Single Chemical Modifications of the C-1027 Enediyne Core, a Radiomimetic Antitumor Drug, Affect Both Drug Potency and the Role of Ataxia-Telangiectasia Mutated in Cellular Responses to DNA Double-Strand Breaks

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Abstract
The radiomimetic enediyne C-1027 induces almost exclusively DNA double-strand breaks (DSB) and is extremely cytotoxic. Unique among radiomimetics, ataxia-telangiectasia mutated (ATM) is dispensable for cellular responses to C-1027-induced DNA damage. This study explores the biological activity of three recently bioengineered C-1027 analogues: 7'-desmethyl-C-1027 (desmethyl), 20'-deschloro-C-1027 (deschlo), and 22'-deshydroxy-C-1027 (deshydroxy). Each compound maintains the characteristic ability of radiomimetics to cleave DNA in cell-free systems, varying in activity from 2-fold (deschlo) to 53-fold (desmethyl) less than C-1027. The induction of cellular DNA breaks based on pulsed field gel electrophoresis, comet analysis, and γH2AX activation was in the same rank order as cell-free DNA break induction, although the amount of breaks induced by desmethyl is greatly reduced compared with the other analogues. Despite the disparity in inducing DNA DSBs, all of the analogues produced G2-M cell cycle arrest and activated DNA DSB damage response proteins, such as p53-Ser15 and Chk2-Thr68, at concentrations in concordance with their ability to inhibit cell growth. Interestingly, of the three analogues, only the desmethyl-induced DNA damage response was similar to C-1027, as it did not cause hypersensitive cell growth inhibition in the absence of ATM nor require the kinase to phosphorylate p53 or Chk2. These findings show that simple modifications of the chromophore of C-1027 can result in varied induction of, and cellular response to, DNA DSBs.

Introduction
Induction of DNA double-strand breaks (DSB) by ionizing radiation (IR) is often used as a treatment option in cancer therapy. In response to DNA DSBs, cells activate DNA damage response pathways to impede progression of the cell cycle, allowing cells additional time to either repair the damaged DNA or activate apoptotic pathways (1–3). Following IR treatment, the histone variant H2AX at Ser139 (commonly known as γH2AX), which recruits DNA DSB repair proteins to the sites of the breaks, is also activated via ATM (6). Cells without ATM function cannot efficiently activate checkpoint responses to IR-induced DNA strand breaks and are hypersensitive to IR treatment (7–9). Moreover, tumor cells are generally more susceptible than normal cells to IR treatment in part because they frequently are defective in their DNA repair and cell cycle checkpoint functions (10).

In addition to IR, DNA DSBs can be induced by radiomimetic enediyynes, which generate free radicals that abstract hydrogen atoms from the backbone of DNA while undergoing a Bergman cycloaromatization reaction (11). There are two major groups of enediynes: the protein chromophore family, which includes neocarzinostatin and C-1027, where the chromophore is surrounded by 100 single-strand breaks (SSB) for every 55-fold (desmethyl) less than C-1027. The induction of cellular DNA breaks based on pulsed field gel electrophoresis, comet analysis, and γH2AX activation was in the same rank order as cell-free DNA break induction, although the amount of breaks induced by desmethyl is greatly reduced compared with the other analogues. Despite the disparity in inducing DNA DSBs, all of the analogues produced G2-M cell cycle arrest and activated DNA DSB damage response proteins, such as p53-Ser15 and Chk2-Thr68, at concentrations in concordance with their ability to inhibit cell growth. Interestingly, of the three analogues, only the desmethyl-induced DNA damage response was similar to C-1027, as it did not cause hypersensitive cell growth inhibition in the absence of ATM nor require the kinase to phosphorylate p53 or Chk2. These findings show that simple modifications of the chromophore of C-1027 can result in varied induction of, and cellular response to, DNA DSBs.

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rational manipulations of the C-1027 biosynthetic machinery in *Streptomyces globisporus* (26). The C-1027 chromophore is derived biosynthetically from four basic biochemical units: a benzoxazolinate, a deoxyamino hexose, a \( \beta \)-amino acid, and an enediyne core (26). Systematical introduction of permutations into the genes that encode the biosynthesis of these units resulted in the isolation of recombinant strains that produce various C-1027 analogues. For example, inactivation of the *sgcC* or the *sgcC3* gene, which encode a hydroxylase and halogenase, respectively, yielded the mutant strains that produce the deshydroxy and deschloro analogues, generating structural variations at the \( \beta \)-amino acid moiety of the C-1027 chromophore (Fig. 1A; refs. 26, 27). Meanwhile, inactivation of *sgcD4*, which encodes a methyltransferase, afforded a mutant strain that produces the desmethyl analogue, exemplifying structural alteration at the benzoxazolinate moiety (Fig. 1A).5 Little is known about the biological activity of the analogues, except that they are active against *Micrococcus luteus* (26).5

In this study, we have systematically evaluated the three novel analogues in comparison with the parent C-1027 for their ability to induce DNA strand breaks into cell-free and cellular DNA, verifying that these compounds maintain this quintessential properties of enediyne (11). Analogue-induced cell growth inhibition and interference with cell cycle progression were also determined along with the ability to activate the DNA damage response proteins γH2AX, Chk2-\( \text{Thr}^{68} \), and p53-\( \text{Ser}^{15} \). Finally, we establish whether DNA damage responses are ATM dependent or independent.

Figure 1. **A**, structures of the C-1027 chromophore and the locations (enclosed rectangles) of the substitutions on the chromophore that produce 7'-desmethyl-C-1027 on the benzoxazolinate moiety and 20'-deschloro-C-1027 and 22'-deshydroxy-C-1027 on the \( \beta \)-amino acid moiety, respectively. **B**, C-1027 analogues cleave cell-free plasmid DNA based on forms conversion analysis. Plasmid DNA was treated with either a standard enediyne (neocarzinostatin (NCS) or C-1027) or one of the analogues for 30 min at 37°C. The DNA was then electrophoresed on a 1% agarose gel and visualized by staining with ethidium bromide to assess conversion of the forms of the plasmid DNA from superhelical form I to circular nicked form II or linear form III DNA. **C**, each enediyne induces conversion of form I to II or III in a concentration-dependent manner. The percentages of the three forms relative to the total DNA are plotted as a function of drug concentration for neocarzinostatin, C-1027, and the three analogues. The DNA was visualized by staining with ethidium bromide and quantitated as described in Materials and Methods.

5 Unpublished data.
Materials and Methods

**Chemicals.** Fermentation, production, isolation, and purification of C-1027, desmethyl, deschloro, and deshydroxy were carried out as described previously (26, 27). The yields for C-1027 from the S. globisorpus wild-type strain, desmethyle analogue from the S. globisorpus SB1009 strain (i.e., AsgC3 mutant), deschloro analogue from the S. globisorpus SB1008 strain (i.e., AsgC2 mutant; ref. 27), and deshydroxy analogue from the S. globisorpus SB1006 strain (i.e., AsgC mutant; ref. 26) were estimated to be 60, 20, 60, and 60 mg/L, respectively. Neocarzinostatin was a gift from Bristol-Myers Squibb Co. (Syracuse, NY). All compounds were stored at 200 μmol/L in double-distilled water at −20°C.

**Cells.** Isogenic ATM-null (AT169A) and ATM-restored (YZ510B) human fibroblast cell lines (gift of Drs. P. Lu and Y. Shihoh) were grown at 37°C and 5% CO2 in DMEM supplemented with 10% fetal bovine serum plus 50 μg/ml/µL hygromycin to maintain selective pressure on the transfected constructs (28). All experiments were done with the YZ510B cell line unless otherwise specified.

**Forms conversion.** pBR322 plasmid DNA (100 ng; New England Biolabs, Ipswich, MA) was incubated with C-1027, desmethyl, deschloro, deshydroxy, or neocarzinostatin in 10 mmol/L Tris (pH 7.5) for 30 min at 37°C. DTT (0.5 mmol/L) was included only in the neocarzinostatin reaction buffer as an activating agent, as we found it was not necessary for C-1027 (29) or for these analogues (data not shown). Samples were electrophoresed on a 1% agarose gel and then the gels were stained with ethidium bromide, photographed using the Gel Doc XR (Bio-Rad Corp., Hercules, CA), and analyzed by ImageQuant software (Molecular Dynamics, Piscataway, NJ).

**Growth inhibition assay.** Cells were seeded at 2.5 × 10⁵ per well in six-well dishes and drug treated 24 h later. Following a 3-day incubation, dishes were washed and cells were counted by Coulter counter (Beckman Coulter, Inc., Fullerton, CA). Cell growth inhibition was calculated by comparing the number of treated to nontreated control cells.

**Pulsed field gel electrophoresis.** Cells were treated with drugs for 2 h, washed, and resuspended in 0.5% low melting temperature agarose, poured into a plug former, and kept at 4°C until solidified. Plugs containing approximately 3 × 10⁷ cells were incubated with 1 mg/ml proteinase K in lysis buffer [10 mmol/L Tris, 2% sodium sarcosinate, 0.5 mmol/L EDTA (pH 8.0)] for 24 h at 50°C. The plugs were loaded into wells of a 0.8% agarose gel. Pulsed field gel electrophoresis was conducted in 0.5 × Tris borate/EDTA for 18 h at 200 V using a ChefDR apparatus (Bio-Rad), and damage was quantitated using ImageQuant software.

** Comet analysis.** Following a 30-min drug treatment, cells were resuspended in PBS at a concentration of 1 × 10⁷/ml and then analyzed by Comet as described elsewhere with minor modifications (30). Briefly, cells were diluted into low melting temperature agarose and placed on an agarose-covered slide for 2 h at 4°C. The slide was then immersed in alkaline lysis buffer overnight at 4°C and then placed in electrophoresis buffer for 20 min before electrophoresis at 27 V for 25 min at 4°C. The slides were then placed in 0.1% sodium citrate, 0.4% NP40, and 0.2 mg/ml RNase A, and analyzed by flow cytometry on a FACScan instrument (Becton Dickinson, San Jose, CA) using WinList software (Verity Software House, Inc., Topsham, ME).

**Results**

**C-1027 analogues cleave cell-free DNA.** An intrinsic property of enediyynes is their ability to cleave DNA in a cell-free system. The C-1027 analogues (Fig. L4) were compared with the parent compound and another protein chromophore enediyne, neocarzinostatin, for their ability to cleave an isolated supercoiled plasmid DNA. In this assay, supercoiled DNA (form I) can be converted to a nicked circular form (form II) by a SSB, whereas a DSB results in a full-length linear duplex (form III) as shown by neocarzinostatin and C-1027 (Fig. 1B). The rapid increase in the appearance of form III DNA after treatment with C-1027, but not neocarzinostatin, indicates that a high percentage of the total C-1027-induced DNA breaks were DSBs (Fig. 1B and C), consistent with the observation that C-1027 induces primarily DSBs whereas neocarzinostatin induces a mixture of SSBs and DSBs (29). Similar to the parent compound, the three analogues also rapidly convert the plasmid into form III DNA, indicating that they also induce a high percentage of DSBs (Fig. 1B and C). With regard to activity, each of the analogues was able to induce cell-free DNA breaks, with a potency of approximately 55X, 2X, and 20X for desmethyl, deschloro, and deshydroxy, respectively, where X is the concentration of C-1027 required to reduce the amount of form I DNA by 50% (Fig. 1C).

At very high drug concentrations that result in extensive DNA cleavage, deschloro and deshydroxy induce mobility retardation in all three forms of the plasmid DNA (Fig. 1B). The levels at which the mobility changes first appear are 600 and 2,000 mmol/L for deschloro and deshydroxy, respectively (Fig. 1B), and the mobility retardation increased in a concentration-dependent manner for those analogues (data not shown). Furthermore, the desmethyl compound also induced mobility retardation in a concentration-dependent manner, starting ~14,000 mmol/L (data not shown). This binding may be related to a reversible DNA interaction with the postactivated chromophore that has previously been described for the parent at very high concentrations (32), but a complete

6We also examined the ability of the analogues to induce abasic sites, which are a common lesion induced by enediyynes, including C-1027 (14). To measure abasic sites, the drug-treated DNA was incubated with 100 mmol/L putrescine for 1 h at 37°C, which results in conversion of abasic sites to DNA strand breaks (31). Similar to the parent compound, putrescine treatment resulted in a 15% to 20% increase in the amount of DSBs induced by each of the analogues, suggesting that they all induce a similar ratio of abasic sites to direct DNA DSBs (data not shown).
work-up of thermodynamic DNA-binding properties would be necessary to determine this conclusively.

**C-1027 analogues inhibit cellular growth.** To determine whether the analogues are active in a cellular environment, we tested their ability to induce cell growth inhibition. Cells were treated with C-1027 analogues, and growth inhibition was measured after 3 days of continuous drug treatment. The IC50s for growth arrest were 24 pmol/L for C-1027, 1,412 pmol/L for desmethyl, 72 pmol/L for deschloro, and 174 pmol/L for deshydroxy, which is approximately 60-, 3-, and 7-fold higher than C-1027, respectively (Fig. 2). The enediyne neocarzinostatin itself, a potent inhibitor of cell growth inhibition, was included in this study and showed that even the weakest C-1027 analogue retains considerable potency in cells.

**C-1027 analogues induce cellular DNA DSBs.** Neutral pulsed field gel electrophoresis, which provides a direct measure of DNA DSBs by fractionating DNA according to molecular weight, was used to test the ability of C-1027 and the analogues to induce cellular DNA breaks. In this assay, undamaged cellular DNA is typically too large to enter the gel and is retained in the wells. However, induction of a small amount of DNA DSBs results in a band of high molecular weight (HMW) material at the top of the gel (Fig. 3A. arrow), although further induction of DSBs reduces this band to a range of smaller and therefore more rapidly migrating pieces of DNA that resolve as a smear below the HMW band. Treatment of cells with the deschloro or deshydroxy analogue for 1 h showed a DNA cleavage pattern comparable with 1 nmol/L C-1027 (Fig. 3A), although as in the cell-free DNA study higher drug concentrations, 8 and 36 times, respectively, were needed. Interestingly, although desmethyl initially resulted in limited amounts of DNA leaving the well, the amount of DNA did not increase significantly with drug concentration nor was DNA migrating below the HMW band (Fig. 3A). Thus, even at the highest concentration (800 nmol/L), desmethyl-induced DSBs were far less than what was observed with 1 nmol/L C-1027 even if normalized for cytotoxicity. To test if the low levels of DNA DSBs were a result of a slower rate of DNA cleavage by the desmethyl analogue, increased treatment times of up to 4 h were examined. However, the proportion of damaged DNA remained similar to that of the parent (data not shown).

C-1027 analogue treatment of cells induces primarily DNA DSBs. C-1027 has a SSB/DSB ratio of approximately 2:1, so neutral pulsed field gel electrophoresis is a suitable measure of total DNA breaks (15). However, because the ratio of SSB/DSB produced by the analogues in cells is unknown, we also determined total DNA breaks (SSB and/or DSBs) based on alkaline comet analysis (Fig. 3B). In the comet assay, the nucleus can be seen as the head of the comet, and on alkaline electrophoresis, DNA that was damaged by either a SSB or DSB migrates out of the nucleus, forming a tail. Smaller, more rapidly migrating DNA fragments move further away from the nucleus than do less damaged fragments so that the amount of DNA damage is proportional to the length of the comet tail. After treatment with C-1027, deschloro, or deshydroxy, the total DNA breaks were consistent with the pulsed field analysis (DSBs alone), as the comet tails formed after an initial limited amount of damage and then the comet tails lengthen in a concentration-dependent manner (Fig. 3). Similarly, treatment with the desmethyl analogue also was consistent with the pulsed field study, as it resulted in an initial limited amount of damage at 50 nmol/L, which did not seem to increase even at 800 nmol/L (Fig. 3B). Thus, the disparity of cellular DNA DSBs induced by

![Figure 2. C-1027 analogues induce cell growth inhibition. After 3 d of treatment with C-1027, an analogue or neocarzinostatin at 37°C, the percentage growth inhibition of YZ510B cells was calculated based on cell number relative to the control at each drug concentration. Insert, IC50 value for each compound examined and their relative potencies compared with C-1027.](Image 314x196 to 536x462)

![Figure 3. A, C-1027 analogues induce cellular DNA DSBs. The ability of C-1027 analogues to cleave cellular DNA was measured by pulsed field gel electrophoresis. DNA DSBs were measured after treatment of YZ510B cells for 1 h at 37°C with either C-1027 or an analogue over a range of concentrations that was expected to induce similar damage profiles. Following pulsed field gel electrophoresis to size fractionate the DNA, the gels were stained with ethidium bromide to visualize the DNA that entered the lane versus total DNA (DNA in the lane + DNA in the well). Arrow, HMW band of DNA entering the gel after drug treatment. B, C-1027 analogues induce a low ratio of SSBs/DSBs in cells. Following C-1027 or analogue treatment under the concentrations used above, total DNA strand breaks were assessed by alkaline comet analysis. After drug treatment, YZ510B cells were harvested and placed on a microscope slide, lysed, and electrophoresed, and after addition of ethidium bromide, DNA comets were detected by fluorescence microscopy.](Image 75x601 to 243x710)
desmethyl is not because it is inducing a higher ratio of SSBs to DSBs in a cellular environment.

C-1027 analogue treatment induces cellular DNA damage responses. Similar to IR or other radiomimetics, treatment of cells with C-1027 has previously shown that H2AX is phosphorylated to γH2AX and recruited to sites of DSBs (33). To test whether treatment of cells with the C-1027 analogues also induces γH2AX, cells were treated with approximately equitoxic concentrations of either C-1027 or an analogue for 30 min and analyzed by Western blotting. At low levels of C-1027 treatment (0.1 nmol/L), γH2AX is detected and increases in a concentration-dependent manner (Fig. 4A). At low, approximately equitoxic levels of deschloro or deshydroxy, γH2AX is activated to a similar extent as the parent compound and the amount of activation increased in a concentration-dependent manner (Fig. 4A).

Treatment with a low equitoxic level of desmethyl showed an initial activation of γH2AX, but the level did not increase with concentration (Fig. 4A). Because γH2AX localization is necessary for recruitment of DNA repair proteins, such as 53BP1, NBS1, and BRCA1, to DNA DSBs, focalization of γH2AX after analogue treatment was also examined (34). To test for γH2AX focalization, cells were treated for 30 min with the lowest and highest concentration of each drug used in Fig. 4A and analyzed by microscopic immunofluorescence. After treatment with low, approximately equitoxic levels of deschloro or deshydroxy, γH2AX is activated to a similar extent as the parent compound and the amount of activation increased in a concentration-dependent manner (Fig. 4A). Treatment with a low equitoxic level of desmethyl showed an initial activation of γH2AX, but the level did not increase with concentration (Fig. 4A).

Because γH2AX localization is necessary for recruitment of DNA repair proteins, such as 53BP1, NBS1, and BRCA1, to DNA DSBs, focalization of γH2AX after analogue treatment was also examined (34). To test for γH2AX focalization, cells were treated for 30 min with the lowest and highest concentration of each drug used in Fig. 4A and analyzed by microscopic immunofluorescence. After treatment with low, approximately equitoxic levels of deschloro or deshydroxy, γH2AX is activated to a similar extent as the parent compound and the amount of activation increased in a concentration-dependent manner (Fig. 4A). Treatment with a low equitoxic level of desmethyl showed an initial activation of γH2AX, but the level did not increase with concentration (Fig. 4A).

C-1027 analogue treatment induces cell cycle checkpoints. Typically, cell cycle checkpoints are activated to impede cell cycle progression following induction of DNA damage. At low concentrations, C-1027, similar to IR and other radiomimetic treatments, induces a strong G2-M arrest 24 h after treatment (Fig. 5A; refs. 3, 21). To test if C-1027 analogues also induce G2-M arrest, cells were treated with equitoxic concentrations of C-1027, desmethyl, deschloro, or deshydroxy. Based on fluorescence-activated cell sorting analysis, the desmethyl and deschloro analogues induce a strong G2-M block. Although deshydroxy also blocks cells at G2-M, a somewhat greater proportion of cells resided in G1 compared with the other compounds (Fig. 5A).
Activation of the G2-M checkpoint in response to DNA DSBs involves the activation of damage response proteins, including phosphorylation of Chk2 at Thr68 (2, 3). At low equitoxic concentrations of drug treatment, modest levels of Chk2-Thr68 phosphorylation were observed for all the compounds (Fig. 5B). The amount of Chk2-Thr68 phosphorylation increased in a concentration-dependent manner even after treatment with the desmethyl analogue, which did not induce γH2AX in a concentration-dependent manner (Figs. 4 and 5B).

Phosphorylation of p53 at Ser15, another downstream DNA damage response protein, is involved in both G1-S and G2-M arrest (3). Phosphorylation of p53-Ser15 was consistent with that of Chk2-Thr68 after treatment with C-1027 or desmethyl, deschloro, and deshydroxy, as low equitoxic concentrations result in modest levels of p53 activation (Fig. 5B). In addition, similar to Chk2-Thr68, increasing drug concentrations of each of the analogues resulted in a concentration-dependent increase of p53-Ser15 phosphorylation (Fig. 5B). These results show that all three C-1027 analogues induce cell cycle arrest at G2-M and robustly activate cell cycle checkpoint proteins (Fig. 5).

DNA DSB damage responses to C-1027 analogue treatment vary in their ATM dependence. Cells typically activate DNA DSB damage response proteins through an ATM-dependent kinase cascade after treatment with IR and radiomimetics, such as calicheamicin and neocarzinostatin (22, 23, 35). However, we have previously shown that, in response to C-1027-induced damage, ATM-null cells can phosphorylate both Chk2-Thr68 and p53-Ser15 as robustly as ATM wild-type cells (24, 25), a result that is recapitulated in Fig. 6A. Similarly, ATM-null cells treated with desmethyl phosphorylate Chk2-Thr68 and p53-Ser15 as readily as ATM wild-type cells (Fig. 6A). In contrast, treatment of ATM-null cells with deschloro resulted in a notable decrease in the activation of both Chk2 and p53 (Fig. 6A) and a very large decrease in the phosphorylation of these proteins after deshydroxy treatment (Fig. 6A).

The relative ratio of Chk2 and p53 phosphorylation between ATM wild-type and ATM-null cells was determined by dividing the phosphorylation level of each protein in the wild-type by the level of the ATM null in response to each drug. The ratio of C-1027 of ~1 is indicative for ATM independence with regard to phosphorylation of both Chk2 and p53 (Fig. 6B). Similarly, desmethyl treatment also activates these DNA DSB damage response proteins independently of ATM as it also resulted in a ratio of ~1 for both proteins (Fig. 6B). In contrast, the relative ratio of neocarzinostatin (~2) was determined to use as a reference point for a typical ATM-dependent damage response (Fig. 6B). Deschloro treatment also resulted in a ratio of ~2, establishing that, like neocarzinostatin, this analogue induces ATM-dependent damage responses (Fig. 6B). Furthermore, in response to treatment with deshydroxy, a ratio of 4 was determined for both proteins, suggesting that this agent induces more extreme ATM-dependent damage responses in comparison with other radiomimetics (Fig. 6B).

Because cells lacking ATM are unable to completely induce DNA damage responses to DNA DSBs induced by IR and radiomimetics (except C-1027), they are more susceptible to growth inhibition or cell death (4). We therefore tested whether hypersensitivity to growth inhibition would vary according to the ATM dependence of checkpoint activation. The IC50 for growth inhibition of ATM wild-type and ATM-null cells were measured after treatment with C-1027 analogues. The IC50 for the ATM wild-type was divided by the IC50 for ATM-null cells to determine a hypersensitivity ratio. The ratio of C-1027 of ~1 is indicative for ATM independence with regard to cell growth inhibition (Fig. 6C). Similar to C-1027, the desmethyl analogue also has a hypersensitivity ratio of ~1 (Fig. 6C). Ratios >1, such as that induced by neocarzinostatin (~2.4), are typical of DNA damage responses that are dependent on ATM. Treatment with either deschloro or deshydroxy resulted in cellular hypersensitivity with regard to cell growth inhibition with ratios of 2.2 and 3.6, respectively (Fig. 6C). Thus, the growth inhibition data are consistent with the ATM-dependent activation of DNA damage response proteins Chk2 and p53. Therefore, both deschloro and deshydroxy act like IR and other radiomimetics in that they induce a classic ATM-dependent damage response, whereas desmethyl, like C-1027, induces an ATM-independent damage response to DNA DSBs (Fig. 6).
Discussion

This study examined the DNA cleavage activity of, and cellular DNA damage responses to, three biosynthetic analogues of the enediyne C-1027, a potent inducer of DNA DSBs (14). Although the biological activity of truncated versions of the enediynes calicheamicin and esperamicin has previously been characterized (36, 37), this is the first study to examine single substitutions on an enediyne chromophore. All of the analogues maintain the ability to cleave cell-free DNA as measured by forms conversion analysis of drug-treated superhelical plasmid DNA (Fig. 1B). C-1027 was the most potent followed by deschloro, deshydroxy, and, finally, desmethyl, with cleavage activity varying by up to 55-fold. Furthermore, the DNA cleavage activities compare favorably with the truncated calicheamicin and esperamicin analogues, which were 10- to 1,000-fold weaker than their respective parent compounds (36, 37).

Substitutions on the C-1027 chromophore may alter the location of the biradical within the DNA minor groove, which previous studies have shown can change not only the potency of a compound but also the ratio of SSBs/DSBs induced (32, 38). The proportion of SSBs to DSBs induced by each enediyne are related to the efficiency of the two radicals in abstracting hydrogen from DNA. For example, the biradicals of enediynes that induce a high proportion of DSBs, such as C-1027 and calicheamicin, are positioned in a conformation favorable for each radical to abstract hydrogen from the DNA backbone (39). In contrast, neocarzinostatin induces primarily SSBs because one radical is positioned such that abstraction of hydrogen from other sources, such as water, is more favorable than from the DNA sugar (40, 41). Similar to the parent compound, all three analogues maintain a low ratio of cell-free DNA SSBs/DSBs because form III DNA is readily observable in drug-treated lanes, whereas an abundant amount of form I DNA still remains, implying that the chromophore substitutions induce little, if any, changes in the positioning of the biradical (Fig. 1C).

Neutral pulsed field gel analysis of DNA isolated from drug-treated cells provides direct evidence that all of the analogues were also capable of inducing DNA DSBs in cells. Based on the appearance of the HMW band of DNA (Fig. 3A, arrow), which appears when sufficient DNA DSBs are induced to allow the DNA to enter the gel, the rank order of activity was consistent with the cell-free experiments. Similar to other radiomimetics, increasing levels of deschloro and deshydroxy treatment continued to reduce DNA size, resulting in a range of lower molecular weight material that appears as a smear below the HMW band within each lane (Fig. 3A; ref. 29). Because the cellular DNA strand scission activity of these two analogues reflects their strand scission activity under cell-free conditions, the uptake and stability of these compounds are likely similar in cells.

However, unlike C-1027 and the other analogues, increasing concentrations of desmethyl did not result in further degradation of the HMW DNA band (Fig. 3A). In fact, even at longer treatment times, or levels >100-fold higher (2,000 nmol/L) than those expected to result in sufficient DSBs to reduce the HMW band, we found no evidence of further DNA damage (data not shown). Consistent with the pulsed field measurements, all of the analogues induced detectable γH2AX phosphorylation and focalization at concentrations even lower than those at which the HMW band was first detected (Figs. 3A and 4). Similarly, increasing concentrations of C-1027, as well as deschloro and deshydroxy, showed a corresponding increase in the amount of γH2AX phosphorylation and focalization; however, desmethyl did not (Figs. 3A and 4). DNA DSBs induced by IR and radiomimetics increase linearly with drug concentration, so the finding that desmethyl-induced DNA DSBs do not increase with drug concentration is atypical (29).

Analysis of cellular DNA breaks by alkaline comet, which detects total DNA strand breaks (DSBs + SSBs), was found to be consistent with the neutral pulsed field gel analysis and γH2AX data (Fig. 3B). Therefore, the drugs all induce primarily DSBs under both cell-free and cellular conditions (Figs. 1C and 3). This is consistent with another enediyne, calicheamicin, which also produces primarily DSBs under cellular conditions, mimicking what is observed in cell-free systems (17). Thus, the unusual mechanism of action of the desmethyl compound is likely not explained by it producing a greater amount of SSBs in cells.

The cytotoxicity of the analogues was C-1027>deschloro>deshydroxy>desmethyl, which is in the same rank order as their ability to induce DNA DSBs (Fig. 2). This is unsurprising, as, regardless of differences in the DNA damage induced by treatment with IR or a radiomimetic, such as C-1027, calicheamicin, neocarzinostatin, and macromomycin/auromomycin, it is the induction of DSBs that best correlates with the induction of cytotoxicity (17–19, 29). However, the relationship between desmethyl-induced DSBs and cytotoxicity is more complex, as, even when normalized for cytotoxicity, desmethyl failed to induce a similar level of DNA DSBs in comparison with the other analogues (Fig. 3A). Moreover, treatment of cells with increasing amounts of desmethyl resulted in an increase in cellular cytotoxicity (Fig. 2), yet the amount of DSBs induced did not increase accordingly (Fig. 3A). Nevertheless, the desmethyl compound is still extremely potent at inducing growth inhibition, as its IC50 was an order of magnitude lower than neocarzinostatin (Fig. 2). Furthermore, desmethyl, like the other analogues, induces a G2/M cell cycle block at low equitoxic concentrations7 (Fig. 5A) despite its limited ability to induce cellular DNA DSBs. Therefore, it is possible that the cellular cytotoxicity and cell cycle block induced by desmethyl treatment is not limited to DSB induction but could involve other mechanisms. For example, some enediynes can also cleave RNA, albeit at concentrations well above those needed to cleave DNA (42). Thus, perhaps certain modifications on the benzoxazolinone moiety (desmethyl analogue) of the C-1027 chromophore increase its affinity for non-DNA targets.

Consistent with the activation of a G2-M cell cycle block, it was not surprising that each analogue induces Chk2-Thr68phosphorylation and focalization; however, desmethyl did not (Figs. 3A and 4). DNA DSBs induced by IR and radiomimetics increase linearly with drug concentration, so the finding that desmethyl-induced DNA DSBs do not increase with drug concentration is atypical (29).

Distinctly from C-1027 and from the other analogues, a notable G1 population remained after deshydroxy treatment (Fig. 5A). Perhaps deshydroxy induces both G1/S and G2/M arrest at approximately equal concentration of treatment as opposed to the other analogues, which require 3- to 5-fold higher concentrations to activate the G1/S checkpoint (data not shown; ref. 22).

7 Distinctly from C-1027 and from the other analogues, a notable G1 population remained after deshydroxy treatment (Fig. 5A). Perhaps deshydroxy induces both G1/S and G2/M arrest at approximately equal concentration of treatment as opposed to the other analogues, which require 3- to 5-fold higher concentrations to activate the G1/S checkpoint (data not shown; ref. 22).
was activated in a concentration-dependent manner after desmethyl treatment (Figs. 4A and 5B). This is consistent with recent studies that suggest that Chk2 can be activated in response to cellular cytotoxicity, not just by DNA DSBs (8, 45, 46). Moreover, for desmethyl, the observation that Chk2 and p53 are activated and cell growth inhibition is induced in a concentration-dependent manner without a correlating increase of DNA DSBs is further evidence that the DNA damage induced by desmethyl is likely not limited to induction of DSBs.

The role of the phosphatidylinositol 3-kinase–like protein kinase (PIKK) family member ATM in activating other DNA damage response proteins and avoiding cellular hypersensitivity to IR- or drug-induced DNA DSBs is well established (8, 24, 47). The notable exception is that, in response to C-1027-induced DNA damage, cells activate Chk2-Thr68 and p53-Ser15 similarly regardless of ATM status, and cells lacking the kinase are not hypersensitive to C-1027 treatment with regard to cell growth inhibition or cell death (24, 25). Surprisingly, descloro and deshydroxy, which generate comparable amounts of DNA DSBs with C-1027 when normalized for cytotoxicity, induce ATM-dependent damage responses, behaving similar to IR and other radiotherapists, such as necrozominatin (Fig. 6). In contrast, desmethyl induces ATM-independent DNA damage responses, yet it induced very low levels of DNA DSBs (Fig. 6). Thus, it is not yet known how both C-1027, which is the most potent DNA DSB inducer of the C-1027 family, and desmethyl, which is the weakest, activate ATM-independent damage responses, whereas the other analogues do not. Possibly, additional types of DNA damage could trigger activation of DNA damage response proteins in the absence of ATM. For example, under anaerobic conditions in cell-free systems, C-1027 has been shown to induce DNA intranest cross-links (ISC), whereas other enediyynes (necrozominatin and calicheamicin) do not (41). The ability of C-1027 to induce cellular DNA ISCs has not yet been studied. However, cells use another PIKK family member, the ATM- and Rad3-related (ATR) kinase, to induce damage responses to other agents that induce ISCs (48). Thus, the observation that cells can use ATR to activate Chk2 and p53 while avoiding hypersensitivity in response to C-1027 treatment is consistent with the idea that this radiomimetic induces cellular DNA ISCs in addition to DNA DSBs (25).

Furthermore, if desmethyl treatment results in cellular DNA ISCs, it would also help explain the ability of the analogues to induce DNA damage response protein activation and cell growth inhibition in a concentration-dependent manner (Figs. 2 and 5B) without inducing a correlating increase in the amount of DNA DSBs (Figs. 3A and 4). Whether DNA ISCs are induced in cells by C-1027 and possibly by the other analogues is under study, as well as the relative importance of cellular DNA ISCs versus DSBs with regard to their reliance on ATM and ATR to activate DNA damage responses. Selective modifications of the C-1027 chromophore will help define the structure-activity relationship of the ATM-dependent and ATM-independent DNA damage response pathways.

Overall, the current work clearly showed that structural permutations on the C-1027 chromophore have a profound effect on the mode of action of the resultant C-1027 chromoprotein complex. It further suggests that the location, not just the presence, of a single modification to the C-1027 chromophore is important in determining not only the ability of the drug to induce DNA DSBs but also the ATM dependency of the cellular DNA damage response. Experiments are in progress to produce additional C-1027 analogues to further decipher the mechanism(s) of action of the C-1027 family of antitumor antibiotics and their ability to invoke cellular DNA damage responses.

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References


Responses to C-1027 Analogue-Induced DNA Damage