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Title page

Doxorubicin inhibits DNMT1 resulting in conditional apoptosis

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Running title page

Running title: Inhibition of DNMT1 by a DNA intercalating drug doxorubicin

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AdoMet, S-adenosyl-L-methionine; AdoHcy, S-adenosyl-L-homocysteine; 5-AdC, 5-aza-2'-deoxycytidine.

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Abstract

Chemotherapy utilizing DNA intercalators is one of the most successful approaches to cancer treatment. Although DNA intercalators are believed to inhibit DNA polymerases and topoisomerases resulting in the induction of apoptosis in tumor cells, other factors potentially inhibited by the anthracycline antibiotics remain to be elucidated. Here we show that the enzymatic activity of DNMT1, the primary DNA methyltransferase in mammalian cells, is inhibited by DNA intercalators such as doxorubicin in an *in vitro* assay. Enzymatic analyses indicate that doxorubicin inhibits the catalytic activity of DNMT1 via DNA intercalation. We also found that apoptosis was induced in *DNMT1*$^{+/+}$ HCT116 cells by only a limited range of doxorubicin dose, meaning that apoptotic cell death is "conditional" with respect to the concentration of the DNA intercalating drug. Interestingly, conditional apoptosis is not observed in human colorectal cancer cells lacking DNMT1 but can be induced in *DNMT1*$^{-/-}$ cells by transfection of a plasmid expressing DNMT1. Our results suggest that DNMT1 is one of the major targets of doxorubicin resulting in drug-induced apoptosis in human cancer cells. We propose that expression levels of DNMT1 in tumor cells may affect the effectiveness of doxorubicin in chemotherapy.

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Introduction

DNA intercalating agents, such as amsacrine (m-AMSA), actinomycin, mitoxantrone, and doxorubicin, have been employed as anti-cancer drugs and are in routine clinical use as chemotherapeutic agents (Brana et al., 2001). DNA intercalators share common structural motifs - the presence of planar polyaromatic systems that bind by insertion between DNA base-pairs. Doxorubicin (Adriamycin), an anthracycline-based DNA intercalator, is one of the most typical anticancer drugs commonly employed in the clinic. It has been well accepted that the anti-tumor activity of doxorubicin is due to the formation of a cleavable complex of topoisomerase II, resulting in apoptosis (Hickman, 1992; Kiechle and Zhang, 2002). Doxorubicin is indicated in the treatment of a broad spectrum of solid tumors (e.g. breast, bladder, endometrium, thyroid, lung, ovary, stomach, and sarcomas of the bone) and in the treatment of lymphoma, as well as acute lymphoblastic and myeloblastic leukemias (Carter, 1975). One of the most important and clinically relevant side effects of doxorubicin is the induction of cardiomyopathy (Lenaz and Page, 1976). A number of mechanisms have been proposed to explain this effect of doxorubicin, including oxidative stress (Myers et al., 1977), the induction of mitochondrial damage (Wallace, 2003), and changes in gene expression in cardiac myocytes and muscle cells in general (Boucek et al., 1999; Kurabayashi et al., 1994). Therefore, doxorubicin likely impacts on the activity of other important proteins or pathways and these need to be elucidated to better understand and to take advantage of its anti-tumor activity.

DNA methyltransferase 1 (DNMT1) is the primary enzyme responsible for maintenance of DNA methylation on genomic DNA (Bestor et al., 1988; Pradhan et al., 1999; Yoder et al., 1996; Yokochi and Robertson, 2002). Disruption of DNMT1 function causes chromosome instability and dysregulation of transcription, and ultimately leads to apoptotic cell death

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(Ehrlich, 2002; Jones and Baylin, 2002; Robertson, 2001). Cre-mediated deletion of the *Dnmt1* gene resulted in demethylation of genomic DNA in cultured murine fibroblasts and led to p53-dependent cell death (Jackson-Grusby et al., 2001), suggesting that loss of DNMT1 induces apoptosis. However, other studies have shown that deletion of the *DNMT1* gene is compatible with cell viability in the HCT116 cell line (Rhee et al., 2002; Rhee et al., 2000). The *DNMT1*$^{-/-}$ HCT116 cells exhibited normal morphology except for a slightly slower growth rate compared to the parental or *DNMT1*$^{+/-}$ cells (Rhee et al., 2000), indicating that loss of DNMT1 did not induce apoptosis in this cell line. Therefore, it was of great interest to determine whether DNMT1 is associated with apoptosis mediated by doxorubicin. Previous work has suggested that DNA methyltransferase activity in crude cell extracts could be inhibited by DNA intercalators (Adams and Rinaldi, 1987), however the molecular mechanism of intercalator-dependent inhibition of DNMT1 functions *in vitro* has not been reported.

In this report, we first investigated the effect of the inhibition of DNMT1 activity by DNA intercalating drugs including doxorubicin in an *in vitro* assay system. Enzymatic studies demonstrate that doxorubicin is a potent inhibitor of DNMT1 activity via DNA intercalation. We also examined doxorubicin-induced cytotoxicity utilizing the HCT116 human colorectal cancer cell line as a model system. Our data show that doxorubicin treatment of HCT116 cells results in massive cell killing by apoptosis at only a particular drug dose (referred to as conditional apoptosis). Lower, and remarkably, higher doses of doxorubicin do not result in significant apoptosis induction. This conditional apoptosis is absent in cells lacking the major DNA methyltransferase DNMT1. However, *DNMT1*$^{-/-}$ HCT116 cells can be made apoptosis-inducible by reintroduction of DNMT1. These results suggest for the first time that DNMT1

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is one of the important targets for doxorubicin and that interaction of DNMT1 and doxorubicin may contribute to the induction of apoptosis in cancer cells.

Materials and Methods

Recombinant protein preparation and DNA methyltransferase assay. Expression and purification of recombinant DNMT1 has been described previously (Yokochi and Robertson, 2002). All experiments in a given panel in Fig. 1 were performed with the same enzyme preparations. DNA methyltransferase activity of DNMT1 was measured using the DNMT-magnetic beads assay (Yokochi and Robertson, 2002). A typical methylation reaction (40 μl) contained 125 nM DNA oligonucleotides (hemimethylated DNA conjugated with a biotin molecule, 34 base-pairs (Yokochi and Robertson, 2002)), 30 nM DNMT1, and 600 nM tritium-labeled AdoMet (Amersham Bioscience, 1 mCi/ml) in reaction buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonylfluoride). Experiments were done independently at least three times and the mean values are shown. Radioactive materials were purchased from Amersham Bioscience. General chemicals were purchased from Sigma, Invitrogen, and Roche.

Cell culture, drug treatment, and transient transfection. HCT116 and *DNMT1*$^{-/-}$ HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Trypsinized cells were plated onto 100 mm (10 ml medium) and 35 mm (2 ml medium) diameter dishes and standard 96-well flat bottom plates (100 μl medium) and incubated for 24 hours prior to drug treatment. Since growth rates of HCT116 cells and its knockout derivative differ slightly (Rhee et al., 2000), initial cell numbers were adjusted so that the cell concentrations were typically 1×10^5 cells/ml at the time of drug addition. *DNMT1*$^{-/-}$ HCT116 cells were transfected with GFP or GFP-DNMT1 expression vectors

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using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Each experiment was repeated three times independently, in which at least 10 cells expressing green fluorescence were examined and were confirmed to be consistent in terms of doxorubicin sensitivity.

Cell viability assay, Trypan Blue staining, and apoptosis-related assays. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-4-sulfophenyl)-2H-tetrazolium, inner salt) assay and Caspase-Glo 3/7 assay (Promega) were performed in accordance with the manufacturer's instructions. A fixed concentration of cells (1×10^5 cells/ml at the time of drug addition) was treated with various concentrations of drugs, and the cell viability or caspase activity were measured after 48 h. Parallel reactions were carried out without cells and this value was subtracted from the experimental values. Results were expressed as the mean of four independent experiments and error bars represent the standard deviation (S.D.). DNA fragmentation in cells treated with doxorubicin was detected as described (Yeung, 2002). For Trypan Blue staining, cells in 2 ml of medium were treated with various concentrations of doxorubicin for 48 hours, trypsinized, resuspended in fresh medium, and then an equal volume of Trypan Blue stain was added. The number of live and dead cells was counted on pictures taken with a microscopy system with a digital camera (Nikon). Results were expressed as the mean of four independent experiments.

Western blotting and methyl acceptance assay. The DNMT1 antipeptide antibody has been previously described (Yokochi and Robertson, 2002). The PCNA and β-actin antibodies were purchased from Santa Cruz Biotechnology. Genomic DNA was extracted from HCT116 cells that were treated with various concentrations of doxorubicin as follows (0, 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 2×10^{-7}, 5×10^{-7}, 1×10^{-6}, 2×10^{-6}, 5×10^{-6}, 1×10^{-5}, 2×10^{-5}, 5×10^{-5}, 1×10^{-4} M) for

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48 hours. Methylation acceptance capability of genomic DNA was measured by *in vitro* methylation reaction utilizing bacterial CpG methylase *Sss*I (New England Biolabs). A reaction solution contained genomic DNA (2 μg), *Sss*I (1 μl), and 900 nM tritium-labeled AdoMet in reaction buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonylfluoride). Following ethanol precipitation, tritium incorporation into genomic DNA was measured by a liquid scintillation counter. Results were presented as the mean of three independent experiments. S.D. was shown as error bars.

Results

Doxorubicin inhibits the enzymatic activity of DNMT1 *in vitro* via intercalation into DNA. We employed an *in vitro* enzymatic assay system (Yokochi and Robertson, 2002), utilizing highly purified recombinant DNMT1 to investigate the effects of DNA intercalators on DNMT1 catalytic activity. Doxorubicin, as well as other DNA intercalating agents, significantly inhibited DNMT1 activity *in vitro* (Fig. 1A). We next compared dose-response plots of DNMT1 activity against the DNA substrate in the presence of a fixed concentration of doxorubicin, S-adenosyl-L-homocysteine (AdoHcy), or methylated DNA as enzymatic inhibitors. Both AdoHcy and methylated DNA are product inhibitors of DNMT1 and they require direct binding to the enzyme to inhibit its catalytic activity. Consistent with the velocity equations for an enzymatic reaction under the effect of an inhibitor (Copeland, 2000; Segel, 1993), DNMT1 activity yielded hyperbolic curves in the presence of either AdoHcy or methylated DNA (Fig. 1B). In contrast, DNMT1 inhibition yielded a sigmoidal curve under the effect of doxorubicin inhibition, suggesting that the inhibitory mechanism by doxorubicin might not be explained by a simple “inhibitor-enzyme” interaction model (Copeland, 2000). To identify which factor (DNMT1, DNA, or AdoMet) interacts with doxorubicin to inhibit

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the DNMT1 catalytic reaction, we employed conditions in which one of the three factors was varied to examine whether it would alter the doxorubicin-dose dependency of DNMT1 activity on the inhibition plots (Fig. 1C). The slope in the relative comparison plot of the inhibition curves was significantly affected only when the amount of DNA was varied (Fig. 1C, left panel, inset), indicating that the inhibitory effect of doxorubicin is dose dependent with respect to DNA. These results suggest that the formation of a drug-DNA complex is important for the inhibition of DNMT1 by doxorubicin and exclude the possibility that doxorubicin binds directly to DNMT1 to cause inhibition. Thus, we conclude that doxorubicin inhibits DNMT1 activity via DNA intercalation.

Doxorubicin demonstrates a specific cytotoxicity in the presence of DNMT1. We next sought to examine the possible consequence of the inhibition of DNMT1 by doxorubicin in human cancer cells. As a reference, we utilized a different type of anticancer drug, etoposide (VP-16-213). Etoposide is a non-DNA intercalating drug that inhibits topoisomerase II specifically resulting in p53-dependent apoptosis (Ross et al., 1984) but does not inhibit DNMT1 *in vitro* (Fig. 1A). A fixed number of HCT116 human colorectal carcinoma cells (*DNMT1*$^{+/+}$ and *DNMT1*$^{-/-}$) were treated with various concentrations of anticancer agents (doxorubicin and etoposide) for 48 hours, and the cell viability was measured by MTS assay. A particular dose (1×10^{-6} M) of doxorubicin specifically decreased cell viability (Fig. 2A), while etoposide yielded a simple dose-dependent decrease in cell viability (Fig. 2B). Interestingly, the *DNMT1*$^{-/-}$ HCT116 cells (Rhee et al., 2000) did not demonstrate the specific cytotoxicity following doxorubicin treatment (Fig. 2C). Similar specific cytotoxicity was observed with other DNA intercalators such as actinomycin D and actinomycin V in *DNMT1*$^{+/+}$ HCT116 cells, and in other cell lines such as HeLa and 293 with doxorubicin (data not shown). The large peak of caspase-3/7 activation at 1×10^{-6} M doxorubicin (Fig. 2D,

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etoposide control in Fig. 2E) is consistent with the decrease in cell viability (Fig. 2A), suggesting that the cytotoxicity at 1×10^{-6} M doxorubicin is due to apoptotic cell death. A slight activation of caspase-3/7 was observed (~two-fold) in a broad, but non-specific range of doxorubicin doses between 1×10^{-7} M and 1×10^{-5} M in *DNMT1*$^{-/-}$ cells (Fig. 2F). This is consistent with previous findings that the other major targets of doxorubicin are DNA polymerases and topoisomerases, and that the inhibition of these DNA-binding proteins with doxorubicin leads to apoptosis (Hickman, 1992; Kiechle and Zhang, 2002).

Apoptosis is induced by only a particular concentration of doxorubicin. To further confirm that apoptosis is associated with the decreasing cell viability induced by doxorubicin in *DNMT1*$^{+/+}$ HCT116 cells, the numbers of live and dead cells were counted after Trypan Blue staining (Fig. 3A). A large fraction of cells died under the treatment with 1×10^{-6} M doxorubicin, suggesting that the decrease in cell viability is related to massive cell killing. Ten-fold higher (1×10^{-5} M) or ten-fold lower (1×10^{-7} M) doses of doxorubicin, however, did not lead to significant cell death. DNA laddering analysis also supports this notion (Fig. 3B). The improved DNA laddering assay described by Yeung (Yeung, 2002) clearly demonstrated nucleosomal fragmentation, which is a well documented characteristic of apoptosis, at the 1×10^{-6} M doxorubicin dose, while random genomic DNA digestion occurred at higher doses (1×10^{-5} M and above). Cells treated with 1×10^{-6} M doxorubicin appeared shrunken and developed blebs on their cell surface, typical morphological hallmarks of apoptosis (Budihardjo et al., 1999) (Fig. 3C). Our results therefore suggest that apoptosis induced by doxorubicin is "conditional", meaning that only a particular concentration of drug specifically induces apoptosis in *DNMT1*$^{+/+}$ HCT116 cells, but higher or lower concentrations do not. It should be noted that, at the highest dose (1×10^{-4} M) of doxorubicin, cells blackened, swelled, and burst (Fig. 3C), and showed neither caspase-3/7 activation (Fig. 2F) nor DNA laddering

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(Fig. 3B). These characteristics are consistent with cell death due to necrosis (Kroemer et al., 1998).

Conditional apoptosis induced by doxorubicin depends on DNMT1 in HCT116 cells. Consistent with the results of cell viability (Fig. 2C) and caspase activity (Fig. 2F), *DNMT1*$^{-/-}$ HCT116 cells yielded a simple dose-dependency with regard to doxorubicin concentration in the Trypan Blue staining assay (Fig. 4A). Since *DNMT1*$^{-/-}$ HCT116 cells grow more slowly than *DNMT1*$^{+/+}$ HCT116 cells, differences between these two cell lines may be explained by the difference of cell growth rates. To rule out this possibility, the cell cycle of *DNMT1*$^{+/+}$ HCT116 cells was first arrested by other growth inhibitors (e.g. Colcemid (Fig. 4B), nocodazole, hydroxyurea, and aphidicolin (data not shown)). Doxorubicin-mediated conditional apoptosis was induced even in these growth-arrested cells (Fig. 4B). This result suggests that the difference of doxorubicin sensitivity between *DNMT1*$^{+/+}$ and *DNMT1*$^{-/-}$ cells is not related to cell growth rates and that cell proliferation and DNA replication are not essential for conditional apoptosis. To further confirm the direct contribution of DNMT1 to conditional apoptosis, transient transfection of *DNMT1*$^{-/-}$ HCT116 cells was performed with either empty GFP (green fluorescent protein) expression vector or GFP-tagged DNMT1 expression vector. After 48 hours of transfection, cells were treated with 1×10^{-6} M doxorubicin (the dose inducing apoptosis in *DNMT1*$^{+/+}$ cells, but not in *DNMT1*$^{-/-}$ cells) for an additional 48 hours. Expression of GFP-DNMT1 caused apoptosis in *DNMT1*$^{-/-}$ HCT116 cells following treatment with 1×10^{-6} M doxorubicin (Fig. 4C, top panels), whereas expression of GFP alone did not (Fig. 4C, bottom panels). Total protein levels of DNMT1 in *DNMT1*$^{+/+}$ HCT116 cells were greatly reduced following treatment with 1×10^{-6} M doxorubicin (Fig. 4D), further supporting the notion that DNMT1 in cells is related to

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conditional apoptosis induced by doxorubicin. Taken together, we conclude that DNMT1 contributes to the conditional apoptosis in HCT116 cells.

Changes in global methylation status of genomic DNA are not associated with conditional apoptosis. Results presented in Fig. 3A from the Trypan blue cell counting assay clearly indicated that one of the effects of doxorubicin was to cause cell cycle arrest. This effect occurred over a relatively wider range of drug concentration than the conditional apoptosis effect, beginning at 1×10^{-7} M doxorubicin. Since demethylation due to inhibition of DNMT1 is believed to occur by a passive mechanism, which requires active cell division to dilute out the methylated parental DNA strands, it was not clear whether doxorubicin treatment and conditional apoptosis would be associated with detectable genomic demethylation. In order to determine this, global DNA methylation levels in doxorubicin-treated *DNMT1*$^{+/+}$ HCT116 were examined using the methyl-acceptance assay. Results of this assay (Fig. 4E) revealed that the cellular DNA methylation status of HCT116 cells was not significantly altered following treatment with 1×10^{-6} M doxorubicin. Although there appeared to be some hypomethylation occurring at very high doses of doxorubicin, the significance of such results is difficult to ascertain because cells are undergoing high levels of cell death by necrosis under these conditions.

Discussion

Two non-exclusive molecular mechanisms have been proposed as the trigger of apoptosis induction by DNA methyltransferase inhibitors: genomic demethylation and enzyme-mediated DNA damage. Loss of genomic methylation as the consequence of DNA methyltransferase inhibition causes dysregulation of gene expression and apoptosis. Depletion of Dnmt1 from *Xenopus* embryos causes embryonic lethality and inappropriate

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gene expression (Stancheva and Meehan, 2000). Furthermore, Cre-*lox* mediated depletion of *Dnmt1* in mouse fibroblasts caused loss of Dnmt1 protein, genomic hypomethylation, and induction of p53-dependent apoptosis (Jackson-Grusby et al., 2001). Since cytosine methylation within the promoter regions of genes can cause transcriptional silencing, demethylation may activate the expression of genes that in turn activate p53. In this report, however, we found that conditional apoptosis was induced only when DNMT1 protein and 1×10^{-6} M doxorubicin coexist. Therefore, the latter mechanism - loss of genomic methylation - may not represent the most plausible cause of conditional apoptosis. An alternative mechanism is that catalytically inactive DNMT1 bound to doxorubicin-treated DNA may be perceived by the cell as a form of DNA damage. The cytotoxic effects of 5-aza-2'-deoxycytidine (5-AdC) are believed to be mediated through the irreversible covalent binding of DNA methyltransferase to 5-AdC-substituted DNA, which is then recognized as DNA damage by the cell, resulting in apoptosis (Juttermann et al., 1994). Indeed, it has been shown that 5-AdC treatment causes p53 activation through a traditional DNA damage response pathway, which is consistent with this model (Karpf et al., 2001). Other studies have shown that the introduction of *DNMT1*$^{-/-}$ *DNMT3B*$^{-/-}$ double mutations into HCT116 cells did not cause apoptosis, even though these cells exhibited marked genomic hypomethylation (a roughly 95% reduction in methylated cytidine content) (Rhee et al., 2002). In addition, we demonstrated that doxorubicin could induce conditional apoptosis only in *DNMT1*$^{+/+}$ HCT116 cells, but not in *DNMT1*$^{-/-}$ HCT116 (this work) or *DNMT1*$^{-/-}$ *DNMT3B*$^{-/-}$ HCT116 (data not shown) cells. These results suggest that DNMT1 itself, rather than the secondary demethylation of genomic DNA, is the primary mediator of drug-induced cytotoxicity.

Total soluble DNMT1 protein levels were decreased specifically in *DNMT1*$^{+/+}$ HCT116 cells treated with the apoptosis-inducible concentration of doxorubicin (Fig. 4D). The reduced

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DNMT1 protein levels may be due to the formation of an irreversible DNMT1-DNA-doxorubicin complex such that the DNMT1 becomes trapped in an insoluble chromatin fraction during protein extraction. Although there is no direct evidence at this time, we speculate that doxorubicin may stabilize the covalent link between DNMT1 and DNA. The DNMT1-DNA complex could be recognized as DNA damage resulting in apoptosis. The degradation or removal of the complex may be facilitated by the cellular DNA repair machinery. Analysis of these, and other possibilities, will be the subject of future work.

It has been thought that the anti-tumor activity of DNA intercalators, including doxorubicin, is closely related to DNA cleavage depending on topoisomerase II and inhibition of DNA replication (Brana et al., 2001; Hickman, 1992; Kiechle and Zhang, 2002). Our data indicate that DNMT1 is also required for conditional apoptosis induced by a particular concentration of doxorubicin, suggesting that DNA methyltransferase is one of the targets of doxorubicin for apoptosis induction in cancer cells. We propose that the expression levels of DNMT1 in tumor cells may be important criteria that should be taken into account to evaluate the selective cytotoxicity of the drug (Pratt et al., 1994) and to determine the optimal dose regimen.

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Figure legends

Fig. 1. Doxorubicin inhibits the enzymatic activity of DNMT1 *in vitro* via intercalation into DNA. (A) Dose-response plots of DNMT1 activity as a function of inhibitor concentration. Catalytic activity of DNMT1 (30 nM) was significantly inhibited in the presence of DNA intercalating drugs such as doxorubicin (closed circle), actinomycin D (closed square), and actinomycin V (closed diamond). Dose-response plots of AdoHcy (open diamond) and etoposide (open circle) are also shown. (B) Dose-response plots of DNMT1 activity as a function of DNA substrate concentration in the absence (open circle) or presence of DNMT1 inhibitors (doxorubicin at 0.2 μM, closed circle; AdoHcy at 0.5 μM, closed triangle; fully methylated DNA at 0.02 μM, closed square). (C) Dose-response plots of DNMT1 activity as a function of doxorubicin concentration under conditions where the amount of either DNA (left), DNMT1 (middle), or AdoMet (right) is varied. The standard reaction mixture contains 125 nM DNA, 30 nM DNMT1, and 600 nM AdoMet. Concentrations of each varying factor are shown above the corresponding panel. The inset panel shows the data re-plotted relative to the reaction with no doxorubicin (set at 1).

Fig. 2. Specific cytotoxicity of doxorubicin in the presence of DNMT1. Doxorubicin at 1×10^{-6} M specifically decreases the cell viability of *DNMT1*$^{+/+}$ HCT116 human colon cancer cells (A) (indicated by an arrow), while etoposide shows a simple dose-dependency (B). (C) *DNMT1*$^{-/-}$ HCT116 cells do not demonstrate conditional apoptosis when treated with doxorubicin. (D) Specific cytotoxicity of 1×10^{-6} M doxorubicin is associated with caspase-3/7 activation. (E) Stimulation of caspase-3/7 by etoposide is also shown as a reference. (F) Caspase-3/7 activation in *DNMT1*$^{-/-}$ HCT116 cells treated with various concentrations of doxorubicin is shown.

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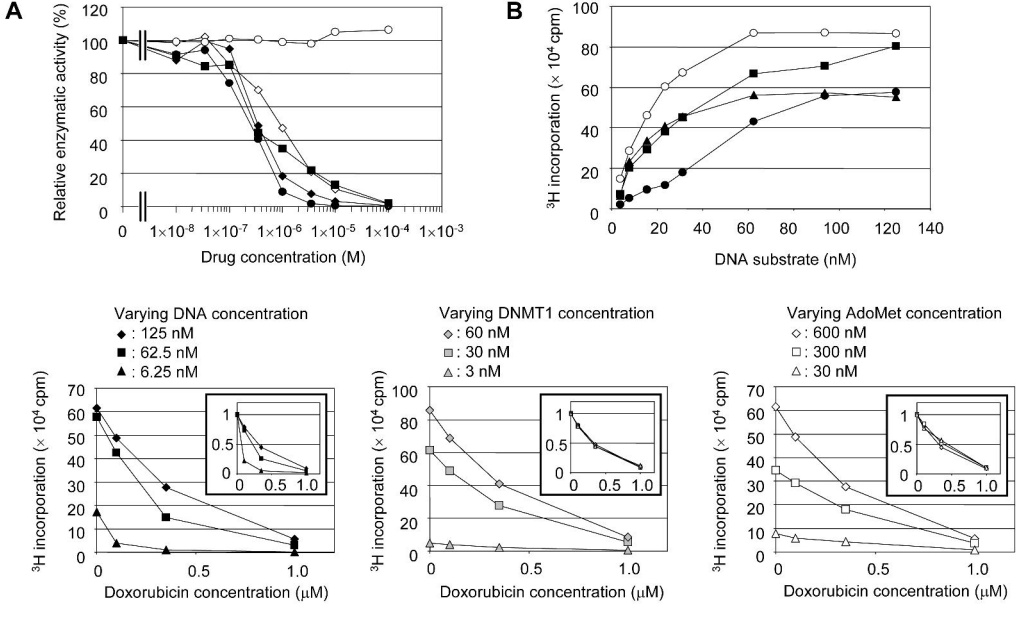
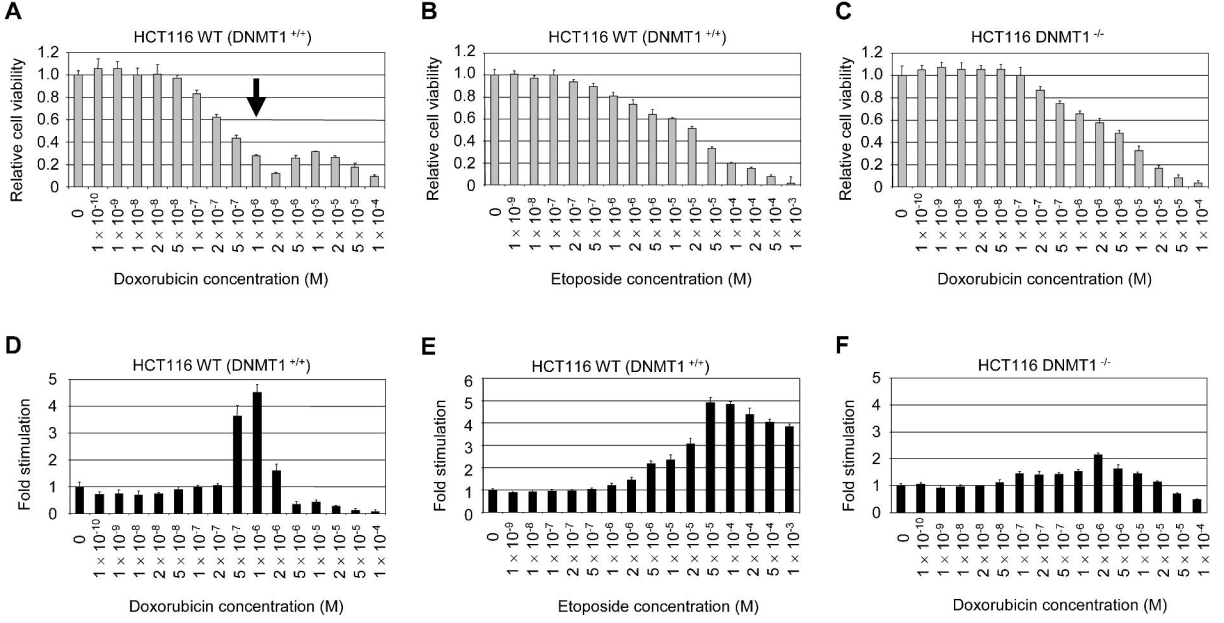
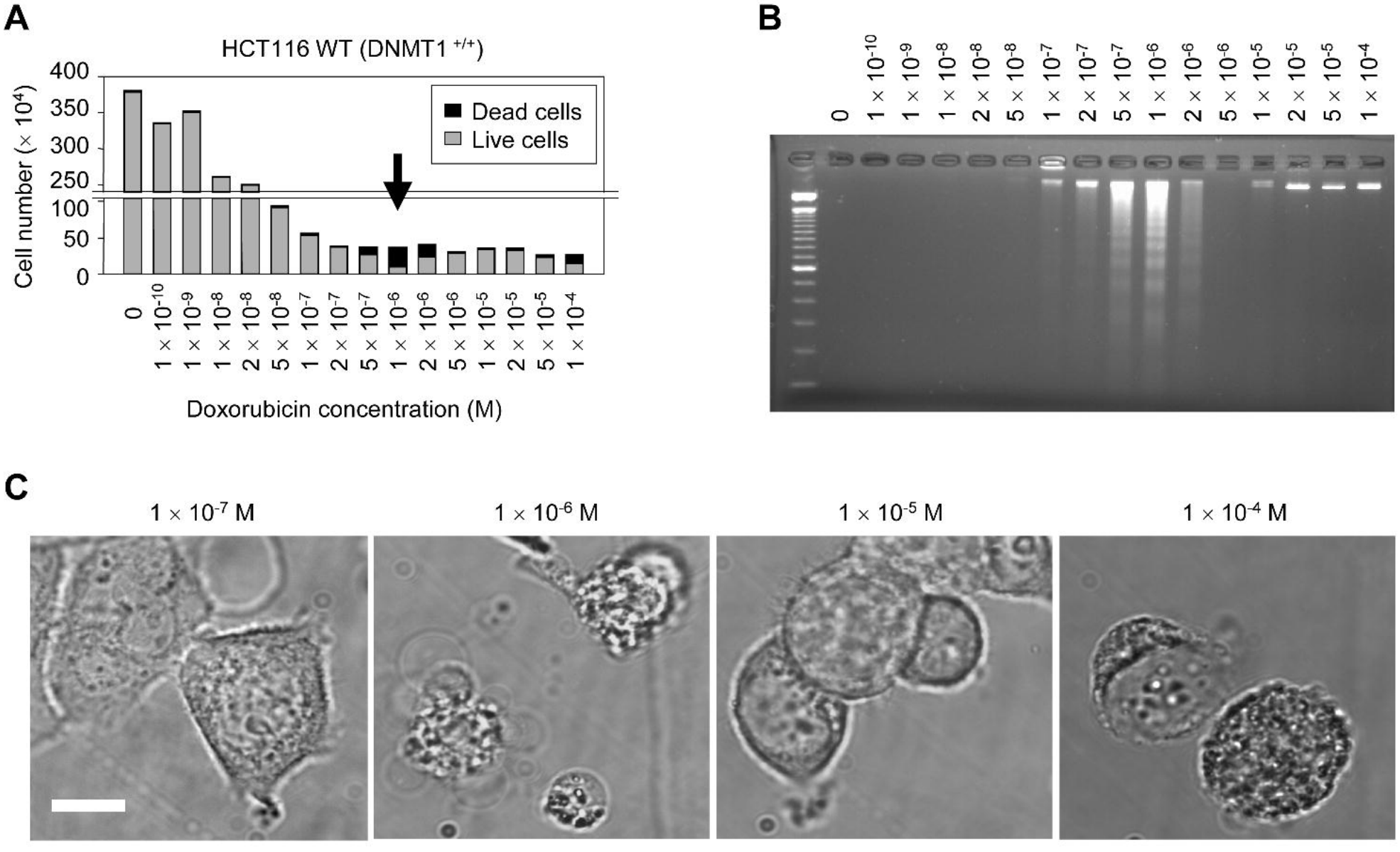
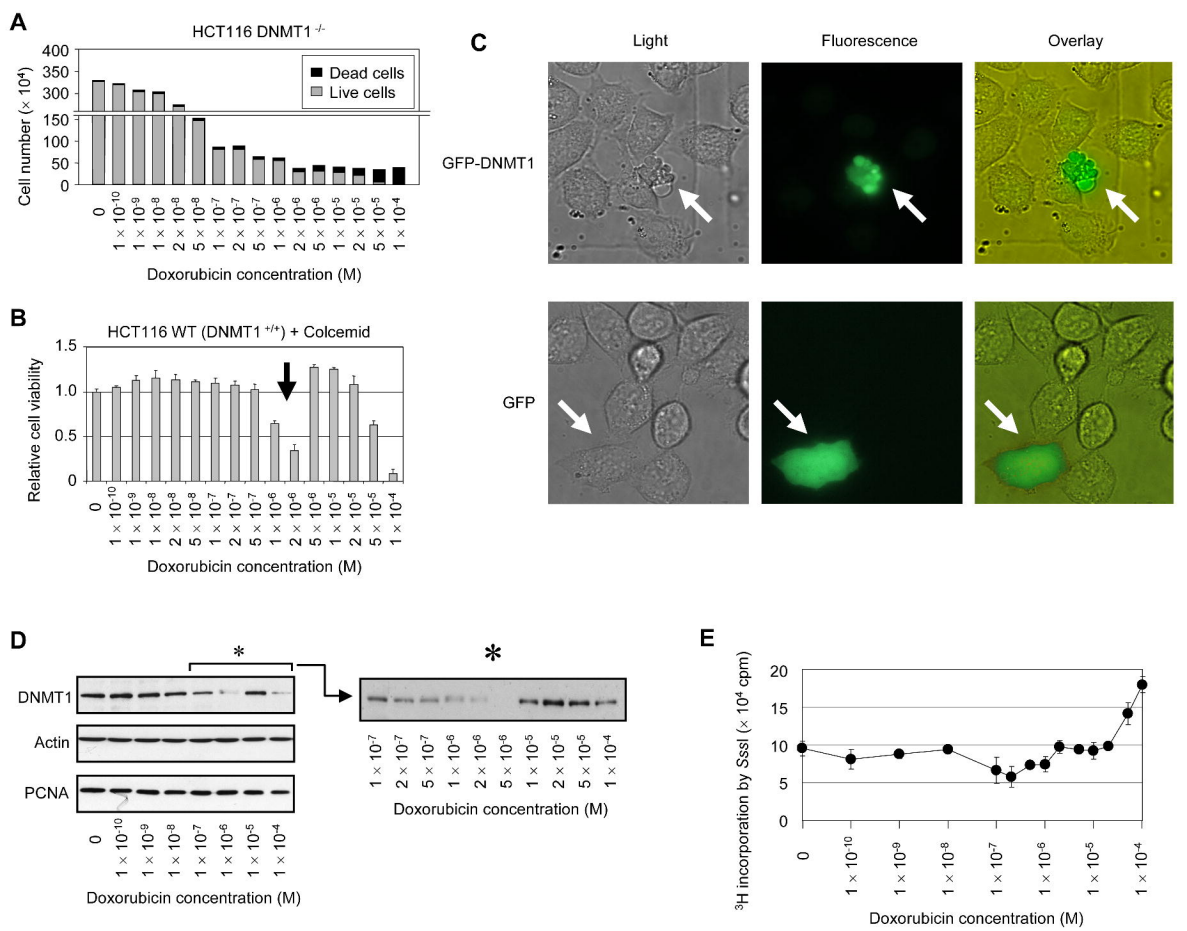
Fig. 3. Specific decrease in cell viability is related to apoptosis. Results of Trypan Blue stain (A) (maximal cell killing indicated by an arrow) and internucleosomal DNA fragmentation (B) are shown. (C) Morphological changes in cells treated with apoptosis-inducible (1×10^{-6} M) and non-inducible (1×10^{-7}, 1×10^{-5}, and 1×10^{-4} M) concentrations of doxorubicin. Bar = 5 microns.

Fig. 4. DNMT1 is associated with conditional apoptosis in HCT116 cells. (A) *DNMT1*$^{-/-}$ HCT116 cells do not demonstrate conditional apoptosis when treated with doxorubicin in a cell number count assay with Trypan Blue. (B) Conditional apoptosis (indicated by an arrow) in cell-cycle arrested cells. *DNMT1*$^{+/+}$ HCT116 cells were treated with 100 nM Colcemid for 24 h prior to doxorubicin addition. Cell viability was measured by MTS assay after an additional 48 h with doxorubicin treatment. (C) Expression of GFP-DNMT1 protein induces conditional apoptosis in doxorubicin treated *DNMT1*$^{-/-}$ HCT116 cells. At 48 h after transfection with GFP-DNMT1 (top) or GFP (bottom), cells were treated with doxorubicin (1×10^{-6} M) for an additional 48 h. (D) Total levels of DNMT1 protein are reduced in *DNMT1*$^{+/+}$ HCT116 cells treated with the apoptosis-inducible concentration (1×10^{-6} M) of doxorubicin. β-Actin and PCNA are loading controls. A more detailed analysis of total DNMT1 levels at the region indicated by an asterisk is shown in the right panel.
(E) Global DNA methylation status in doxorubicin-treated *DNMT1*$^{+/+}$ HCT116 monitored using the methyl acceptance assay. Note that in this assay the degree of tritium incorporation is inversely related to the level of genomic DNA methylation.


A
Relative enzymatic activity (%)
120
100
80
60
40
20
0
0
1×10-8
1×10-7
1×10-6
1×10-5
1×10-4
1×10-3
Drug concentration (M)
B
3H incorporation (× 10⁴ cpm)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
DNA substrate (nM)
C
Varying DNA concentration
◆ : 125 nM
■ : 62.5 nM
▲ : 6.25 nM
Varying DNMT1 concentration
◇ : 60 nM
▒ : 30 nM
△ : 3 nM
Varying AdoMet concentration
◇ : 600 nM
□ : 300 nM
△ : 30 nM
3H incorporation (× 10⁴ cpm)
Doxorubicin concentration (μM)
0
0.5
1.0


A
HCT116 WT (DNMT1 +/+)
Relative cell viability
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1 × 10-10
1 × 10-9
1 × 10-8
2 × 10-8
5 × 10-8
1 × 10-7
2 × 10-7
5 × 10-7
1 × 10-6
2 × 10-6
5 × 10-6
1 × 10-5
2 × 10-5
5 × 10-5
1 × 10-4
Doxorubicin concentration (M)
B
HCT116 WT (DNMT1 +/+)
Relative cell viability
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1 × 10-9
1 × 10-8
1 × 10-7
2 × 10-7
5 × 10-7
1 × 10-6
2 × 10-6
5 × 10-6
1 × 10-5
2 × 10-5
5 × 10-5
1 × 10-4
2 × 10-4
5 × 10-4
1 × 10-3
Etoposide concentration (M)
C
HCT116 DNMT1 -/-
Relative cell viability
1.2
1.0
0.8
0.6
0.4
0.2
0
0
1 × 10-10
1 × 10-9
1 × 10-8
2 × 10-8
5 × 10-8
1 × 10-7
2 × 10-7
5 × 10-7
1 × 10-6
2 × 10-6
5 × 10-6
1 × 10-5
2 × 10-5
5 × 10-5
1 × 10-4
Doxorubicin concentration (M)
D
HCT116 WT (DNMT1 +/+)
Fold stimulation
5
4
3
2
1
0
0
1 × 10-10
1 × 10-9
1 × 10-8
2 × 10-8
5 × 10-8
1 × 10-7
2 × 10-7
5 × 10-7
1 × 10-6
2 × 10-6
5 × 10-6
1 × 10-5
2 × 10-5
5 × 10-5
1 × 10-4
Doxorubicin concentration (M)
E
HCT116 WT (DNMT1 +/+)
Fold stimulation
6
5
4
3
2
1
0
0
1 × 10-9
1 × 10-8
1 × 10-7
2 × 10-7
5 × 10-7
1 × 10-6
2 × 10-6
5 × 10-6
1 × 10-5
2 × 10-5
5 × 10-5
1 × 10-4
2 × 10-4
5 × 10-4
1 × 10-3
Etoposide concentration (M)
F
HCT116 DNMT1 -/-
Fold stimulation
5
4
3
2
1
0
0
1 × 10-10
1 × 10-9
1 × 10-8
2 × 10-8
5 × 10-8
1 × 10-7
2 × 10-7
5 × 10-7
1 × 10-6
2 × 10-6
5 × 10-6
1 × 10-5
2 × 10-5
5 × 10-5
1 × 10-4
Doxorubicin concentration (M)


A
HCT116 WT (DNMT1 +/+)
Cell number (× 10⁴)
400
350
300
250
100
50
0
Dead cells
Live cells
0
1 × 10⁻¹⁰
1 × 10⁻⁹
1 × 10⁻⁸
2 × 10⁻⁸
5 × 10⁻⁸
1 × 10⁻⁷
2 × 10⁻⁷
5 × 10⁻⁷
1 × 10⁻⁶
2 × 10⁻⁶
5 × 10⁻⁶
1 × 10⁻⁵
2 × 10⁻⁵
5 × 10⁻⁵
1 × 10⁻⁴
Doxorubicin concentration (M)
B
0
1 × 10⁻¹⁰
1 × 10⁻⁹
1 × 10⁻⁸
2 × 10⁻⁸
5 × 10⁻⁸
1 × 10⁻⁷
2 × 10⁻⁷
5 × 10⁻⁷
1 × 10⁻⁶
2 × 10⁻⁶
5 × 10⁻⁶
1 × 10⁻⁵
2 × 10⁻⁵
5 × 10⁻⁵
1 × 10⁻⁴
C
1 × 10⁻⁷ M
1 × 10⁻⁶ M
1 × 10⁻⁵ M
1 × 10⁻⁴ M


A
HCT116 DNMT1 -/-
Dead cells
Live cells
Cell number (× 10⁴)
Doxorubicin concentration (M)
B
HCT116 WT (DNMT1 +/+) + Colcemid
Relative cell viability
Doxorubicin concentration (M)
C
Light
Fluorescence
Overlay
GFP-DNMT1
GFP
D
DNMT1
Actin
PCNA
Doxorubicin concentration (M)
E
³H incorporation by SssI (× 10⁴ cpm)
Doxorubicin concentration (M)