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Brief Communication

The radiomimetic enediyne, 20′-deschloro-C-1027 induces inter-strand DNA crosslinks in hypoxic cells and overcomes cytotoxic radioresistance☆

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ABSTRACT

The ability of the radiomimetic anti-tumor enediyne C-1027 to induce DNA inter-strand crosslinks (ICLs), in addition to the expected DNA strand breaks, is unique among traditional DNA targeted cancer therapies. Importantly, radiation therapy and most radiomimetic drugs have diminished effect in hypoxic environments due to decreased induction of DNA strand breaks, which is an oxygen requiring process. However, C-1027's induction of ICLs is enhanced under hypoxia and it is actually more potent against hypoxic cells, overcoming this common tumor resistance mechanism. In this study, an analog of C-1027, 20′-deschloro-C-1027 was examined for its ability to induce DNA ICLs under hypoxic conditions. Deschloro-induced ICLs were detected under hypoxic cell-free conditions, with a concomitant reduction in the induction of DNA strand breaks. In cells deschloro behaved similarly, inducing cellular ICLs under hypoxic conditions with a reduction in DNA breaks. The cytotoxicity of deschloro treatment was similar in normoxic and hypoxic cells, suggesting that the ICL induction allows deschloro to retain its cytotoxic activity under hypoxia. It appears that rational engineering of the C-1027 family of radiomimetics holds promise toward overcoming the radioresistance associated with the hypoxic environment associated with solid tumors.

1. Introduction

IR is a major treatment option for patients diagnosed with any of a wide variety of cancers. Although IR produces a plethora of DNA lesions, the predominant cytotoxic lesion is DNA double strand breaks [1]. To induce a DNA strand break, IR produces OH radicals leading to multiple single strand breaks, and subsequently double strand breaks, when two single strand breaks align sufficiently close on opposite DNA strands. The production of the OH radicals requires molecular oxygen, thus the therapeutic effect of IR and radiomimetics on tumor cells is significantly decreased under hypoxic conditions [2]. Furthermore, tumor resistance to IR treatment is known to correspond to induction of hypoxic microenvironments, which often arise over the course of treatment due to radiation induced damage to the vascular system [3]. Radiomimetic compounds also induce DNA strand breaks by abstracting hydrogen atoms from the sugar backbone of DNA. The resultant deoxyribose radical(s) will be converted to a DNA single strand (one radical) or double strand (diradical) break in the presence of sufficient oxygen levels [4–6]. In general, radiomimetic compounds suffer the same limitations to hypoxic cells as IR, since in low oxygen environments the diradicals formed on the DNA sugar backbone have a reduced ability to convert to double strand breaks, resulting in a marked reduction in cytotoxicity [7].

C-1027 acts uniquely amongst radiomimetics due to its ability to directly induce both DNA strand breaks and inter-strand crosslinks (ICLs) into cells [7]. The ratio of DNA strand breaks to ICLs is dependent on oxygen levels: when oxygen levels decrease, strand breaks diminish but ICLs increase [7]. Moreover, the increased production of ICLs under hypoxia appears to compensate for the diminished induction of DNA DSBs since C-1027 is 3-fold more cytotoxic to

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hypoxic cells in comparison to normoxic cells [7,8], while other radiomimetics like neocarzinostatin (NCS) or esperamicin demonstrate reduced cytoxicity in the range of 4–15 fold [7,9].

Since C-1027 appears to represent a new class of enediyne that has the potential to overcome the resistance to treatment inherent in hypoxic cells, we sought to explore whether analogs of C-1027 would share this ability. C-1027 is a protein-chromophore enediyne produced by *Streptomyces globisporus* that is composed of four basic biochemical units, a benzoxazolinate, a deoxyaminohexose, a β-amino acid, and an enediyne core [10]. Utilization of gene manipulation techniques on the *S. globisporus* species has resulted in the isolation of recombinant strains that produce various C-1027 analogs [11,12]. Of the previously engineered C-1027 analogs, 20′-deschloro-C-1027 (deschloro) was chosen because it retained the most cytotoxicity, with an IC50 of ~174 pM, about 7-fold less potent that C-1027 [13].

In this study, we evaluate DNA lesions induced by deschloro treatment of cell-free DNA under both normoxic and hypoxic conditions. Studies are then extended into cellular systems to determine the oxygen dependence of deschloro-induced cellular DNA strand breaks and ICLs. Finally, the sensitivity of normoxic and hypoxic cells to deschloro treatment is compared.

2. Materials and methods

2.1. Chemicals and drug preparations

Fermentation, production, isolation, and purification of C-1027 from the *S. globisporus* wild-type strain and deschloro from SB1008 strain (i.e., ΔsgcC3 mutant) were carried out as previously described [10].

2.2. Hypoxic conditions for detection of cell-free DNA strand breaks and ICLs

For ICL detection, pBR322 DNA was linearized with the EcoR1 restriction enzyme (Fermentas, Inc.), incubated with drug under normoxic or hypoxic conditions, denatured and electrophoresed as described previously [7]. For DNA break detection, tubes containing 100 ng of supercoiled pBR322 plasmid DNA incubated with drug under normoxic or hypoxic conditions, and electrophoresed visualized, imaged and quantified as described above as described previously [7].

2.3. Cells and cell culture

HCT116 human colon carcinoma cells (a gift from Dr. B. Vogelstein, Sidney Kimmel Comprehensive Cancer Center, John Hopkins University, Baltimore, MD) were grown under normoxic and hypoxic conditions as described previously [14].

2.4. Cellular analysis

2.4.1. IR treatment of cells

After drug treatment, medium was removed and replaced with 0.5 mL cold PBS. Cells were immediately irradiated on ice under normoxic conditions using a Philips RT 250 Orthovoltage X-ray Unit (GE Healthcare) with a 0.5-mm Cu filter at 20 Gy.

2.4.2. Comet analysis

After enediyne incubations with or without IR treatment, HCT116 cells were analyzed as described previously [7,8].

3. Results

3.1. The oxygen dependence of deschloro-induced cell-free ICLs

To evaluate whether deschloro induces cell-free ICLs under low oxygen conditions, linearized plasmid DNA was drug treated, denatured and subsequently electrophoresed on an agarose gel to resolve DNA ICLs. DNA ICLs will prevent double stranded DNA (dsDNA) from denaturing, so the percentage of DNA containing ICLs is reflected by the percentage of dsDNA [7,8]. Fig. 1A depicts a representative gel, while Fig. 1B quantitates 3 independent trials. After treatment with 200 nM deschloro, cell-free ICLs were detected at 0.5% oxygen as approximately 25% of the DNA was crosslinked[1] (Fig. 1A and B). As a positive control, we also treated linear DNA with 25 nM C-1027, which induced readily detectable ICLs at 0.5% oxygen, consistent with our previous findings (Fig. 1A and B) [8].

3.2. The oxygen dependence of deschloro-induced cell-free DNA strand breaks

Enediynes require oxygen to induce DNA breaks and the percentage of breaks induced diminishes as oxygen levels decrease [4,15,16]. To assess the effect of oxygen levels on deschloro's ability to induce DNA breaks, we performed DNA forms conversion assessments. Cell-free plasmid DNA was treated with deschloro under both normoxic and hypoxic (0.5% oxygen) levels. The rate at which DNA plasmid will migrate during gel electrophoresis is altered if supercoiled DNA (form 1) is converted to a plasmid where one strand of DNA is broken (form II, single strand break) or linearized (form III, double strand break). Comparing normoxic versus hypoxic conditions, the levels of DNA break induction was clearly repressed under hypoxic conditions. 200 nM deschloro induced a DNA double strand break in approximately 25% of the total plasmid DNA, as measured by the amount of form III DNA, while loss of form I DNA, which signifies the percentage of total DNA strand breaks induced was almost 80% (Fig. 1C). Under hypoxic conditions, deschloro-induced double strand breaks are reduced to 20% of the total DNA and the total loss of form I DNA was reduced to 50% (Fig. 1C). C-1027 induction of DNA breaks, shown as a positive control, is similarly reduced compared to the normoxic control in both the formation of DNA double strand breaks and total strand breaks (Fig. 1C and D). DNA double strand breaks are reduced from approximately 25% to 17% under hypoxic conditions and total strand breaks is reduced from nearly 75% to 45% (Fig. 1D).

3.3. Deschloro-induced cellular DNA strand breaks under hypoxia

Under normoxic conditions, enediyne induction of cell-free DNA damage is generally is predictive of an ability to inflict cellular DNA damage [17]. We have previously shown that at least with C-1027, this correlation extends to hypoxic conditions, as C-1027 induction of both cell-free and cellular DNA breaks is decreased [7]. To determine the effects of hypoxia on deschloro-induced DNA damage, we compared DNA breaks at the cellular level under normoxic and hypoxic conditions.

DNA breaks were measured at the individual cell level by an alkaline single cell gel electrophoresis assay, or Comet analysis [18]. Fig. 2A consists of representative images of normoxic

[1] Deschloro's induction of cell-free ICLs was not observed at 3% oxygen levels (data not shown) and only occurred at very low oxygen levels (0.5%). This helps explain an apparent contradiction with our previous results [8] where deschloro-induced cell-free ICLs were only minimally detected compared to C-1027, as it is unlikely we were able to reach oxygen levels of 0.5% in our previous study, which was not performed under controlled but rather reduced oxygen conditions.