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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.

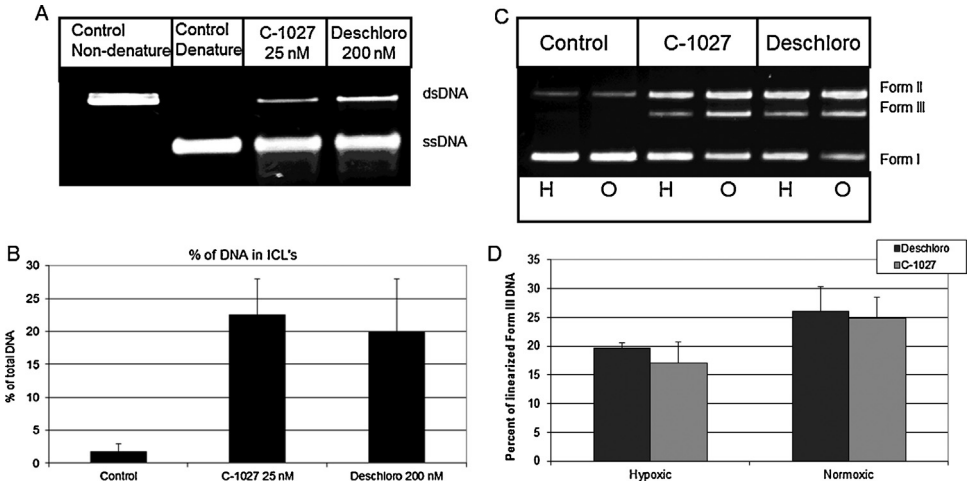

Fig. 1. The impact of oxygen levels on deschloro-induced cell-free DNA ICLs and strand breaks. Crosslink activity was based upon treatment of linearized pBR322 DNA under hypoxic conditions as previously described [7]. A representative gel is shown in (A), while 3 independent experiments are quantitated and graphed in (B). The impact of oxygen levels on cell-free DNA strand breaks is shown in (C and D). DNA break activity of deschloro or C-1027 is based upon damage of a plasmid DNA under conditions of hypoxic (0.5% oxygen), symbolized by "H" and normoxic oxygen levels, symbolized by "O" in (C). A representative gel is shown in (C), while 3 independent experiments are quantitated and graphed in (D).

and hypoxic cells treated with 5 nM deschloro. As anticipated, a markedly greater percentage of DNA breaks are induced under the normoxic conditions in comparison to hypoxic conditions. Fig. 2B quantitates the changes in comet tail length following treatment with increasing concentrations of deschloro under normoxic conditions. Treatment with 0.5 nM deschloro resulted in a tail intensity of about 10%, which increased to almost 30% at 1.5 nM deschloro and 45% at 5 nM deschloro. In Fig. 2C, we examined the comet tails induced by deschloro under hypoxic conditions, using higher concentrations of drug to compensate for the expected decrease in strand break induction as previously shown with C-1027 and other enediynes [7]. Interestingly, increasing levels of deschloro decreased the intensity of the comet tails as a tail intensity of 25% was observed with 2.5 nM deschloro, which decreased to 18% at 5 nM deschloro and then 12% 10 nM of treatment (Fig. 2C). While decreasing amounts of DNA breaks are expected under hypoxic versus normoxic conditions, it is unexpected that the tail intensities for the hypoxic samples would actually decrease with increasing drug concentration.

3.4. Deschloro-induced cellular ICLs under hypoxia

The induction of ICLs is known to prevent DNA denaturation by covalently linking the two strands of DNA together. Thus, the inversely proportional drug-comet tail intensity observed in Fig. 2C, combined with the cell-free ICLs induction of deschloro in Fig. 1, is consistent with the notion that cellular ICLs are being formed in a deschloro dose-dependent manner, restricting the migration of

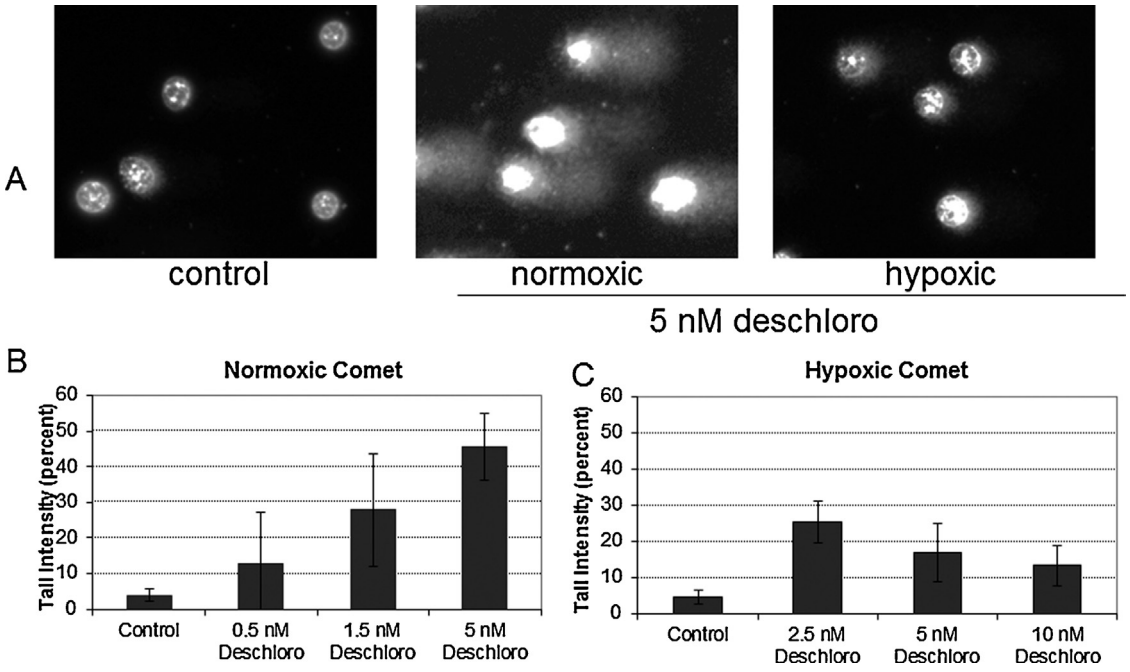

Fig. 2. Oxygen dependence of deschloro induced cellular DNA breaks by Comet assay. (A) Representative alkaline Comet images of HCT116 cells incubated with deschloro for 1 h at 37 °C under normoxic or hypoxic (0.5% oxygen) conditions. (B and C) Quantitation of at least 3 Comets tail experiments where tail intensity reflects the dose-dependent extent of DNA breaks induced by deschloro, C-1027 or NCS under normoxic (B) and hypoxic conditions (C).

DNA under hypoxic conditions. To confirm that deschloro is inducing ICLs under hypoxic conditions, we adapted the alkaline comet assay, as described by Olive et al. [19], and used routinely in our laboratory [7,8], as well as many others [20–22].

Essentially, cells are pretreated with a known (or suspected) DNA ICL inducing agent and then irradiated with 20 Gys in order to produce a moderate comet signal. The comet signals of the drug pretreated + IR cells are normalized to that of cells exposed only to IR and the level of ICL induction is correlated to the reduction in comet tail intensity. Fig. 3A depicts the results of pretreatment of cells under normal levels of oxygen with 200 nM NCS (an agent known to only induce DNA breaks and not ICLs), 1 nM C-1027 (which induces both DNA breaks and ICLs) or various levels (1.5 or 5 nM) of deschloro. As expected for an agent that induces only DNA breaks, NCS + IR leads to an increased comet signal in comparison to IR alone (Fig. 3A). In contrast, C-1027 + IR induces a similar comet signal to IR alone, indicative of a moderate level of ICL production (Fig. 3A). Deschloro + IR follows a similar pattern as NCS + IR, indicating ICL formation is not significant under these conditions (Fig. 3A).

However under hypoxic conditions (Fig. 3B), pretreatment of cells with either 2.5 nM or 10 nM deschloro significantly reduces the observed comet tail intensity in a concentration dependent manner from that of IR alone. Pretreatment of cells with 2.5 nM deschloro reduced the IR induced comet tail intensity by 25% under hypoxic conditions and it was reduced to 50% after pretreatment with 10 nM deschloro (Fig. 3B). Pretreatment of cells with 1 nM C-1027 decreased the tail intensity from IR alone, while pretreatment with 500 nM NCS revealed no reduction in the size of the comet tail (Fig. 3B). The deschloro mediated reduction of the IR-induced comet tail demonstrates that under hypoxic conditions, like C-1027 but not NCS, induces cellular ICLs [8]. C-1027 + IR also demonstrated increased ICL production compared to cells treated under normoxic conditions, consistent with previous studies, while NCS + IR did not [7,8].

3.5. Hypoxic cells are not resistant to treatment with deschloro

Since deschloro is able to induce ICLs under hypoxic conditions, we examined the extent to which deschloro could overcome the cytotoxic resistance that hypoxic cells display to treatment with IR and most radiomimetics. A colony formation assay was performed following deschloro treatment of HCT116 cells under hypoxic and normoxic conditions as described previously [7]. The LD_{90} for both normoxic and hypoxic cells was about 0.9 nM, thus hypoxic cells are not resistant to deschloro treatment. (data not shown). In our previous studies, hypoxic cells were approximately 3-fold more resistant in the colony formation assay to NCS, typical of what is observed with radiomimetics [7]. In contrast, hypoxic cells were actually 3-fold more sensitive to C-1027 than normoxic cells [7].

4. Discussion

The hypoxic resistance mechanism of tumor cells to IR and radiomimetic drugs is of major consequence in the treatment of cancer. C-1027, which represents a new class of enediyne with its unique ability to directly induce DNA breaks and ICLs concurrently in cells, provides a potential solution to overcome this resistance. Additionally, the recent breakthrough in the creation of C-1027 analogs via gene manipulation provides a means to improve the therapeutic potential of the parent compound, if they can maintain the ability to induce ICLs in hypoxic cells.

The Goldberg group previously determined a mechanism for the induction of C-1027 crosslinks under cell-free anaerobic conditions, where the DNA diradicals are able to interact with the unsaturated bonds from the now inactive C-1027 chromophore, bridging the two strands of DNA [23,24]. It is thought that to form ICLs, the C-1027 benzoid diradicals abstract two hydrogen atoms from the deoxyribose backbone, and the resultant deoxyribose radicals react back to the aromatized C-1027 chromophore, forming the ICLs [8,23,24]. Thus, the efficiency of ICL induction could be influenced by not only the amount of oxygen present but also the proximity, steric availability, and the relative reactivity of the aromatized C-1027 chromophore toward the deoxyribose radicals. Given their high reactivity toward radicals, both the benzoxazolinate and chlorinated phenol moieties serve as potential sites for interaction with the deoxyribose radicals to form ICLs. Our results support this model as the removal of the C-20′ chlorine atom, exemplified by the deschloro analog, would decrease the reactivity of the phenolic moiety toward the deoxyribose radicals in comparison to the parent compound.

Deschloro-induced DNA damage is typical of the pattern displayed by radiomimetics, where under normoxic conditions, increasing levels of drug produced increasing amounts of DNA breaks in both cell-free and cellular systems (Figs. 1 and 2B). Deschloro is also consistent with IR and other radiomimetics, in that the level of breaks it induces is significantly reduced under hypoxic conditions compared to normoxic conditions (Figs. 1 and 2C), [7,13]. The reduction in DNA break induction is supported by our measurement of γH2AX induction by deschloro under both normoxic and hypoxic conditions, where almost a 50% drop in phosphorylation was observed for the latter (data not shown). Where deschloro (and C-1027) diverge from IR, NCS and other radiomimetics is that in cells under hypoxic conditions, increasing amounts of drug do not correlate to increased DNA breaks as determined by Comet tail sizes, as they actually decrease in size (Fig. 2C), [7], due the induction of ICLs (Fig. 3).

The ability of deschloro to induce ICLs in hypoxic cells suggests that it may retain cytotoxicity in hypoxic cells versus normal cells. This is important since the DNA double strand break has long been thought to be the major cytotoxic lesion induced by IR and radiomimetic treatment and the reduced ability of these agents to induce such breaks in hypoxic cells leads to a reduction in cytotoxicity [15,25]. The induction of other lesions that do not require oxygen, such as DNA monoadducts or ICLs has generally been too inefficient to compensate for break loss. For example, the Hunting group has found IR can induce ICLs in DNA that contains bromouracil at specific sites or in other mismatched portions of DNA albeit with low efficiency [26]. While the induction of these types of lesions by IR and other radiomimetics may contribute to cytotoxicity under hypoxia, their effect is insufficient to overcome radioresistence. For example, hypoxic cells are 3-fold more resistant to IR, 4 fold more resistant to NCS and over 15 fold more resistant to esperamicin treatments [7,9]. In stark contrast, hypoxic cells display an enhanced susceptibility of almost 3-fold to C-1027 treatment, due to induction of ICLs [7]. It is interesting that there is neither an enhancement nor reduction in the cytotoxicity of hypoxic cells to deschloro treatment, suggesting that the amount of ICLs induced contributes sufficient cytotoxicity to compensate for the loss of DNA double strand break cytotoxicity.

Our results demonstrate that the ratio of DNA breaks to ICLs induced in cells by deschloro treatment is dependent on the level of oxygen present. The induction of cellular ICLs or DNA monoadducts (where the chromophore only binds to one DNA strand) under hypoxic conditions has not been examined for other enediynes, but their formation could be important when it comes to the activation of DNA damage responses and understanding the nature of drug induced cytotoxicity. For example at high drug levels or extended treatment times, the activation of damage responses to IR and other radiomimetics can involve ATR dependent mechanisms consistent with ICL induction [27]. Considering that oxygen levels in cells are

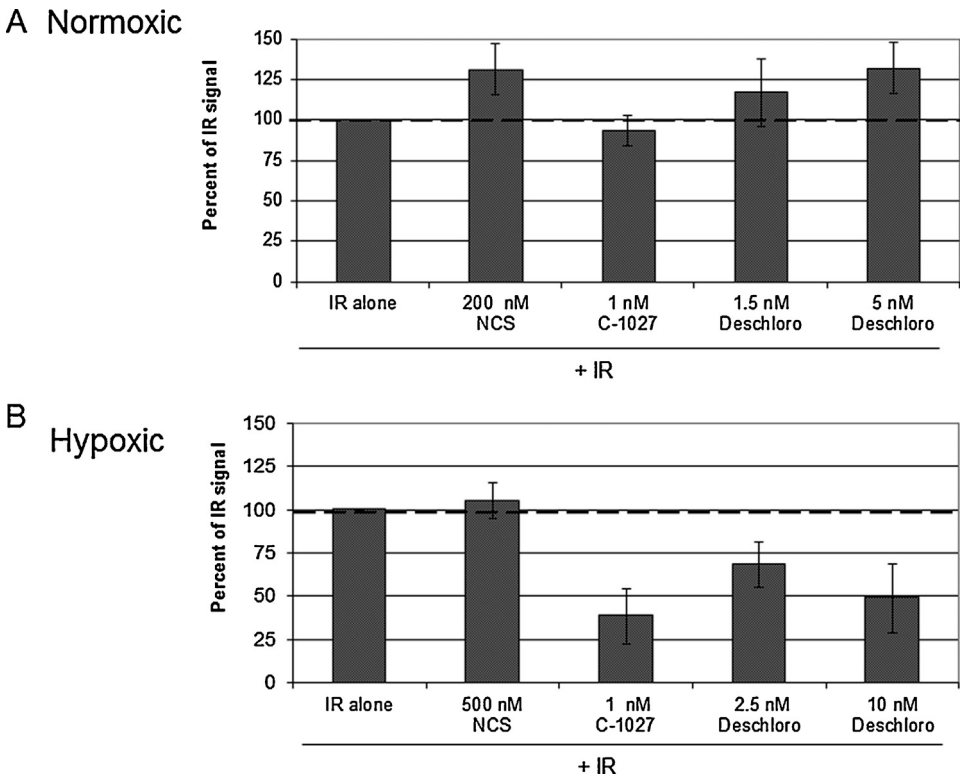

Fig. 3. Induction of ICLs by deschloro, C-1027, or NCS under normoxic or hypoxic conditions. HCT116 cells were pre-treated with either deschloro or C-1027 for 1 h at 37 °C. Cells were subsequently exposed to 20 Gy of IR. Comets were performed and analyzed as described previously [7]. The black dashed line signifies the comet signal of IR alone and induction of ICLs is indicated by the extent of reduction in comet signal.

substantially lower than atmospheric levels [28], and that tumors exist in a microenvironment that is hypoxic in comparison to normal cells [5], the types of DNA lesions induced under low oxygen conditions and their contribution to the activation of DNA damage response may have increased therapeutic relevance.

In this study, we have identified deschloro as a second radiomimetic that does not display the typical resistance displayed by IR and radiomimetics in hypoxic cells [2,29]. That a C-1027 analog retains this unique property of the parent compound suggests that rational engineering of the C-1027 family of radiomimetics holds promise toward overcoming the radioresistance associated with the hypoxic cells found in these solid tumors [30].

Conflict of interest statement

None.

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