C-1027, A Radiomimetic Enediyne Anticancer Drug, Preferentially Targets Hypoxic Cells

Terry A. Beerman,1 Loretta S. Gawron,1 Seulkih Shin,1 Ben Shen,2 and Mary M. McHugh1

1Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY; and 2Division of Pharmaceutical Sciences, University of Wisconsin National Cooperative Drug Discovery Group, and Department of Chemistry, University of Wisconsin, Madison, Wisconsin

Abstract
The hypoxic nature of cells within solid tumors limits the efficacy of anticancer therapies such as ionizing radiation and conventional radiomimetics because their mechanisms require oxygen to induce lethal DNA breaks. For example, the conventional radiomimetic enediyne neocarzinostatin is 4-fold less cytotoxic to cells maintained in low oxygen (hypoxic) compared with normoxic conditions. By contrast, the enediyne C-1027 was nearly 3-fold more cytotoxic to hypoxic than to normoxic cells. Like other radiomimetics, C-1027 induced DNA breaks to a lesser extent in cell-free, or cellular hypoxic, compared with normoxic environments. However, the unique DNA interstrand cross-linking ability of C-1027 was markedly enhanced under the hypoxic conditions that reduced its DNA break induction. Although the unique chemistry of C-1027 allows it to concurrently generate both DNA breaks and cross-links in normoxic cells, a low oxygen environment represses the former and promotes the latter. Thus, treatment with C-1027 offers a facile approach for overcoming the radioresistance associated with poorly oxygenated cells.

Introduction
It has long been recognized that hypoxic cells associated with tumor microenvironments, as well as cells grown under conditions of low oxygen, are more difficult to kill with ionizing radiation (IR), which uses oxygen for its DNA strand scission activity (1). Essentially, the deoxyribose radicals generated by radiation abstraction of hydrogen from DNA require sufficient oxygen levels to allow their conversion along a pathway leading to DNA breaks (2, 3). Radiomimetic enediyne anticancer drugs reportedly suffer the same limitation, as break induction occurs via a similar oxygen-mediated mechanism (4, 5). However, recent findings identified a family of designer enediynes, which can induce an additional type of DNA damage, interstrand cross-links (ICLs). Because oxygen is not required for ICL formation, these enediynes may be exploited for targeting hypoxic cells (6, 7).

Although IR induces a plethora of DNA damage events in addition to double (DSB) and single-strand breaks (SSB), including a variety of DNA degradation products, enediynes generally induce only a subset of these lesions (frank DNA strand breaks; refs. 8–10). The mechanism of action of enediyne DNA break induction was identified primarily from studies with neocarzinostatin (NCS), a holo-form drug, consisting of an apoprotein carrier and an active chromophore, and was assumed to be representative of all agents in this class (11). The NCS chromophore contains a bicyclic enediyne that damages DNA via a Myers-Saito cycloaromatization reaction, resulting in a 2,6-indacene diradical structure capable of hydrogen abstractions from deoxyribose (12, 13). Subsequent to generation of a sugar radical, reaction with oxygen quickly and efficiently leads to formation of hydroxyl radicals that induce DNA-damaging activity in cells: an ability to induce concurrently DSBs/SSBs at a 1:5 ratio. The more recently discovered holo-form enediyne C-1027 (Fig. 1) induces predominately DSBs whereas ICLs owing to the efficiency of hydrogen abstraction by its 1,4-benzenoid diradical that results from a similar Bergman cycloaromatization reaction (14, 15). Although DSBs induced by either NCS or C-1027 are created with a two base pair stagger, variations in alignment of the diradical element results in different spectrums of preferential cleavage sites (16–18).

In addition to DSBs, a little known aspect of certain enediynes is that they can form multifunctional and bifunctional adducts with cell-free oligonucleotides under anoxic conditions (19, 20). Although the mechanism of adduct formation remains unclear, it is thought that the deoxyribose radical cannot convert to a hydroxyl radical in the absence of oxygen. Instead, it binds back to the drug, forming a DNA adduct. That adduct formation under anoxic conditions is limited to certain enediynes was indicated by the fact that C-1027 can induce ICLs, whereas calicheamycin, which also induces primarily DSBs, produced no detectable adducts (19).

In cells, IR and other radiomimetic agents induce cells to respond to DNA DSBs by activation, via the phosphatidylinositol 3-kinase–like kinase ATM, of cell cycle checkpoint proteins that delay cell cycle progression to allow DNA repair and preserve chromosomal integrity (21). However, recent studies with C-1027 and selected analogues revealed that robust activation of cell cycle checkpoints and cell death hypersensitivity was independent of ATM or ATR status (6, 7, 22). In addition to ATM phosphorylation, these enediynes were able to induce activation of the rad3 related kinase, ATR generally associated with DNA damage that leads to replication fork stalling (e.g., ICLs; ref. 23).

This sole exception to ATM-dependent responses to DNA DSBs was explained by the discovery that C-1027 exhibits a novel DNA-damaging activity in cells: an ability to induce concurrently DSBs and ICLs in normoxic cells (24). Cellular ICLs induction by C-1027 was unexpected because deoxyribose radicals should be readily converted to hydroxyl radicals in normoxic cells (25). The presence of both types of cellular DNA lesions explains why either ATM or ATR can regulate DNA damage responses (24).

To date, C-1027 is the only drug known to generate DNA damage that is balanced between DNA breaks and ICLs in a normoxic cellular environment. In a hypoxic environment, C-1027 might be expected to induce fewer DSBs, whereas ICL formation, which
typically is impeded by oxygen, should be robust. This study evaluated the oxygen dependence of C-1027 induction of DNA breaks and cross-links and whether C-1027 would overcome the radioresistance associated with hypoxic cells.

**Materials and Methods**

**Chemicals.** Fermentation, production, isolation, and purification of C-1027 from Streptomyces globisporus were carried out as described (26). NCS was a gift from Bristol-Myers Squibb Co. Stock solutions of all compounds were stored at −20°C and diluted in double-distilled water before use.

**Drug preparations.** For dilutions under hypoxic conditions, aliquots of drugs were placed in a BioSpherix chamber (BioSpherix, Ltd.) pre-equilibrated to gas phase O2 at 0.5% unless indicated otherwise, and diluted immediately before use with water pre-equilibrated in the chamber overnight. Separate dilutions for normoxic samples were prepared at the same time.

**Hypoxic conditions for cell-free assays.** Tubes containing DNA and 1 mmol/L Tris (pH 7.5) were placed in a hypoxia chamber equilibrated to gas phase O2 at 0.5% unless otherwise indicated.

**Cell-free DSB and ICL detection.** For DNA break detection, 100 ng of supercoiled pBR322 plasmid DNA (New England Biolabs) was incubated with drug in the presence of 1 mmol/L Tris (pH 7.5) at 37°C under hypoxic (0.5% O2) or normoxic conditions. After incubation for 30 min, tubes were removed from the chamber and loading dye was added. The DNA was electrophoresed on a 1% agarose gel to separate forms, visualized by staining with SYBR Gold (Invitrogen Corp.), imaged on the GelDoc XR (Bio-Rad Corp.), and quantified using Molecular Dynamics ImageQuant software (GE Healthcare).

For ICL detection, EcoRI restriction enzyme (Fermentas, Inc.) was used to linearize pBR322 DNA. DNA (200 ng) and drug were incubated with 1 mmol/L Tris (pH 7.5) at 37°C under hypoxic conditions for the appropriate time. After incubation, denaturation buffer was added to achieve a final concentration of 60 mmol/L NaOH, 10 mmol/L NaCl, and 0.1 mmol/L EDTA under normoxic conditions for 15 min before the addition of loading dye and electrophoresis of DNA as described above.

**Cells.** HCT116 human colon carcinoma cells (a gift from Dr. B. Vogelstein, Sidney Kimmel Comprehensive Cancer Center, John Hopkins University, Baltimore, MD) were grown in McCoy's 5A, 10% fetal bovine serum and Pen/strep for 24 h in 6-well dishes, 3 × 10⁵ cells per well, at 37°C and 5% CO₂.

**Hypoxic conditions for cell culture.** Fifty milliliters of medium in an uncapped sterile bottle covered with aluminum foil were placed on a stir plate and stirred gently in the hypoxia chamber that had been pre-equilibrated to 0.5% O2 and 5% CO2 unless otherwise indicated. After 1 h, HCT116 cells grown under normoxic conditions were placed in the hypoxia chamber, and the cell medium was replaced with 2 ml of the pre-equilibrated hypoxic medium. Cells were covered with aluminum foil and incubated on a dry block at 37°C. After 2 h,³ enediyne dilutions as described above in hypoxic water were added, and incubation continued for 1 h. Normoxic samples were treated similarly, except they were left in a standard CO2 incubator at 37°C for an identical time period and treated with enediyne dilutions in normoxic water.

**IR treatment of cells.** After drug treatment, medium was removed and cells were immediately irradiated on ice under normoxic conditions using a Phillips RT 250 Orthovoltage X-ray Unit (GE Healthcare) with a 0.5-mm Cu filter at 20 Gy, and harvested.

**Comet analysis.** After enediyne incubations with or without IR treatment, HCT116 cells were analyzed as described previously (7). Briefly, cells were harvested by Trypsin/EDTA detachment, resuspended in PBS at a concentration of 1 × 10⁵/ml, diluted into low-melting-temperature agarose, and placed on an agarose-coated slide. The slides were left on ice for 30 min, then immersed in alkaline lysis buffer (pH 13) and placed at 4°C overnight. Slides were equilibrated for 20 min in alkaline electrophoresis buffer before electrophoresis at 27V for 25 min at 4°C. Subsequently, slides were neutralized by washing in 0.4 mol/L Tris (pH 7.5), thrice for 5 min, and dried after immersion first in methanol and then in ethanol. Cells were visualized with ethidium bromide and imaged. Because the smaller the DNA fragment the more rapid its migration through agarose, the intensity and length of the DNA tail (Comet) extruding from the nucleus was proportional to the level of DNA breaks. Comet signals were quantified by tail intensity expressed as a percentage of the total intensity of the comet using Comet Assay IV software (Perceptive Instruments). Typically, 5 fields were imaged for each treatment condition from which 50 to 60 comets were selected to ensure a representative analysis.

**Immunoblotting.** After drug treatment, total cellular extracts were prepared for Western analysis as described previously (6). Essentially, cell lysates were cleared by centrifugation and equal amounts of protein were electrophoresed on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with the primary antibody Anti–phospho-Histone γH2AX (Ser 139; Millipore) followed by a secondary antibody conjugated with horseradish peroxidase (Sigma). Chemiluminescent protein bands were visualized on X-ray film and quantified using the Gel Doc XR System and ImageQuant software.

**Cytotoxicity.** HCT116 cells were plated in 6-well dishes at 5 × 10⁴ cells per well and incubated overnight. Cells were rendered hypoxic (as described above), or left normoxic, and enediyne was added. After 1 h, cells were detached using trypsin, counted, diluted, and replated in duplicate at 1,000, 300, or 100 cells per well. After 10 d of growth in a CO2 incubator under normoxic conditions, cells were stained and fixed in Methylene Blue/ methanol. Individual colonies of 50 or more cells were counted, and plating efficiency was calculated relative to that of untreated (control) cells. Evaluation of drug cytotoxicity was based on three or more experiments.

**Results**

**Oxygen dependence of C-1027–induced DNA damage under cell-free conditions.** The discovery of the ability of C-1027 to induce ICLs was initially observed by treatment of an oligonucleotide under cell-free anoxic conditions that suppressed DSBs formation (20). To establish whether suppression of DNA breaks occurs under hypoxia, a less stringent oxygen deprivation condition, plasmid DNA was equilibrated to 0.5% gas phase O2 before addition of C-1027. Gel electrophoretic analysis identified

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³ Based on optimal suppression of enediyne-induced cellular DNA breaks, it was determined that 2 h was the minimum time necessary for the cells to achieve a hypoxic state.
DNA breaks based on topological forms conversion of the plasmid. Figure 2A reveals a significant decrease in C-1027 strand scission activity in hypoxic compared with normoxic samples. For example, at 250 nmol/L C-1027, the amount of uncut supercoiled plasmid DNA in the hypoxic sample is 2.2-fold greater than the amount in the normoxic sample. A similar strategy was applied to test damage induction at different O_2 levels. At 3% O_2, full cleavage activity was obtained, whereas concentrations below 0.5% O_2 (e.g., < 0.2%), did not reduce DNA breakage below that observed with 0.5% (data not shown).

Recently, cross-linking of plasmid DNA under anoxic conditions was revealed by showing that C-1027–treated DNA was resistant to strand separation under alkaline conditions (24). Using a similar approach, cross-link formation was measured under hypoxic conditions that repress breaks (0.5% O_2). The electrophoretic gel analysis in Fig. 2B shows C-1027's concentration-dependent induction of cross-links after a 1-hour incubation, based on the amount of linearized plasmid DNA that resisted alkaline induced strand separation. At the highest drug level tested, 150 nmol/L C-1027 for the indicated times at 37°C, followed by alkaline denaturation and electrophoresis as described in B.

Enediyne induction of cellular H2AX signaling under hypoxic conditions. Unlike a cell-free environment in which DNA strand scission and cross-linking activity predominate under normoxic and anoxic/hypoxic conditions, respectively, in cells, C-1027 has a unique ability to induce DSBs and ICLs concurrently (24). How formation of these lesions is influenced by a hypoxic cellular environment was investigated. Based primarily on cell-free studies with IR and radiomimetics, DSB activity should decrease in hypoxic cells because the oxygen required to trap deoxyribose radicals before they can be rendered inactive by interactions with cellular thiols like glutathiones is limiting (1, 19, 25). To test this prediction, cells were equilibrated to 0.5% gas phase O_2, C-1027 treated, and assayed by Western analysis for induction of phosphorylated H2AX, a surrogate marker for DSBs (26). Figure 3A is a representative Western blot that reveals a significant decrease in phosphorylated H2AX signaling, and, hence, DSBs, in hypoxic versus normoxic cells treated with 0.5 to 4.5 nmol/L C-1027. The
The oxygen sensitivity of DSB repression in cells is not unexpected and was similar to that observed with other enediynes, including NCS and calicheamicin (Fig. 3B).

Oxygen dependence of C-1027 induction of cellular DNA breaks. DNA damage response pathways including repair proteins are affected by hypoxia and could influence H2AX signaling (27, 28). The influence of oxygen on C-1027 break activity also was evaluated using a more direct assessment of DNA breaks. Alkaline Comet electrophoretic analysis of single cells can be used to show the extent of DNA breaks, which are proportional to the length of the DNA tail extruding from the nucleus (29). Figure 4A reveals a significant reduction in the Comet signals of cells treated under hypoxic versus normoxic conditions. Quantitation of the extent of DNA break repression as a function of oxygen is in general agreement with that identified by Western analysis. Like the cell-free experiments, significant repression and full restoration of cellular DNA damage induction were observed at 0.5% O2 and 3% O2, respectively (Fig. 4B).

C-1027 induction of cellular ICLs under hypoxic conditions. DSB suppression under low-oxygen conditions suggests that deoxyribose radicals are not efficiently converted to hydroxyl radicals/DNA breaks. If so, the formation of ICLs rather than DSBs may be expected under hypoxic but not normoxic conditions. In a prior study, Comet analysis was used to detect not only C-1027 induced DNA breaks but also cross-links (24). In those experiments, cells were treated sequentially with C-1027 and IR. If C-1027 caused only DSBs, the Comet score resulting from the combination of C-1027 and IR treatment should equal the sum of the scores obtained with each agent individually. If C-1027 induced not only strand breaks but also ICLs, the Comet signal would be noticeably less than the sum of the individual scores because cross-links would render IR-fragmented DNA resistant to alkaline-induced DNA strand separation. For example, when cells were treated with the non cross-linking member of the C-1027 family, deschloro-C-1027, followed by IR, a significant increase in the IR-induced Comet signal was observed due to the DNA breaks induced by 5nmol/L deschloro-C-1027 (e.g., 148% of IR alone). However, treatment with C-1027 produced a modest decrease in the expected C-1027 plus IR Comet signal (24).

To test whether hypoxic conditions alter C-1027 cross-linking potential, cells equilibrated with 0.5% gas phase O2 were treated with C-1027 for 1 h, removed from the chamber, and irradiated before assessing DNA breaks via Comet. Figure 5A shows representative Comet images that reveal the ability of C-1027 to reduce the size of IR-generated Comets. A smaller C-1027–induced Comet was observed under hypoxic compared with normoxic conditions. Also, irradiated cells pretreated with C-1027 formed Comets that were dramatically reduced in size. Based on measurements of the Comet tail moment, increasing concentrations of C-1027 resulted in correspondingly more severe reductions in the combined drug plus IR Comet signal consistent with substantial increases in ICLs (Fig. 5B). Notably, the higher concentrations of C-1027, (i.e., 5.0 and 10 nmol/L) dramatically reduced the combined drug plus IR Comet to 10% or less of the IR alone value (Fig. 5C). In striking contrast, in a previous study under normoxic conditions, C-1027 plus IR Comets were 90% the intensity of those induced by IR alone (24). For comparison, NCS
(as well as calicheamicin; data not shown), which lacks an ability to cross-link, produced a corresponding increase in the drug plus IR signal to 160% of the IR alone signal under hypoxic conditions (Fig. 5C).

**Cytotoxicity of enediyynes under normoxic and hypoxic conditions.** Treatment with IR or radiomimetics such as esperamicin or NCS results in diminished cytotoxicity in hypoxic compared with normoxic cells, presumably due to reduced DSBs formation (1, 30). To corroborate these findings, we treated HCT116 cells with NCS under hypoxic conditions that we determined would suppress break induction (data not shown) and then measured cytotoxicity based on a colony formation assay carried out under normoxic conditions. Figure 6B shows that survival was increased when cells were treated with NCS under hypoxic compared with normoxic conditions. By contrast, a similar experiment showed that survival was decreased when cells were treated with C-1027 under hypoxic compared with normoxic conditions (Fig. 6A). Overall, hypoxic cells were nearly 4-fold more resistant to NCS, but 3-fold more sensitive to C-1027 cytotoxicity, than were normoxic cells. Thus, killing of hypoxic cells by C-1027 is enhanced about an order of magnitude over that of the conventional radiomimetic drug NCS.

**Discussion**

The paradigm for the interaction of aromatized enediyne core diradicals with DNA depicts deoxyribose radicals generated from hydrogen abstraction reactions culminating in DNA breaks through an oxygen mediated mechanism (31). The unprecedented ability of C-1027 to induce both DSBs and ICLs in cells, an apparent contradiction to the current model, prompted an investigation of the oxygen dependence of its DNA-damaging activities. Implicitly, the fate of C-1027 generated sugar radicals seems balanced between completing the reactions required to induce DNA breaks or binding back to the drug to form a cross-link. If the DNA damage shifts toward ICLs in low-oxygen environments, then C-1027 unlike IR has the potential to target cells within hypoxic microenvironments associated with many solid tumors (1).

The inverse relationship between the DSB and ICL activity of C-1027 is apparent from cell-free experiments in which breaks are suppressed under the hypoxic conditions (0.5% O\textsubscript{2}) required for cross-link formation (Fig. 2A). The findings are consistent with observations from the Goldberg laboratory that under anoxic conditions, C-1027 generated deoxyribose radicals can rebind to portions of the aromatized enediyne core positioned within the DNA minor groove leading to ICL formation (19, 20). Although the 50% reduction of DSBs under hypoxic conditions is less than the decreases (>80%) reported for anoxia, it is apparently sufficient to allow ICLs to form (20, 31). Also, like DNA breaks, ICLs form rapidly (Fig. 2C), which supports the notion that both are generated from unstable deoxyribose radicals, which need to commit to either type of DNA damage to avoid inactivation (25).

Similar to cell-free experiments, cells treated with C-1027 show substantial decreases in DSBs under hypoxic conditions based on the \gamma\textsubscript{H}2AX activation or tail Comet intensity (i.e., 45% and 60% decreases, respectively; Figs. 3 and 4). At the same time, ICL induction in hypoxic cells is increased and is well above levels detected in earlier studies carried out in normoxic cells (Fig. 5; 24). In both cell-free and cellular environments, hypoxia results in decreased oxidation of deoxyribose radicals, which preserves their potential to rebind the drug and form ICLs (19, 25). It had been presumed that even in cellular environments with available deoxyribose radicals, the presence of hydrogen donors such as glutathiones would readily lead to quenching. Although conditions promoting deoxyribose radicals rebinding to the drug are not well-understood, positioning of the drug within the DNA minor groove, availability of oxygen, as well as the presence of reducing factors are considered critical (19). The oxidizing environment within normoxic cells seems compatible with generation of both C-1027–induced breaks and cross-links (24). The repressed oxidative state in hypoxic cells apparently promotes the ICL activity of the drug at the expense of DNA breaks. Although C-1027 provides a novel agent for targeting DNA in low-oxygen environments, the basis for its unique DNA-damaging ability in normoxic and hypoxic cells is unclear. All enediyynes, including C-1027, trigger a DNA breakage reaction by going through a cycloaromatization process via a Myers-Saito or Bergman-type rearrangement resulting in biradicals capable of position-specific hydrogen abstraction from the deoxyribose backbone of DNA (32). At present, predicting whether an enediyne has the potential to induce DSBs, ICLs, or both is not possible. Although all known enediyynes (with the exception of the engineered C-1027 analogue, desmethyl-C-1027) induce robust amounts of DSBs, an accompanying ICL activity may depend on the reactivity as well as the proximity and steric availability of the aromatized enediyne chromophore to the deoxyribose radicals (7, 45).
19, 24). The precise mechanistic reasons for the differing activities of C-1027 and its designer analogues are also unclear. However, that the desmethyl-C-1027 analogue predominately induces ICLs indicates the potential for fine tuning the DNA-damaging activity of enediyynes by introducing minimal structural perturbation. Similarly, other C-1027 analogues with modifications of the enediyne core, such as deschloro-C-1027, induce primarily cellular DSBs and not ICLs (24). Interestingly, the degree to which C-1027 and its analogues induce DSBs, ICLs, or both correlates with whether DNA damage responses are regulated by ATM, ATR, or both, respectively (6, 7, 24).

Treatment of hypoxic cells with NCS, as with IR or other radiomimetics, decreased DNA breaks, and a 4-fold drop in cytotoxicity was observed (Fig. 6b). Although it is difficult to judge the cytotoxic potential of DSBS versus ICLs, the DNA damage induced by C-1027 revealed decreased breaks and elevated cross-links and a corresponding 2.7-fold increase in cytotoxicity in hypoxic compared with normoxic cells (Fig. 6a). Thus, increased ICLs production seemed to correlate with increased cytotoxicity. That C-1027 DNA damage shifts toward ICLs under hypoxic conditions that approximate a radioreistant environment (1% O2 or lower) for many types of human tumors suggests a potential for using C-1027 based enediyynes to target hypoxic cells within tumors (1, 33). These findings also highlight the potential for rational engineering of C-1027 as well as other enediyne biosynthetic pathways based on SAR studies to yield new generations of enediyne analogues that could be explored as antitumor (34).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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