Cancer therapy primarily consists of surgery whenever possible, chemotherapy and radiotherapy. Chemotherapy and radiotherapy cytotoxicity is at least in part due to unrepaired DNA damage and the ability of cancer cells to recognize DNA damage and initiate repair is an important mechanism of resistance (1–6). Pharmacologic inhibition of DNA repair has the potential to make cancer cells more vulnerable to the damaging effects of therapy, therefore increasing the response to treatment (7). Over the last decade, numerous academic laboratories and pharmaceutical companies have developed molecules to modulate DNA repair by targeting proteins involved in different repair pathways. Briefly, two families of inhibitors were tested: (a) damage signaling inhibitors, targeting poly(ADP-ribose) polymerase (8–10) and ataxia telangiectasia mutated (ATM; ref. 11) and (b) base excision repair via O6-methylguanine DNA methyltransferase (12), homologous recombination repair via RAD51, or nonhomologous end joining via the DNA-dependent protein kinase (DNA-PK; refs. 13–18). All inhibition strategies were designed to target one key enzyme and therefore may be thwarted by mutations in the target or overactivation of an alternative repair pathway.

DNA double-strand breaks (DSB) are one of the most severe types of DNA damage, which if left unrepaired are lethal to the cell. Two main DNA repair pathways combat DSBs: homologous recombination and nonhomologous end joining, the latter being the most important in mammalian cells (1). Recruitment of DNA damage signaling and repair proteins to the sites of genomic damage constitutes a primary event required for repair. Many components of the DNA damage signaling and repair complex, including ATM, DNA-PK, and BRCA1, are targets for small-molecule inhibitors. Therefore, we hypothesized that inhibiting DNA damage repair would sensitize tumors to radiation-induced DNA damage.

**Purpose:** Enhanced DNA repair activity is often associated with tumor resistance to radiotherapy. We hypothesized that inhibiting DNA damage repair would sensitize tumors to radiation-induced DNA damage.

**Experimental Design:** A novel strategy for inhibiting DNA repair was tested. We designed small DNA molecules that mimic DNA double-strand breaks (called Dbai) and act by disorganizing DNA damage signaling and DNA repair. We analyzed the effects of Dbai in cultured cells and on xenografted tumors growth and performed preliminary studies of their mechanism(s) of action.

**Results:** The selected Dbai molecules activate H2AX phosphorylation in cell culture and in xenografted tumors. In vitro, this activation correlates with the reduction of Nijmegen breakage syndrome 1 and p53-binding protein 1 repair foci formation after irradiation. Cells are sensitized to irradiation and do not efficiently repair DNA damage. In vivo, Dbai induces regression of radioresistant head and neck squamous cell carcinoma (Hep2) and melanoma (SK28 and LUI205) tumors. The combination of Dbai32Hc treatment and fractionated radiotherapy significantly enhanced the therapeutic effect. Tumor growth control by Dbai molecules depended directly on the dose and was observed with various irradiation protocols. The induction of H2AX phosphorylation in tumors treated with Dbai suggests that it acts in vivo through the induction of “false” DNA damage signaling and repair inhibition.

**Conclusions:** These data validate the concept of introducing small DNA molecules, which mimic DNA damage, to trigger “false” signaling of DNA damage and impair DNA repair of damaged chromosomes. This new strategy could provide a new method for enhancing radiotherapy efficiency in radioresistant tumors.

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**Abstract**

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**Cancer Therapy: Preclinical**

**Small-Molecule Drugs Mimicking DNA Damage: A New Strategy for Sensitizing Tumors to Radiotherapy**

Maria Quanz,1,4,5 Nathalie Berthault,1,2 Christophe Roulin,4 Maryline Roy,1,4,5 Aurélie Herbette,1,4,5 Céline Agrario,1,4,5 Christophe Alberti,1,3 Véronique Josserand,6 Jean-Luc Coll,6 Xavier Sastre-Garau,7 Jean-Marc Cosset,8 Lionel Larue,1,3 Jian-Sheng Sun,5,9,10,11 and Marie Dutreix1,2,4

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M. Quanz and N. Berthault contributed equally to the work.

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**Translational Relevance**

The use of inhibitors of DNA damage repair or signaling pathways appears to provide a novel opportunity for targeting genetic differences between tumor and normal cells. To date, few kinase inhibitors are in preclinical development to treat cancer. We designed Dbait molecules that offer an alternative avenue for enhancing radiotherapy by interfering with DSB repair activity. They act as a DSB pathway inhibitor by interfering at all levels of the repair pathway: damage sensing, signaling and repair, in a bona fide manner. They exhibit interesting pharmacokinetic properties: stable inside tumor cells and long-lasting bioactivity. It is worthy to note that the standalone activity of Dbait at higher dose reveals its dual cytotoxic and cytostatic tumor-specific features, which are of great interest in molecular therapy. The experimental protocols used in three xenograft tumors were designed by radiation oncologists to achieve proofs of concept with clinical relevance.

---

**Table 1. Comparison of the survival of xenografted animals treated with various protocols**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Irradiation</th>
<th>Dbait concentration</th>
<th>No. mice</th>
<th>Cured mice*</th>
<th>Median survival time (d)</th>
<th>RR (P)</th>
<th>Mean TGD</th>
<th>Standard TGD</th>
<th>Range TGD</th>
<th>Mean % TGD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LU1205</td>
<td>—</td>
<td>—</td>
<td>21</td>
<td>24</td>
<td>C</td>
<td>0</td>
<td>2.8</td>
<td>-4</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>LU1205</td>
<td>6 × 5 Gy</td>
<td>32Hc 6 × 60 μg (3 nmol)</td>
<td>10</td>
<td>41</td>
<td>0.25 (P &lt; 1 × 10^-5)</td>
<td>10</td>
<td>2.8</td>
<td>1</td>
<td>12</td>
<td>208</td>
</tr>
<tr>
<td>SK28</td>
<td>—</td>
<td>—</td>
<td>31</td>
<td>57</td>
<td>C</td>
<td>0</td>
<td>7.3</td>
<td>-16</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>SK28</td>
<td>6 × 5 Gy</td>
<td>32Hc 6 × 60 μg (3 nmol)</td>
<td>15</td>
<td>88</td>
<td>0.29 (P &lt; 1 × 10^-5)</td>
<td>17</td>
<td>15</td>
<td>-3</td>
<td>65</td>
<td>191</td>
</tr>
<tr>
<td>SK28</td>
<td>6 × 5 Gy</td>
<td>32Hc 6 × 60 μg (3 nmol)</td>
<td>32</td>
<td>1</td>
<td>75 (C-IR)</td>
<td>12</td>
<td>16.4</td>
<td>-4</td>
<td>84</td>
<td>162</td>
</tr>
<tr>
<td>SK28</td>
<td>6 × 5 Gy</td>
<td>32C 6 × 60 μg (3 nmol)</td>
<td>8</td>
<td>76</td>
<td>0.56 (P &lt; 0.21)</td>
<td>16</td>
<td>7.8</td>
<td>1</td>
<td>24</td>
<td>184</td>
</tr>
<tr>
<td>Hep2</td>
<td>—</td>
<td>—</td>
<td>10</td>
<td>68</td>
<td>0.4 (P &lt; 9 × 10^-3)</td>
<td>34</td>
<td>21.3</td>
<td>7</td>
<td>69</td>
<td>421</td>
</tr>
<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 60 μg (3 nmol)</td>
<td>17</td>
<td>61</td>
<td>C-IR</td>
<td>13</td>
<td>14.2</td>
<td>-3</td>
<td>45</td>
<td>218</td>
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<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 60 μg (3 nmol)</td>
<td>23</td>
<td>1</td>
<td>123 (0.30 (P &lt; 3.9 × 10^-4))</td>
<td>59</td>
<td>40.7</td>
<td>0</td>
<td>139</td>
<td>652</td>
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<tr>
<td>Hep2</td>
<td>—</td>
<td>—</td>
<td>10</td>
<td>55</td>
<td>0.62 (P &lt; 0.24)</td>
<td>7</td>
<td>6.7</td>
<td>-3</td>
<td>17</td>
<td>169</td>
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<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 60 μg (3 nmol)</td>
<td>11</td>
<td>1</td>
<td>58 (0.41 (P &lt; 1.6 × 10^-2))</td>
<td>39</td>
<td>41.6</td>
<td>-5</td>
<td>139</td>
<td>460</td>
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<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 120 μg (6 nmol)</td>
<td>11</td>
<td>1</td>
<td>&gt;150 (0.14 (P &lt; 10^-5))</td>
<td>56</td>
<td>38.7</td>
<td>-3</td>
<td>160</td>
<td>624</td>
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<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 20 μg (1 nmol)</td>
<td>15</td>
<td>86</td>
<td>0.43 (P &lt; 1.9 × 10^-2)</td>
<td>49</td>
<td>26.6</td>
<td>8</td>
<td>134</td>
<td>561</td>
</tr>
<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 60 μg (3 nmol)</td>
<td>20</td>
<td>5</td>
<td>1.29 (P &lt; 2.2 × 10^-3)</td>
<td>&gt;56</td>
<td>45.2</td>
<td>-3</td>
<td>139</td>
<td>707</td>
</tr>
<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 120 μg (6 nmol)</td>
<td>20</td>
<td>4</td>
<td>&gt;150 (0.13 (P &lt; 10^-5))</td>
<td>&gt;86</td>
<td>43.6</td>
<td>1</td>
<td>139</td>
<td>903</td>
</tr>
<tr>
<td>Hep2</td>
<td>—</td>
<td>—</td>
<td>12</td>
<td>46</td>
<td>0.7 (P &lt; 0.3)</td>
<td>6</td>
<td>4.9</td>
<td>0</td>
<td>19</td>
<td>156</td>
</tr>
<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 60 μg (6 nmol)</td>
<td>12</td>
<td>80</td>
<td>0.54 (P &lt; 0.12)</td>
<td>29</td>
<td>19.8</td>
<td>3</td>
<td>59</td>
<td>374</td>
</tr>
</tbody>
</table>

*NOTE: C and C-IR reference groups for statistics in nonirradiated and irradiated tumor group, respectively.

*Cured mice are animals surviving >300 d after the beginning of treatment.

TGD calculation and statistical analysis described in Materials and Methods.
mass, 20,153) is ~ 20 µg. All Dbait molecules were made by automated solid-phase oligonucleotide synthesis (Eurogentec) as described in Supplementary Fig. S1. Sequences are 5'-GCTTGCCCACCAACCG-CAAAACAGCCTAGA-(H)-TCTAGGCTGGTGGTGGTGGTGGTG- GGCCAGCG-3' Dbait32Hc, where H is a hexaethyleneglycol linker; 5'ACGCCAGGTTGTTGTTGTTGTTGATCT-(H)-AGATCCAA- CAAACGCAACACCGCTGCGG-3' Dbait32H; 5'AGCCAGCGHG-(H)- CCCTGCGC-3' DbaitH; and 5'ACGCCAGGTTGTTGTTGTTGATCT- (H)-GTCGATCT-3' Dbait32s.

**Kinase assay.** DNA-PK activity was monitored using the kit SignaTECT DNA-PK Assay System (Promega). The biotinylated peptide substrate, various amounts of nuclear extract (cleaned of endogenous DNA by DEAE-Sepharose filtration), and 20 mmol/L Dbait molecules were incubated for 5 min at 30°C with [γ-32P]ATP according to the manufacturer's instructions. The biotinylated substrate was captured on a streptavidin membrane, washed, and counted in a scintillation counter. Percentage of phosphorylation is calculated by dividing the bound radioactivity by the total count of [γ-32P]ATP per sample.

**Single-cell gel electrophoresis comet assay.** MRC5 cells, mock transfected or transfected by 2 µg(Dbait32H), were suspended in 0.5% low melting point agarose in DMEM and transferred onto a frosted glass microscope slide precoated with a layer of 0.5% normal melting point agarose. Slides were irradiated or not and incubated in DMEM under cell growth conditions for various times after irradiation. Slides were immersed in lysis solution [2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% sodium lauryl sarcosinate, 10% DMSO, 1% Triton X-100 (pH 13)] at 4°C for 1 h, placed in a electrophoresis tank containing 0.3 mol/L NaOH (pH 13) and 1 mmol/L EDTA for 40 min, electrophoresis for 25 min at 25 V [300 mA], washed with neutral buffer [400 mmol/L Tris-HCl (pH 7.5)], and stained with 20 µg/mL ethidium bromide. The variables of the "comets" were quantified with the use of the software Comet Assay 2 (Perceptive Instrument). Duplicate slides were processed for each experimental point. The tail moment is defined as the product of the percentage of DNA in the tail and the displacement between the head and the tail of the comet (28).

**Histology and immunodetection.** At various times, animals were sacrificed and tumors were excised and divided in two parts: one half was stored in frozen nitrogen to be used for immunodetection and the other half was placed in neutral buffered formalin before to be embedded in paraffin. Sections (7 µm) were cut and stained with hematoxylin, eosin, and safran. The extent of necrosis (indicated by increased cell size, indistinct cell border, eosinophilic cytoplasm, loss or condensation of the nucleus, or associated inflammation) is expressed as the proportion [% of the surface area of the tissue section analyzed that was necrotic]. The number of mitotic cells and apoptotic cells were estimated from representative nonnecrotic fields of ~1,000 cells analyzed at high power. Sections of frozen sample were fixed with 4% paraformaldehyde for 20 min before immunodetection by overnight incubation with mouse monoclonal anti-phospho-histone H2AX (Ser139; Upstate) diluted 1:200 in 1× PBS, 2% bovine serum albumin. For immunostaining of cells grown on coverslips, we used rabbit polyclonal anti-53BP1 (Cell Signaling Technology), rabbit polyclonal anti-NBS1 (Novus Biologicals), and mouse monoclonal anti-phospho-histone H2AX (Ser139; Upstate) diluted 1:200, 1:100, and 1:500, respectively. All secondary antibodies conjugated with Alexa 555, 633, or 488 (Molecular Probes) were used at a dilution of 1:200 and DNA was stained with 4',6-diamidino-2-phenylindole.

**Dbait and irradiation treatments in mice.** Hep2, SK28, and LLI1205 xenograft tumors were obtained by injecting 10³ tumor cells into the flank of adult female nude mice (Charles River). The animals were housed in the laboratory at least 1 week before beginning experiments. There were 6 animals per cage under controlled conditions of light and dark cycles (12 h:12 h), relative humidity (55%), and temperature (21°C). Food and tap water were available ad libitum. After 8 days for Hep2 and LLI1205 and 12 days for SK28, when the subcutaneous tumors measured 150 to 200 mm³, the mice were separated into homogeneous groups to receive different treatments.

Irradiation was done in a 137Cs unit (0.5 Gy/min) with a shield designed to protect about two-thirds of the animal's body. Doses were controlled by theroluminescence dosimetry. A total dose of 30 Gy was delivered in 15 sessions at intervals of 3 sessions of 2 Gy/wk during 5 weeks (Hep2) or in 6 sessions at intervals of 3 sessions of 5 Gy/wk during 2 weeks (LL1205, SK28). Dbait molecules with in vivo jet polyethyleneimine (PEI) reagent (Polyplus Transfection) at the N/P ratio 6 were diluted in 100 µL of 5% glucose. The Dbait-PEI mixture was incubated for 15 min at room temperature before injection. Intratumoral injections of the indicated amount of Dbait were done 5 h before each radiotherapy session. Mock treated animals were injected with 100 µL of 5% glucose according to the protocol of the associated assays. Tumor size was assessed by caliper measurements every 3 days and size was calculated by the formula: 0.5 × length × width². Mice were weighed and pictures of tumors were taken every week. For ethical reasons, the animals were sacrificed when tumors reached 2,000 mm³. This endpoint used in survival analysis was death day. The Local Committee on Ethics of Animal Experimentation approved all experiments.

**Statistical analysis.** Descriptive analyses of the tumor response were done for each treatment and each tumor type for 150 days after the first treatment session (day 1). Median lifetime was estimated according to the Kaplan-Meier method. Tumor growth delay (TGD) was calculated by subtracting the mean tumor volume quadrupling time of the control group from tumor volume quadrupling times of individual mice in each treated group. The mean TGD was calculated for each treated
group using the individual measurements. Overall survival curves were assessed by Kaplan-Meier estimates and compared using the nonparametric log-rank test because the data do not follow a normal distribution. The analysis was done using S-Plus 6.2 version software (MathSoft) and statEL (ad Science). A global log-rank test was first done for each group with the same tumor type, and treatments with Dbait were compared with the mock-treated control. The number of animals (n), the relative risk (RR), and the P value are reported in Table 1.

**Results**

**Design and in vitro screening of the Dbait molecules.** We designed small DNA molecules (called Dbait) that mimic DSBs. To favor double-stranded DNA structure in cells, the Dbait molecules were hairpin with one blunt end and the two strands of the other end joined by a hexaethyleneglycol linker. The blunt end was protected from exonuclease attack by substituting the three 3’ and 5’ terminal nucleotide residues with phosphorothioate nucleotides (29). Molecules were screened for DNA-PK activation in cell extracts. As observed previously with unmodified DNA (30, 31), we found that both Dbait32Hc and Dbait32H molecules, which are 32 bp in length and differ only in their sequences, efficiently activate DNA-PKcs kinase activity of the purified enzyme and in cell extract (Supplementary Fig. S1). Short (8 bp) molecules (Dbait8H) as well as single-stranded 32-nucleotide oligonucleotides (Dbait32ss) that did not activate kinase activity were used as negative controls.

**Induction of H2AX phosphorylation by Dbait molecules.** One of the earliest steps in the cellular response to DSBs is the phosphorylation of Ser139 of histone H2AX by the phosphatidylinositol 3-kinases (24, 32, 33). The appearance of the phosphorylated form of γ-H2AX in nuclei is often used as an indicator of the presence of DNA DSBs produced by ionizing radiation (34). We therefore assayed for the presence of...
γ-H2AX in cells treated with Dbait32Hc, designed to mimic DSBs. High levels of γ-H2AX with a pan-nuclear distribution was observed in 40% to 60% of cells transfected with Dbait32Hc (Fig. 1A) but not in cells transfected with Dbait8H inactive control, indicating that Dbait32Hc molecules are recognized as DSBs and induce kinase activation in transfected cells. The γ-H2AX mean level was 6- to 10-fold higher in Dbait-transfected cells than after 10 Gy irradiation and persisted for at least 48 h (Fig. 1B and C). Irradiation of Dbait32Hc-transfected cells increased the mean level of γ-H2AX. Similar results were obtained in all cell lines (HeLa, Hep2, MRC5, MO59K, LU1205, and SK28) and primary fibroblasts examined (Fig. 1B and C; data not shown).

ATM and DNA-PK have been shown to function redundantly to phosphorylate H2AX after irradiation (24). To test if both kinases were also involved in the phosphorylation of H2AX after Dbait32Hc transfection, we measured γ-H2AX in ATM (AT5B1) and DNA-PK (MO59J) mutants (Fig. 1C). Induction of γ-H2AX by Dbait32Hc transfection was observed in AT5B1 but not in MO59J, indicating that, for Dbait32Hc-induced signaling, ATM is not able to compensate for the DNA-PK defect in MO59J mutant as it does for irradiation-induced signaling.

Inhibition of repair foci formation by Dbait32Hc. Formation of γ-H2AX foci is important for recruiting and/or stabilizing numerous DSB recognition and repair factors at the break site, including DNA damage checkpoint proteins, chromatin remodelers, and cohesion. Several groups have shown that γ-H2AX foci colocalize with DNA repair proteins including NBS1, 53BP1, MDC1, RAD51, and BRCA1 repair proteins involved in homologous recombination repair pathway (20, 21, 32, 35, 36) in addition to DNA-PKcs and XRCC4. To determine how the Dbait32Hc-induced pan-nuclear distribution of γ-H2AX on chromosomes would affect repair foci formation at DSB sites, we monitored foci formed by 53BP1 and NBS1, a component of the MRE11-RAD50-NBS1 complex (32) after irradiation. In irradiated cells, the number of foci formed by 53BP1 and NBS1 was significantly lower in cells with high γ-H2AX content (in Dbait-transfected cell nuclei) compared with cells with normal level of γ-H2AX in the same population (illustrated in Fig. 2A). To confirm that γ-H2AX immunolabeling did not affect foci detection, we compared the number of cells without 53BP1 or NBS1 foci after irradiation in Dbait32Hc populations treated with γ-H2AX antibodies or not. Results were similar in both cases with at least 20% and 15% of irradiated cells that did not form 53BP1 or NBS1 foci, respectively, in Dbait32Hc-transfected population compared with <1% without foci in populations that were not transfected or transfected with Dbait8H.

Inhibition of damage repair by Dbait32Hc. Because defects in “repair foci” formation have been shown to be associated with DNA repair defects, we tested DNA repair in Dbait32Hc-transfected cells using a single-cell alkaline comet assay that measures DSBs as well as single-strand breaks and base modifications. Dbait32Hc transfection without irradiation did not induce apoptosis (Supplementary Fig. S2). However, after irradiation, repair of DNA damage was delayed in the Dbait32Hc-transfected population and some cells persisted without significant repair for several hours, indicating that Dbait32Hc prevented damage repair in these cells (Fig. 2B; Supplementary Fig. S3).

We analyzed the ability of cells transfected with various amounts of Dbait32H to form clones. Transfection treatment induced a small dose-independent lethality in Dbait32Hc-transfected cells as well as in Dbait8H (Fig. 2C) or Dbait32ss (data not shown) transfected cells. Survival after irradiation decreased in transfected cells according to the amount of Dbait32Hc but was not affected by Dbait8H at any concentration (Fig. 2C). The dose resulting in 50% survival in the absence of Dbait (LD50 = 3 Gy; data not shown) or in the presence of the inactive Dbait8H (LD50 = 2.95 Gy) was 3-fold reduced in the presence of the active Dbait32H (LD50 = 1 Gy; Fig. 2D).
Only Dbait32H and Dbait32Hc molecules that activate DNA-PK were able to sensitize cells to irradiation (Supplementary Fig. S4). This Dbait radiosensitizing effect was probably underestimated because, due to low transfection efficiency under cell culture conditions, only half of the transfected population received enough Dbait to induce H2AX phosphorylation. In vivo assays were designed to analyze the effects of Dbait on tumor radiosensitivity with the aim of increasing the observed radiosensitivity using repeated Dbait treatments.

**Dbait treatment increases Hep2, LU1205, and SK28 xenografted tumor growth control.** We tested the effect of Dbait32Hc on tumor growth using two different protocols of treatment according to the type of tumors treated: head and neck tumors (Hep2) received fractionated treatment in 15 sessions (2 Gy/session) over 5 weeks, whereas the melanoma tumors (SK28 and LU1205) were treated with a hypofractionated protocol of 6 sessions (5 Gy/session) over 2 weeks. Dbait32Hc was combined with PEI (39–42) to facilitate cellular delivery. The Dbait32Hc-PEI combination persisted in the tumor for at least 5 days (Supplementary Figs. S5 and S6). Formulation with PEI was required to observe the Dbait radiosensitizing effect, indicating that Dbait molecules have to enter the cells to be active (Supplementary Fig. S6). In the three tumor models, single treatment with irradiation or with Dbait32Hc (3 nmol/session) inhibited tumor growth but failed to permanently prevent it, whereas combination of both treatments led to efficient growth control (Fig. 3). The median survival of animals that received combined treatment was significantly increased compared with survival of animals treated only with radiotherapy (RR = 0.3; P < 3.9 × 10⁻⁴ in Hep2 and RR = 0.26; P < 6.4 × 10⁻⁵ in SK28) and (RR = 0.47; P < 1.2 × 10⁻² in LU1205; see also Table 1). Interestingly, up to 12% of tumors treated with Dbait combined with irradiation showed complete necrosis followed by a rapid healing and no recurrence of tumor growth over >1 year (Table 1; illustrated in Supplementary Fig. S7). Such efficient regression has not been observed when animals were treated with only radiotherapy or Dbait32Hc treatment.

Histologic analysis revealed large areas of necrosis that cover 30% and 50% of the tumors treated with radiotherapy or Dbait treatment alone, respectively, and up to 75% of the tumors treated with the combination of Dbait32Hc and radiotherapy. These observations were confirmed by magnetic resonance imaging analysis (Supplementary Fig. S8A). The tissue outside the necrotic area showed a significant increase of apoptotic cells and a drastic reduction of the number of mitotic cells (Supplementary Fig. S8B).

**Dbait induces H2AX phosphorylation in tumors.** Tumors were treated with Dbait32Hc fluorescent molecules during 1 week and analyzed for Dbait and γ-H2AX distribution (Fig. 4A). In parallel, cells from nonnecrotic tumoral tissues were dissociated and analyzed by Western blot (data not shown) or flow cytometry (Fig. 4B and C). The three independent methods of analysis indicated a significant increase of the level of phospho-H2AX after Dbait32Hc treatment. Irradiation alone and 8H treatment did not induce a significant increase of γ-H2AX in tumors (Fig. 4C). The H2AX phosphorylation induced in tumors treated with Dbait32Hc confirms that the Dbait molecules retain the ability to mimic DSBs and activate damage signaling kinase activity in vivo.
Dbait32Hc. Both molecules enhanced the efficacy of radiotherapy in a similar manner (Table 1), confirming that the sequence is not a significant part of the therapeutic activity of Dbait. These results are in agreement with our in vitro observations that Dbait32H and Dbait32Hc similarly activate DNA-PKcs (Supplementary Fig. S1) and inhibit DNA repair. In vitro radiosensitization of Dbait is dose-dependent. In vitro, the radiosensitivity of the cells is proportional to the amount of Dbait-transfected cells (Fig. 2C). To determine if a similar dose dependence is observed in vivo, we compared the efficacy of various amounts of Dbait32H for treating Hep2 tumors (Fig. 5A). Administration of Dbait32H alone had no effect at a dose of 1 nmol (RR = 0.62; P < 0.24). Tumor growth was reduced at a dose of 3 nmol (RR = 0.41; P < 0.016) and the best efficiency was observed at a dose of 6 nmol (RR = 0.14; P < 10^{-5}; Fig. 5A, left; Table 1). Similarly, in association with radiotherapy, increasing amounts of Dbait improved tumor growth control, resulting in a median survival that increased from 61 days with radiotherapy alone to 86 days (RR = 0.43; P < 1.9 \times 10^{-4}), 129 days (RR = 0.25; P < 2.2 \times 10^{-4}) and 150 days (RR = 0.13; P < 10^{-5}) with doses of Dbait32H of 1, 3, and 6 nmol, respectively (Fig. 5A, right; Table 1). Interestingly, although 1 nmol Dbait had no significant effect when administered alone, it significantly enhanced radiotherapy efficiency when it was administered before irradiation, indicating a synergistic effect of the combined treatment at low doses.

Radiotherapy in fractionated as well as hypofractionated treatments had no detectable effect on the treated healthy skin. Cytology analysis of the treated tissue showed only a slight inflammation similar to the irradiated area treated without or with Dbait injections.

Systemic administration of Dbait. Various authors have shown that DNA formulated with PEI and administered by intraperitoneal injection accumulates in subcutaneous tumors (42, 45). We analyzed the efficacy of intraperitoneal injections of Dbait32Hc formulated with PEI in controlling tumor growth. Animals with subcutaneous SK28 tumors received 6 nmol Dbait32Hc before irradiation (6 sessions). All treated tumors rapidly underwent large central necrosis. Cytologic analysis of the treated tissue showed that injection of the CpG-rich Dbait32H induced a rapid response of IL-6 and a more delayed response of IL-12p70, whereas Dbait32Hc, which is devoid of CpG, did not induce any cytokine production irrespective of whether the delivery was intravenous or subcutaneous (Supplementary Fig. S10). Moreover, the combination of Dbait32Hc and radiotherapy in fractionated as well as hypofractionated treatments had no toxic effect on the treated healthy skin. Cytology analysis of the treated tissue showed only a slight inflammation similar to the irradiated area treated without or with Dbait injections.

Discussion

We have developed new molecules (Dbait) that mimic DSBs and disorganize DNA damage signaling. The molecular characteristics for Dbait activities were similar for activation of the protein kinase, inhibition of DNA repair, and enhanced sensitivity of tumors to γ-irradiation. We found that the optimal Dbait molecules were 32 bp double-stranded DNA with one free end. The sequence had no influence on the activities tested, indicating that the effects of Dbait were due to

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Fig. 5. Dose effect of Dbait32Hc treatment. A, Kaplan-Meier survival representation of animals with Hep2 tumors treated in 15 sessions over 5 wk with increasing amounts of Dbait32H associated (right) or not (left) with radiotherapy (2 Gy/session). The dose of Dbait32H per session was 20 µg (1 nmol; black line), 60 µg (3 nmol; dotted line), and 120 µg (6 nmol; line with crosses). Gray line, untreated control; dotted gray line, radiotherapy with mock injection. B, Kaplan-Meier survival representation of animals with SK28 tumors treated over 2 wk with 6 sessions of 5 Gy irradiation associated to 80 µg Dbait32Hc injected intratumorally (dotted black line) or 120 µg injected intraperitoneally (black line). Gray line, untreated control; dotted gray line, radiotherapy with mock injection. C, tumors treated with irradiation associated (gray) or not (hatched) with Dbait32Hc (intraperitoneal) were sampled, stained with hematoxylin, eosin, and safran, and analyzed using microscopy for extent of necrosis. The mean percentage of necrotic area in each tumor was estimated using the analysis of four different sections per tumor (5 tumors per treatment).
the molecular structure as a mimetic substrate rather than to the targetting of a specific sequence. The phosphorothioate and hexaethyleneglycol added to the DNA molecule did not account for the biological effect because DbaitsH, which contains all, did not show any significant activity. The high level of γ-H2AX in cells treated with DbaitsHc indicates that the Dbaits molecules activate a kinase involved in DNA damage signaling. The increase of cell radiosensitivity on transfection with DbaitsHc may be explained by Dbaits binding and sequestering DNA-PK or by a more complex mechanism in which the "false" damage signaling induced by Dbaits disturbs the organization or functioning of the DSB repair complexes.

In this work, we show that Dbaits radiosensitize different types of radioresistant tumors. The fact that Dbaits molecules trigger histone H2AX phosphorylation in tumors (Fig. 4) suggests that they could act in vivo as observed in vitro (Fig. 2). As Dbaits targets the general mechanism of DNA repair, its application is likely to be applied to irradiation as well as other treatments that induce genotoxic damage. How does Dbaits act in the absence of genotoxic treatment? Dbaits could activate the DNA damage response in tumors that in turn would stop tumor proliferation through cell cycle arrest or death mechanisms (senescence or apoptosis). Alternatively, the stand-alone effect of Dbaits could reflect that tumor cells also require repair for proliferation. Actually, it is well known that tumor cells encounter repetitive chromosome breaks during the cell cycle and require efficient DNA repair to survive. Conversion of this damage and replication stress into fatal lesions (unrepaired DSBs) by Dbaits-induced perturbation of damage signaling and repair is expected to trigger cell death.

Although inhibition of DNA repair kinase has been widely discussed as a strategy for sensitizing tumors to antineoplastic treatments (7, 13, 15, 16, 46), to date, a DNA-PK inhibitor NU7441 and an ATM inhibitor KU55933 are the only DSB repair inhibitors in preclinical development (47). Our Dbaits molecules offer an alternative avenue for the enhancement of radiotherapy by interfering with DSB repair activity. Dbaits differs fundamentally from kinase inhibitors as it acts by disorganizing the spatiotemporal response to damage rather than targeting a key protein of the repair pathway. Moreover, the fact that it targets a whole complex and not a unique enzyme reduces the possibility of developing resistance during treatment.

Disclosure of Potential Conflicts of Interest


Acknowledgments

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