Cancer Therapy: Preclinical

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

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Abstract

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 Dbait) and act by disorganizing damage signaling and DNA repair. We analyzed the effects of Dbait in cultured cells and on xenografted tumors growth and performed preliminary studies of their mechanism(s) of action.

Results: The selected Dbait 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, Dbait induces regression of radioresistant head and neck squamous cell carcinoma (Hep2) and melanoma (SK28 and L1210) tumors. The combination of Dbait32Hc treatment and fractionated radiotherapy significantly enhanced the therapeutic effect. Tumor growth control by Dbait molecules depended directly on the dose and was observed with various irradiation protocols. The induction of H2AX phosphorylation in tumors treated with Dbait 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.

Cancer treatment primarily consists of surgery whenever possible, chemotherapy and radiotherapy. Chemotherapy and radiotherapy cytotoxicity is at least in part due to un repaired 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


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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 D-bait 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 D-bait 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.

response, including ATM, BRCA1, p53-binding protein 1 (53BP1), MDC1, RAD51, and the MRE11/RAD50/Nijmegen breakage syndrome 1 (NBS1) complex, form ionizing radiation-induced foci (19–21). These foci colocalize with γ-H2AX, a phosphorylated form of the H2AX histone (22). The principal mediators of this signaling pathway are the phosphatidylinositol 3-kinase-like family of kinases. At least four phosphatidylinositol 3-kinase-like kinases are involved in the transduction of the signal that originates at broken DNA ends: ATX, ATR, ATM, and DNA-PK, the two later being the most important in the response to irradiation (23, 24). Although γ-H2AX facilitates faithful repair, the biochemical mechanism remains unclear. It is currently admitted that it “tags” the DSBs on chromosomes and facilitates the increase of local concentration of repair factors near the lesion (25–27).

In this study, we propose that introducing short DNA molecules that would be recognized as DSBs by the signaling and repair proteins would hijack them away from the chromosome DSBs to be repaired and consequently increase the sensitivity of the cell to irradiation. To test this hypothesis, we synthesized molecules that were screened for their ability to be recognized by the DNA-PK complex and trigger DNA-PKcs kinase activation. The effect of these molecules, called D-bait (for DSB bait), was first tested in cell culture and then on xenografted tumors.

Materials and Methods

Cell lines and D-bait. Studies of cultured cells were done using Hep2 (head and neck squamous cell carcinoma), HeLa S3 (epithelial carcinoma of the cervix), MO59K and MO59J (glioblastomas), and MRC5 and AT5BI (fibroblasts). Xenograft was done with human tumor cell lines Hep2 and L11205 and SK28 (melanomas). Cells were grown at 37°C in monolayer cultures in complete DMEM containing 10% fetal bovine serum (Invitrogen) and antibiotics (100 µg/mL streptomycin and 100 µg/mL penicillin) under conditions of 100% humidity, 95% air, and 5% CO2.

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Irradiation</th>
<th>D-bait 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>
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<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>10</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>2.9</td>
<td>1; 12</td>
<td>208</td>
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<tr>
<td>LU1205</td>
<td>6 × 5 Gy</td>
<td>Mock</td>
<td>30</td>
<td>99</td>
<td>C-IR</td>
<td>18</td>
<td>19.5</td>
<td>-7; 98</td>
<td>303</td>
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<tr>
<td>SK28</td>
<td>—</td>
<td>—</td>
<td>17</td>
<td>78</td>
<td>0.47 (P &lt; 1.2 × 10^-2)</td>
<td>&gt;27</td>
<td>13.8</td>
<td>-7; 58</td>
<td>400</td>
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<tr>
<td>SK28</td>
<td>—</td>
<td>—</td>
<td>11</td>
<td>63</td>
<td>0.77 (P &lt; 0.46)</td>
<td>30</td>
<td>5.9</td>
<td>16; 39</td>
<td>425</td>
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<tr>
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<td>—</td>
<td>—</td>
<td>31</td>
<td>57</td>
<td>C</td>
<td>0</td>
<td>7.3</td>
<td>-16; 13</td>
<td>100</td>
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<tr>
<td>Hep2</td>
<td>—</td>
<td>—</td>
<td>15</td>
<td>88</td>
<td>0.29 (P &lt; 1 × 10^-5)</td>
<td>17</td>
<td>15</td>
<td>-3; 65</td>
<td>191</td>
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<tr>
<td>SK28</td>
<td>6 × 5 Gy</td>
<td>Mock</td>
<td>32</td>
<td>75</td>
<td>C-IR</td>
<td>12</td>
<td>16.4</td>
<td>-4; 84</td>
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<tr>
<td>SK28</td>
<td>6 × 5 Gy</td>
<td>32Hc 6 × 60 µg (3 nmol)</td>
<td>22</td>
<td>135</td>
<td>0.26 (P &lt; 6.4 × 10^-5)</td>
<td>&gt;39</td>
<td>28.4</td>
<td>2; 127</td>
<td>307</td>
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<tr>
<td>SK28</td>
<td>6 × 5 Gy</td>
<td>8H 6 × 15 µg (3 nmol)</td>
<td>8</td>
<td>76</td>
<td>0.56 (P &lt; 0.21)</td>
<td>&gt;16</td>
<td>7.8</td>
<td>1; 24</td>
<td>184</td>
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</tr>
<tr>
<td>Hep2</td>
<td>—</td>
<td>—</td>
<td>21</td>
<td>49</td>
<td>C</td>
<td>0</td>
<td>3.8</td>
<td>-5; 12</td>
<td>100</td>
<td></td>
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<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; 69</td>
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</tr>
<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>Mock</td>
<td>17</td>
<td>61</td>
<td>C-IR</td>
<td>13</td>
<td>14.2</td>
<td>-3; 45</td>
<td>218</td>
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</tr>
<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 60 µg (3 nmol)</td>
<td>23</td>
<td>123</td>
<td>0.30 (P &lt; 3.9 × 10^-5)</td>
<td>&gt;59</td>
<td>40.7</td>
<td>0; 139</td>
<td>652</td>
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<tr>
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<td>15 × 2 Gy</td>
<td>32Hc 15 × 20 µg (1 nmol)</td>
<td>10</td>
<td>55</td>
<td>0.62 (P &lt; 0.24)</td>
<td>7</td>
<td>6.7</td>
<td>-3; 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>58</td>
<td>0.41 (P &lt; 1.6 × 10^-2)</td>
<td>&gt;39</td>
<td>41.6</td>
<td>-5; 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>11 &gt;150</td>
<td>0.14 (P &lt; 10^-5)</td>
<td>&gt;56</td>
<td>38.7</td>
<td>-3; 139</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^-5)</td>
<td>49</td>
<td>26.6</td>
<td>8; 134</td>
<td>561</td>
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<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 60 µg (3 nmol)</td>
<td>20</td>
<td>129</td>
<td>0.25 (P &lt; 2.2 × 10^-2)</td>
<td>&gt;65</td>
<td>45.2</td>
<td>-3; 139</td>
<td>707</td>
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<tr>
<td>Hep2</td>
<td>15 × 2 Gy</td>
<td>32Hc 15 × 120 µg (6 nmol)</td>
<td>20</td>
<td>&gt;150</td>
<td>0.13 (P &lt; 10^-5)</td>
<td>&gt;86</td>
<td>43.6</td>
<td>1; 139</td>
<td>903</td>
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<td>Hep2</td>
<td>—</td>
<td>32s 15 × 60 µg (6 nmol)</td>
<td>12</td>
<td>46</td>
<td>0.7 (P &lt; 0.3)</td>
<td>6</td>
<td>4.9</td>
<td>0; 19</td>
<td>156</td>
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</tr>
<tr>
<td>Hep2</td>
<td>—</td>
<td>32ss 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; 59</td>
<td>374</td>
<td></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′-GCTGTCGCCACACCCCG-CAAAACAGGCTAGA-(H)-TCTAGGTTTGTGGTGTGGTGTGG- GGACCAAGC3′ Dbait32Hc, where H is a hexaethyleneglycol linker; 5′AGCAGCGGGTTTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGTGGT GTGGTGTGGTGT GG...
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 hexaethylene glycol 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

Fig. 2. Inhibition of DNA repair by Dbait transfection of MRC5 cells. A, immunodetection of 53BP1 (green; top) and NBS1 (green; bottom) foci and γ-H2AX (red) in irradiated cells (10 Gy). Histograms show the quantification (200 cells counted) of the number of foci in nonlabeled (white) or γ-H2AX-labeled (black) cells. B, damage repair after irradiation. MRC5 cells were transfected with 2 μg Dbait32Hc (white) or not transfected (black) and irradiated 10 Gy before analysis at different times after irradiation by alkaline comet assay. Damaged nuclei were nuclei with a comet tail (moment size, >20; see details in Supplementary Fig. S3). Mean from 6 independent experiments (300 nuclei analyzed per condition). C, clonal survival of cells as a function of Dbait concentration. Cells were transfected with various amounts of Dbait32Hc (circle) or Dbait8H (square) without irradiation (dotted lines) or with exposure to 2 Gy irradiation (plain lines). Cell survival was estimated by dividing the number of colony-forming units in treated samples by the number of colony-forming units in untreated samples. D, clonal survival of cells as a function of the irradiation dose. Cells were transfected with 2 μg Dbait32Hc (circle) or Dbait8H (square) for 5 h and exposed to increasing doses of irradiation. Dotted lines, LD50.
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 ATM 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 cohesors. 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 Dbait32Cs (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).

Fig. 3. Radiosensitization of Hep2, LU1204, and SK28 tumors by Dbait32Hc. Tumor volume progression of Hep2 (left), LU1205 (middle), and SK28 (right) subcutaneous tumor xenografts treated as described in Materials and Methods. Four groups of animal for each tumor type received different treatments: NT, no treatment (gray dotted line); 32Hc, Dbait32Hc injections (60 μg/session; gray line); IR, radiotherapy (black dotted line); 32Hc + IR, coupled treatments with irradiation and Dbait32Hc injections (black line). Tumor growth was monitored twice a week. The number of animals included in each group is indicated in Table 1.
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.

**Radiosensitizing effect of Dbait depends on its length of the molecule but not on its sequence.** Dbait molecules that do not activate DNA-PK in vitro such as single-stranded Dbait32ss, and short Dbait8H molecules displayed no radiosensitization activity in vivo (Table 1). To confirm that the sequence is not involved in the radiosensitizing effect of Dbait, we used another molecule, Dbait32H, differing only in the sequence from Dbait32Hc.
Dbaits2Hc. 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 Dbaits. These results are in agreement with our in vitro observations that Dbaits2H and Dbaits2Hc similarly activate DNA-PKcs (Supplementary Fig. S1) and inhibit DNA repair (Supplementary Fig. S3).

Radiosensitization is Dbaits dose-dependent. In vitro, the radiosensitivity of the cells is proportional to the amount of Dbaits-transfected cells (Fig. 2C). To determine if a similar dose dependence is observed in vivo, we compared the efficacy of various amounts of Dbaits2Hc for treating Hep2 tumors (Fig. 5A). Administration of Dbaits2H 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 < 10⁻⁶; Fig. 5A, left; Table 1). Similarly, in association with radiotherapy, increasing amounts of Dbaits 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 × 10⁻⁵), 129 days (RR = 0.25; P < 2.2 × 10⁻³) and 150 days (RR = 0.13; P < 10⁻²) with doses of Dbaits2H of 1, 3, and 6 nmol, respectively (Fig. 5A, right; Table 1). Interestingly, although 1 nmol Dbaits 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.

Radiosensitization by Dbaits is not dependent on fractionation of the radiotherapy and was observed either in association with 10 sessions of 3 Gy (10 × 3 Gy), 6 sessions of 5 Gy (6 × 5 Gy), or 2 sessions of 15 Gy (2 × 15 Gy; Supplementary Fig. S9). With all three radiotherapy protocols, administration of Dbaits increased the efficacy of the radiotherapy with a concurrent increase in median survival, 36 days with 10 × 3 Gy (RR = 0.4; P < 0.13), 79 days with 6 × 5 Gy (RR = 0.28; P < 10⁻⁵), and 20 days with the 2 × 15 Gy radiotherapy protocol (RR = 0.33; P < 0.02).

Lack of toxic effect or immune response in healthy tissues. Large DNA molecules and oligonucleotides (43, 44) carrying CpG sequences have been reported to induce immune responses. To determine if Dbaits induced immune responses, we measured the levels of interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-12P70, IFN-γ, and tumor necrosis factor-α in blood samples at various times after repeated intravenous or subcutaneous injections of Dbaits2Hc or Dbaits2H in BALB/c mice. The molecules differ only in their sequence and were equally efficient in controlling tumor growth (Table 1). Animals received seven injections of 6 nmol Dbaits within 24 days without developing any toxicity or skin inflammation. We found that injection of the CpG-rich Dbaits2H induced a rapid response of IL-6 and a more delayed response of IL-12P70, whereas Dbaits2Hc, 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 Dbaits2Hc and 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 Dbaits injections.

Systemic administration of Dbaits. 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 Dbaits2Hc formulated with PEI in controlling tumor growth. Animals with subcutaneous SK28 tumors received 6 nmol Dbaits2Hc before irradiation (6 sessions). All treated tumors rapidly underwent large central necrosis. Cytology analysis of the tumors sampled 1 week after the end of treatment confirmed areas of large necrosis in tumors treated with intraperitoneal injection of Dbaits (Fig. 5C). Although a lower efficiency than intratumoral administration was observed, systemic delivery of the Dbaits-PEI complex increased the median survival of the treated animals (RR = 0.4; P < 0.009) compared with animals that received only radiotherapy (Fig. 5B).

Discussion

We have developed new molecules (Dbaits) that mimic DSBs and disorganize DNA damage signaling. The molecular characteristics for Dbaits 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 Dbaits 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 Dbaits were due to
the molecular structure as a mimetic substrate rather than to the targeting 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 radiosensitizes 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 standalone 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 kinases 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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