Extraction. Cells were harvested by centrifugation at 4 °C at 2000 rpm for 5 min. The pellets were washed in PBS and lysed in 4 mL of extraction buffer (10% Triton X-100 (v/v), 50 mM phenylmethylsulfonyl fluoride, 2% sodium azide (w/v) in PBS). Pellets were resuspended in 0.2 N hydrochloric acid overnight at 4 °C. Extracts were centrifuged at 4 °C at 2000 rpm for 10 min, and the supernatant was stored at −20 °C. Twenty microgram protein aliquots were boiled and separated by 10% SDS-PAGE.

Western Blotting. Protein samples were size-fractionated by electrophoresis in 10% denaturing polyacrylamide gels and electrotransferred to nitrocellulose membranes (Amersham). 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 *36, 37*), anti-γ histone 2AX (H2AX) (Upstate cat. no. 05-636), anti-dimethylated histone 3 lysine 9 (H3K9) (Upstate cat. no. 05768), anti-PARP antibody (Pharmingen cat. no. 551025), anti-PAR (Pharmingen cat. no. 551813), anti-procaspase-9 (Pharmingen cat. no. 551246), -8 (Oncogene Research cat. no. AM46T), or -3 (Cell Signaling cat. no. 9662) antibodies, anti-p73 antibody (Oncogene Research cat. no. OP108), anti-Chk1 antibody (Cell Signaling cat. no. 2345), and the polyclonal anti-actin antibody (Sigma cat. no. A5060). The membranes were incubated in anti-mouse or anti-rabbit secondary antibodies (Sigma), and the signals were visualized by chemiluminescence.

Quantitative RT-PCR. RNA was isolated using the Qiagen RNeasy Mini Kit. Five micrograms of RNA was used for complementary DNA (cDNA) synthesis using the high-capacity cDNA archive kit (Applied Biosystems). The primer–probes for *bax*, *noxa*, *fas*, *puma* (Applied Biosystems, Celera Discovery Systems Assays on Demand), and *gapdh* (Applied Biosystems predeveloped assay reagents) were utilized for TaqMan PCR using the Applied Biosystems 7500 sequence detection system (Perkin Elmer) as follows: one cycle, 2 min (50 °C); one cycle, 10 min (94 °C); and 40 cycles, 15 s (94 °C) and 1 min (60 °C).

MTT Assay. Sensitivity following treatment with DNA damaging agents was determined using the tetrazolium dye-based microtitration assay. Cells were seeded at 1.25×10^5 cells, attached overnight, and were treated with drug for the indicated times. Following incubation, MTT solution (5 mg mL^{-1} 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 2 h. The cells were centrifuged at 1850 rpm for 5 min at RT, and pellets were resuspended in 0.04 N hydrochloric acid diluted in isopropanol. Samples were incubated for 5 min at RT and centrifuged at 14,000 rpm for 2 min. Supernatant absorbance was read at 550 nm. Data represent percent population of viable cells in each sample relative to the untreated sample.

MultiCaspase Assay. Following treatment, at least 5.0×10^4 of ML-1, K562, DLD-1, or 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).

Immunofluorescence. Immunostaining for poly(ADP-ribosyl)ated or phosphorylated H2AX proteins was performed on cells plated onto glass coverslips. Cells were fixed with 2% paraformaldehyde in PBS (v/v) for 15 min at RT. Cells were permeabilized with PBS containing 0.2% Triton X-100 (v/v) and 1% FBS (v/v) for 5 min at −20 °C, washed three times in 1% FBS–PBS (v/v), and incubated with PAR (ADP-ribose) antibody (BD Pharmingen cat. no. 551025) or monoclonal anti-γ H2AX (Upstate cat. no. 05-636) in 1% FBS–PBS at RT for 1 h. Cover slips were rinsed three times with 1% FBS–PBS solution and incubated with secondary FITC-conjugated goat anti-rabbit antibody (Santa Cruz) or FITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) in 1% FBS–PBS. Cells were rinsed three 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.

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