The DNA Repair Inhibitor Dbait Is Specific for Malignant Hematologic Cells in Blood

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Abstract

Hematologic malignancies are rare cancers that develop refractory disease upon patient relapse, resulting in decreased life expectancy and quality of life. DNA repair inhibitors are a promising strategy to treat cancer but are limited by their hematologic toxicity in combination with conventional chemotherapies. Dbait are large molecules targeting the signaling of DNA damage and inhibiting all the double-strand DNA break pathways. Dbait have been shown to sensitize resistant solid tumors to radiotherapy and platinum salts. Here, we analyze the efficacy and lack of toxicity of AsiDNA, a cholesterol form of Dbait, in hematologic malignancies. We show that AsiDNA enters cells via LDL receptors and activates its molecular target, the DNA dependent protein kinase (DNA-PKcs) in 10 lymphoma and leukemia cell lines (Jurkat-E6.1, MT-4, MOLT-4, 174xCEM.T2, Sup-T1, HuT-78, Raji, IM-9, THP-1, and U-937) and in normal primary human PBMCs, resting or activated T cells, and CD34+ progenitors. The treatment with AsiDNA induced necrotic and mitotic cell death in most cancer cell lines and had no effect on blood or bone marrow cells, including immune activation, proliferation, or differentiation. Sensitivity to AsiDNA was independent of p53 status. Survival to combined treatment with conventional therapies (etoposide, cyclophosphamides, vincristine, or radiotherapy) was analyzed by isobolograms and combination index. AsiDNA synergized with all treatments, except vincristine, without increasing their toxicity to normal blood cells. AsiDNA is a novel, potent, and wide-range drug with the potential to specifically increase DNA-damaging treatment toxicity in tumor without adding toxicity in normal hematologic cells or inducing immune dysregulation.

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Introduction

Hematologic malignancies account for approximately 10% of all newly diagnosed neoplasms. Their common characteristic is the presence of chromosomal translocations, a trait not typically observed in solid tumors (1). This, among other characteristics, makes hematologic malignancies a unique class of neoplasms resulting in a unique set of challenges for treatment and the prevention of relapse. A major underlying complication of hematologic malignancies is the development of refractory disease upon patient relapse. DNA repair systems that can reverse the cytotoxicity of many clinically used DNA-damaging agents are among the more widely studied targets to overcome tumor resistance. Targeting DNA repair for cancer therapy has been well validated in solid tumor oncology. PARP inhibitors are the most advanced repair inhibitors, but their apparent hematologic toxicity (2–4) could limit their use in treatment of hematologic neoplasia.

Human cancers typically arise after a long process of random mutations, accompanied by continual selection of more rapidly adapting and proliferating tumor cells. Several of the founder mutations that lead to cancers involve genetic changes in key DNA repair pathways (5). In addition, epigenetic changes via DNA methylation and/or histone methylation and acetylation at the site of DNA repair genes are found in numerous cancers (6). Thus, cancer treatments that target a specific DNA repair defect can be selectively toxic (lethal) to cancer cells, with various DNA repair capacities, while sparing normal (DNA repair proficient) tissues. Genetic instability plays a critical role in inherited and sporadic leukemia and is a common feature of blood malignancy. Chromosomal abnormalities in chronic lymphocytic leukemia (CLL) are detected in up to 80% of patients (7). In general, clonal chromosome aberrations are found in approximately half of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) patients (8). Moreover, several mutations in repair genes are responsible for hereditary lymphoma susceptibility disorders, including ataxia-telangiectasia (A-T), Nijmegen breakage syndrome (NBS), and Bloom syndrome. In the absence of a genetic predisposition, exposure to toxins or immune system alterations (e.g., such as those that occur in acquired immunodeficiency syndrome) may interfere with the mechanisms that assure genetic safety by resolving DNA breaks. Inherited DNA repair defects that predispose individuals to cancer, such as mutations of the ataxia telangiectasia mutated (ATM) gene, are found in several types of cancer, including acute leukemia (9). CLL is another hematologic malignancy with a poor prognosis in which there are mutations in the ATM gene. Such CLL cells have homologous recombination (HR) defects and are amenable to therapy with PARP1 inhibitors (10). AML are sensitive to PARP1 inhibition because of their...
intrinsic genomic instability, and PARP1 inhibition has been found to increase the cytotoxicity of alkylating agents in this leukemia (11). However, the high toxicity of the combined treatment, mainly myelosuppression (neutropenia and thrombocytopenia), was shown to limit the dose in various trials preventing combined treatment to achieve a complete tumor response (12).

Most conventional cancer treatments, consisting of radio- or chemotherapy, target DNA to induce genotoxicity. However, these chemotherapeutic drugs also target normal proliferating cells in naturally proliferating tissues. These tissues, which include bone marrow, gastrointestinal tract, liver, and hair follicles, are also sensitive to DNA-targeting treatments. This leads to dose limitations and may limit the success of treatment. The difference between treatment efficacy on malignant cells and toxicity in normal tissues determines the so-called "therapeutic index." The key to effective cancer treatment is to target tumor cells while sparing normal tissue. This has led to the idea that the search for cures should focus on "disrupting the broader biological pathways that support cancer growth" (13). Given that DNA repair defects are early drivers of many cancers, there is great interest in developing therapeutics that exploits these potential weaknesses. We have designed a new strategy called Dbaït, which uses short DNA molecules, to inhibit all DNA repair pathways involved in double-strand (DSB) and single-strand break (SSB) repair. Dbaït bind and activate DNA damage signaling enzymes, PARP, and DNA-dependent kinase (DNA-PK). Once activated, the two enzymes modify their targets, generating a false DNA damage signal. This inhibits recruitment of key repair proteins, such as BRCA1 and XRCC1, to the site of damage (14, 15), (15, 16). Thus, repair enzymes do not aggregate at damage site and do not form foci after irradiation in Dbaïttreated cells with a pan nuclear phosphorylation of H2AX induced by DNA-PK activation. Repair of single-strand and double-strand DNA breaks [via SSB repair (17), nonhomologous end joining (15), and homologous recombination (18)] is impaired in Dbaïttreated cells. AsidNA is the clinical form of Dbaït. It is composed of the active part of Dbaït, a stable 32-bp long double-stranded DNA with a blunt end, covalently bound to a cholesterol, to allow molecules to enter the cells without the help of transfection vectors. AsidNA (called DT01 for the first-in-man trial) was recently tested in a phase I clinical trial on melanoma skin metastases in association with radiotherapy (19). The results demonstrated the lack of toxicity of locally administered molecules and suggested an increase in radiotherapy efficacy. AsidNA activity was never tested in vitro. Here, we demonstrate its ability to enter hematologic cancer cells and treat lymphoma and leukemia without toxic effects on hematopoietic cells. We explore the specificity of the uptake and target activation and toxicity of AsidNA in lymphoma and leukemia models as well as in normal human blood cells.

Materials and Methods

Culture of malignant hematologic cell lines

Ten hematogenic malignant cell lines including THP-1 and U-937 myeloid leukemia cells;Jurkat-E6.1, MOLT-4, CRL-1582, MOLP-4, CRL-1582, 174xCEM.T2, CRL-1992; U-937, CRL-1593.2, and THP-1, TIB-202 respectively, except for MT-4, which was a kind gift from Dr. Olivier Delelis (CNRS UMR-1021, ENS de Cachan, France). Cell lines were authenticated at the beginning and at the end of the study by short tandem repeat profiling (Geneprint 10, Promega) at 9 different loci (TH01, D5S818, D13S317, D7S820, D16S539, CSF1PO, AMEL, vWA, and TPOX). Cell lines were verified to be negative for mycoplasma contamination using the VenomGeM Advance Kit (Biovalley). Cells were grown according to the supplier's instructions in RPMI1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere at 5% CO2. Reagents for cell culture were purchased from Gibco Invitrogen.

Culture of primary blood-derived cells

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors (Etablissement Français du Sang Cabanel, Paris, France). Written informed consent to use the cells for research was obtained from each donor. PBMCs were extracted by Ficoll–Hypaque density gradient, washed twice in RPMI1640 medium, and directly cultured. The isolation of resting CD4+ T cells, negatively selected from PBMCs, was performed using the Isolation Kit from Miltenyi Biotec according to the manufacturer's protocol with the addition of bead-conjugated antibodies against both early (CD69 and CD25) and late (HLA-DR) activation markers. Cells were depleted using LS columns as recommended by the manufacturer (Miltenyi Biotec) with a purity >98%. When necessary, CD4+ T cells were activated using the T-Cell Activation/Expansion Kit from Miltenyi Biotec and expanded in culture for 10 days. Resting and activated CD4+ T cells were cultured in RPMI1640 supplemented with 10% human serum (Gibco Invitrogen) and 10 to 20 ng/mL rhIL-2. Plasmacytoid dendritic cells (pDC) were purified as described previously (20). Briefly, PBMCs were isolated using Ficoll gradient (Amerham). The pDCs were isolated by flow cytometry (21) as Lin-CD11c+CD4+ cells with a purity >98%. The cells were cultured at a density of 10⁶ cells/mL in complete RPMI Glutamax (Gibco) containing 10% FBS (HyClone).

Dbaït molecules

AsidNA is the clinical form of Dbaït, a 64-nucleotide (nt) oligodeoxyribonucleotide consisting of two complementary 32-nt strands, connected through a 1,19-bis(phospho)-8-hydrazone-2-hydroxy-4-oxa-9-oxo-nondecanecarboxyl linker, with a cholesterol at the 5’-end and three phosphorothioate internucleotide linkages at each of the 5’ and the 3’ ends (Agilent). The sequence is: 5’-X GGSCTs GTG CCC ACC ACC CAG AA CAG CTA GA L - CGTCT ACG CIT GTT TGC GTG GTG GCC AC aGsGc-3’, where L is an amino linker, X a cholesterol tetaoctylencglycol, C a carboxylic (hydroxymycanoic) acid linker and s a phosphorothioate linker. The Cpg-Dbaït molecule only differs from AsidNA by its sequence, which includes seven CpGs (in bold): 5’-X aGsGc CAT GCC TTC TGG GTG TGC GTG TGC CAT CT L - CLA TCC GAA CAA ACG ACC CAA CAC CCG TGA TGC GT-3’ (Agilent).

Chemicals and antibodies

4-hydroxycyclophosphamide (the active form of the cyclophosphamide prodrug) was purchased from Santa Cruz Biotechnology SA (#sc-206885) and dissolved in cell culture
medium to make a stock solution. Etoposide (#E1383) and vincristine sulfate (#V8388) were both purchased from Sigma-Aldrich. Etoposide was dissolved in DMSO to a stock concentration of 500 mmol/L. All chemicals were directly dissolved in the appropriate solvent before the addition of one tenth of the final volume to the cell culture medium. Anti-LDL receptor (#ab60107), anti-LDL receptor Alexa-Fluor-488 (#ab636977), anti-calreticulin PE (#ab83220), and anti-H2AX-pS139 Alexa-Fluor-647 (#ab560447) were purchased from Abcam. Anti-CD4 APC (#130-098-901), anti-CD25 PE (#130-098-861), anti-HLA-DR PerCP (#130-098-179), anti-CD80 PE (#130-099-200), anti-CD83 APC (#130-098-889), and anti-CD86 FITC (#130-098-182) were purchased from Miltenyi Biotec. Anti-CD80 PECy7 (#305217), anti-CD86 PECy5 (#305407), was purchased from eBioscience.

Cell survival and proliferation

Relative cell survival was measured by the mitochondrial MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay or live-cell monitoring. MTT assay was modified for cells in suspension as described previously (22). Briefly, one tenth of the culture volume of MTT solution [5 mg/mL in PBS was added to the cultures and the cells incubated at 37°C in 5% CO2 for 30 minutes to 4 hours (depending on the cells)]. Cells were lysed by adding one culture volume of lysis buffer (50% dimethylformamide, 20% SDS, and 0.22% acetic acid) directly to the medium and incubated for 2 hours at 37°C before reading at 550 nm (Victor-X3, PerkinElmer). For proliferation studies, cells were cultured in 96-well plates: the MT-4, MOLT-4, 174xCEM.T2, Jurkett-E6, 1, Sup-T1, U-937, IM-9, and Raji cell lines were seeded at 2 × 10^3 cells/mL, HuT-78 at 4 × 10^4 cells/mL, and THP-1, PBMCs, and activated and resting CD4+ T cells at 10^6 cells/mL. For the combined treatments, AsiDNA was added to the cell cultures 24 hours before the addition of other drugs or irradiation. MTT assays were performed 4 days after the last treatment except for irradiations that were performed 6 days after. Cell proliferation and cell death were monitored after staining with 0.4% trypan blue (Sigma Aldrich) by visual counting using a Burker chamber. Cell survival was calculated as the ratio of living treated cells to living mock-treated cells. Cell death was calculated as the number of dead cells divided by the total number of cells.

Colony-forming units of granulocytes and/or macrophages assay

The colony-forming units of granulocytes and/or macrophages (CFU-GM) assay was performed as described previously (23, 24). Briefly, 2,000 human blood CD34+ progenitors from bone marrow (Hu-BM-CD34+, #70002.3, Stemcell Technologies) were plated for methylcellulose assays in triplicate in 1.5 mL MethoCult (H4535-Enriched-without-EPO, #04535, Stemcell Technologies) in a 35-mm dish with or without AsiDNA. Cells were cultured for 14 days before colonies were scored for proliferation and differentiation. Differentiation was based on cell shape and proliferation estimated by counting colonies according to the manufacturer’s protocol. Results are expressed as the percentage of differentiated cells and colonies following treatment relative to those of cells that were not.

Annexin V/propidium iodide assay

Cell death analyses was performed on 10^6 living cells harvested and resuspended in 100 μL MACS buffer in the presence of a 1:50 dilution of Annexin V-APC (Miltenyi Biotec), for 20 minutes at room temperature. The viability dye, propidium iodide (from Sigma Aldrich), was then added to a final concentration of 5 mg/mL before flow cytometry analyses.

Analysis of immune cell activation

The membrane activation markers CD69 and CD25, for CD4+ T cells, and CD80 and CD86, for pDCs, were analyzed by flow cytometry according to the manufacturers’ protocols. Flow cytometry analysis was performed on a FACSCalibur, Aria III, or LSRII (BD-Biosciences). At least 10,000 events were collected. All data were analyzed using Flowjo software (TreeStar). Cytokine secretion was quantified by ELISA for human IFNγ (#SIF50, R&D Systems) according to the manufacturer’s protocol.

Regression trendlines, EC_{50} and synergy calculations

Nonlinear regression trendlines were generated using GraphPad (Prism) software. The EC_{50} was calculated as the inflection point of the curves to generate Hill equation values, taking into account the standalone effect of the second drug for the drug combinations. Synergy, additivity, and antagonism were evaluated by the dose reduction index (DRI); combination index, according to the Lowe nonlinear regression model (CI_{Lowe}); and Chou linear regression model (CI_{Chou}) as described previously (25). The DRI was calculated as the ratio between the EC_{50}, obtained for the combination compared with that of the single agents alone, expressed as the fold change. The CI_{Chou} and isobolograms were calculated using the SynerDrug interface based on R software as described previously. Drug-drug interactions can be additive (DRI = 1; CI_{Chou} = 1; CI_{Lowe} = 0), antagonistic (DRI < 1; CI_{Chou} > 1; CI_{Lowe} > 0), or synergistic (DRI > 1; CI_{Chou} < 1; CI_{Lowe} < 0). Isobolograms were calculated as described previously (26) and consist of two parts: a two-dimensional scatterplot of the combinations of inhibitory concentrations and an envelope of additivity determined by two possible additive mechanisms, the first (mode-I) implying independent effects of the two agents and the second (mode-II) implying an interaction between the effects of the two agents: if the combined effect of the two agents falls within this envelope, their effects are additive; if it is below, they are synergistic; and if it is above, they are antagonist.

Statistical analysis

Comparisons between different conditions in experiments were performed using the Wilcoxon test (nonparametric paired test). Correlations were evaluated using the Spearman test (nonparametric test). P < 0.05 was considered to be significant for all statistical tests applied.

Availability

SynerDrug interface based on R software Maps is an open-source collaborative initiative available in the Institut Curie repository (https://github.com/bioinfo-pf-curie/synerdrg), as described previously (25).

Results

AsiDNA is toxic to malignant hematologic cells

We tested the toxicity of AsiDNA monotherapy on a wide range of hematologic cancer cells, including two myeloid leukemias,
four acute T-cell leukemias, two Burkitt B-cell lymphomas, and two T-cell lymphomas (Table 1). We evaluated the half maximal effective concentration (EC_{50}) of AsiDNA for all cell lines by measuring the survival 4 days after treatment with various doses, ranging from 0.16 to 48 \( \mu \text{mol/L} \), the maximum concentration close to the level observed in monkeys after intravenous injection (Fig. 1A and B). AsiDNA was toxic for eight of the 10 tested cell lines, with EC_{50} inferior to 16 \( \mu \text{mol/L} \) for the most sensitive (class 'S': U-937, IM-9, MOLT-4, and Sup-T1) and ranging from 16 to 48 \( \mu \text{mol/L} \) for the cells with intermediate sensitivity (class 'I': MT4, Jurkat-E6.1, 174xCEM.T2, and HuT-78). Two cell lines were resistant up to the maximum tested dose of 48 \( \mu \text{mol/L} \) (class 'R': Raji and THP-1; Table 1). Similar results were obtained with the colorimetric MTT assay (Fig. 1), or direct counting of viable cells by trypan blue enumeration (Supplementary Fig. S1).

Sensitivity to AsiDNA was independent of the cellular subset (myeloid, B cells, T cells) and the type of disease, either leukemia or lymphoma (Table 1). Unexpectedly, the efficacy of AsiDNA in the various cell lines did not appear to depend on the level of p53 activity, as the most sensitive (U-937 and Sup-T1) and resistant cells (Raji and THP-1) were all p53 deficient \( P = 0.3387 \), ns.

AsiDNA is not toxic to normal peripheral blood cells

AsiDNA was toxic to most of the cancer blood cells tested. Thus, we wanted to gain further insights on its effects on normal blood cells. We used fresh whole PBMCs from healthy donors to test global blood cell toxicity. We first performed an \textit{in vitro} AsiDNA dose escalation study similar to that performed for the cancer cells. No significant toxicity was observed at any tested dose, with more than 80% survival relative to nontreated cells at the highest dose of 48 \( \mu \text{mol/L} \) (Fig. 1B). We tested whether cell cycling is required for AsiDNA toxicity by analyzing the fate of resting T cells (CD4r), as well as their activated and cycling \textit{ex vivo} counterpart (CD4a), for up to 11 days after treatment and observed no toxicity at any time point (Fig. 1B).

We analyzed the effect of AsiDNA on bone marrow progenitors using the \textit{ex vivo} CFU-GM assay to confirm its lack of toxicity to normal cells. CFU-GM assays are generally used to investigate the direct adverse effects of xenobiotics on the proliferative and differentiation capacities of blood-forming multipotential stem cells (CD34^+), depending on the humoral growth factors and local cytokines in their specific microenvironment (23, 24). We treated cells with various doses of AsiDNA from 0.48 to 48 \( \mu \text{mol/L} \) and analyzed their ability to form colonies, either differentiated or not, based on their morphology. There was no statistically significant decrease either in proliferation or differentiation under these conditions (Fig. 1C), indicating the absence of acute or delayed toxicity of Dbait on bone marrow human progenitors at doses sufficient for antitumor efficacy in most leukemia and lymphoma tested cell lines (Fig. 1A). These results are in accord with the absence of toxicity found in mice after systemic administration of AsiDNA (personal data).

AsiDNA triggers programmed necrotic and mitotic death in malignant hematologic cells

The observed effects of AsiDNA on malignant hematologic cells were related to the DNA portion of the molecule, as cholesterol alone did not induce any toxicity in the same range of doses (Fig. 2A). This confirms that the effect is due to the capacity of AsiDNA to mimic DSBs. The decrease in the number of colonies for AsiDNA standalone was additive (Table 1). The CI calculated with the two methods (Lowe and Chou) were identical, which suggests that the effect is due to the direct action of AsiDNA on the target cells.

### Table 1.

effects of AsiDNA on hematologic malignant cell lines

| Name status | time (h) | EC_{50} (\mu mol/L) | Sensitivity
|-------------|----------|---------------------|-------------
| Raji M, DEL | 18       | 0.046               | Syn         |
| IM-9        | 19       | 0.219               | Syn         |
| Full-8      | 36       | 0.041               | Syn         |
| Sup-T1      | 24       | 0.021               | Syn         |
| HuT-78      | 24       | 0.010               | Syn         |
| MT4         | 24       | 0.234               | Add         |
| Jurkat-E6.1 | 24       | 0.023               | Syn         |
| 174xCEM.T2  | 24       | 0.003               | Syn         |
| HuT-78      | 24       | 0.003               | Syn         |
| U-937       | 24       | 0.002               | Syn         |
| THP-1       | 48       | nd                  | Add         |
| MOLT-4      | 24       | 0.034               | Add         |
| Sup-T1      | 24       | 0.034               | Add         |
| MT4         | 24       | 0.034               | Add         |
| U-937       | 24       | 0.034               | Add         |
| THP-1       | 48       | nd                  | Add         |
| Raji M      | 18       | 0.046               | Syn         |
| IM-9        | 19       | 0.219               | Syn         |
| Full-8      | 36       | 0.041               | Syn         |
| Sup-T1      | 24       | 0.021               | Syn         |
| HuT-78      | 24       | 0.010               | Syn         |
| MT4         | 24       | 0.234               | Add         |
| Jurkat-E6.1 | 24       | 0.023               | Syn         |
| 174xCEM.T2  | 24       | 0.003               | Syn         |
| HuT-78      | 24       | 0.003               | Syn         |
| U-937       | 24       | 0.002               | Syn         |
| THP-1       | 48       | nd                  | Add         |
| MOLT-4      | 24       | 0.034               | Add         |
| Sup-T1      | 24       | 0.034               | Add         |
| MT4         | 24       | 0.034               | Add         |
| U-937       | 24       | 0.034               | Add         |
| THP-1       | 48       | nd                  | Add         |

**NOTE:** All experiments were performed in triplicate. **CI:** Concentration index. **CI_Lowe:** Lowe nonlinear regression. **CI_Chou:** Chou linear regression.
of living cells was associated with a corresponding increase in the number of dead cells, characteristic of the cytotoxic activity of AsiDNA, rather than an arrest of division. The absence of a perturbation of the cell cycle was confirmed by analysis of the distribution of the treated cells in the cycle (sensitive or resistant to Dbait), which was unaffected after 12, 24, or 48 hours of Dbait treatment, regardless of the dose (Supplementary Fig. S2).

However, a global increase of cells in the G2 phase started to appear after 3 days of treatment and increased by the fourth day in cell lines that displayed sensitivity to AsiDNA (Fig. 2B). This increase of cells in G2 was concomitant with cell death. The most sensitive cells (U-937) showed the strongest increase in the proportion of cells in G2. In contrast, the resistant and intermediate sensitive cell lines, which did not die at these doses of AsiDNA did not show any short- or long-term variations of the cell cycle (Fig. 2B). By 4 days of treatment, the number of polyploid cells and those in the sub-G0–G1 subset, increased slightly, but this increase was significant only for p53-proficient IM-9 cells (Fig. 2B). Annexin V/propidium analysis after 4 days of treatment revealed a large increase of necrosis and, to a lesser extent, late apoptosis in cells treated with AsiDNA (Fig. 3C). Cholesterol, without the DNA moiety to activate DNA-PK, had no effect on cell survival or the cell cycle. Calreticulin exposure at membrane of dying cells (ecto-CRT) is observed in all the cells treated by AsiDNA in a dose-dependent manner as a marker of an endoplasmic reticulum stress and programmed cell death (27).

Altogether, these results strongly suggest that AsiDNA induces mitotic cell death in malignant hematologic cells independently of their p53 status.

Cell membrane LDL receptors control AsiDNA uptake

The lack of sensitivity of normal and resistant cells could be due to the poor uptake of AsiDNA by these cells. We analyzed intracellular AsiDNA levels 24 hours after treatment using Cy5.5-labeled fluorescent AsiDNA (Fig. 3A). All cell lines displayed AsiDNA uptake with 80% to 100% showing Cy5.5 fluorescence. The individual cell fluorescence intensity varied little among different cells in the same cell culture. However, the mean value of fluorescence intensities (MFI) varied by up to 10-fold between cell lines, indicating that all cell lines can uptake AsiDNA, but with different efficiencies. The MFI did not significantly reflect the variation of cell size (Spearman correlations: cell volume/Cy5.5 MFI, \(P = 0.1237\) ns). AsiDNA cellular uptake is promoted by its cholesterol moiety (28). We thus evaluated the cell membrane concentration of the low-density lipoprotein receptors (LDLRs) on cell surfaces of different cell lines using a fluorescent LDLR-labeled antibody.

Figure 1.

Survival of malignant hematologic and normal human primary immune cells in response to AsiDNA dose escalation. Cell survival was determined by MTT assay after 4 days of treatment. A, Malignant hematologic cells were classified according to their sensitivity to AsiDNA. Sensitive (green): U-937 (cross), Sup-T1 (asterisk), MOLT-4 (diamond), IM-9 (triangle); intermediate (blue): Jurkat-E6.1 (circle), MT-4 (square), 174xCEM.T2 (rectangle), HuT-78 (star); resistant (red): Raji (triangle), THP1 (cross). Data are the mean of at least six independent experiments. B, Normal human primary blood cells: PBMCs (black diamonds), activated CD4 T cells (blue circles), and resting CD4 T cells (red squares). Cell survival was determined by MTT assay after 4 (plain lines), 7 (dashed lines), and 11 days of treatment (dotted lines). Data are the mean of three to four independent experiments with cells from different donors. C, CFU-GM assay on bone marrow stem cells. Proliferation (squares) and differentiation (diamonds) were assayed after 14 days of treatment with AsiDNA. Data are the mean of three to four independent experiments. Error bars, SD.
lipoprotein receptor (LDL-R), which plays a key role in cholesterol-rich LDL endocytosis. The LDL-R is differentially expressed in malignant hematologic cells (29, 30), and its membrane concentration varied by approximately 10-fold within our set of cell lines (Fig. 3A). The respective mean concentration of AsiDNA and the level of LDL-R at the membrane were highly correlated (Spearman $P < 0.0001$; Fig. 3C). LDL-R expression is induced by IL2 in NK cells (31). Here, the level of LDL receptors at the membranes of T cells activated by IL2 and anti-CD3/CD28 TCR were 5-fold higher than in

![Figure 2](image_url)

**Figure 2.** AsiDNA triggers necrotic and mitotic death of malignant cells. Four cell lines were treated with cholesterol or AsiDNA at 4.8 $\mu$mol/L for Sup-T1, MOLT-4, and M-9, or 0.48 $\mu$mol/L for the highly sensitive U-937. A, Proliferation after 2, 3, and 4 days of treatment measured by counting live (solid black) and dead (grey) cells in untreated sample (circles) or treated with cholesterol (triangles) or AsiDNA (squares). Flow cytometry analysis. B, Cell cycle after 2, 3, or 4 days of treatment: percentage of cells in polyploidy (black), G2-M (dark gray), S (light gray), G0-G1 (white), and sub-G0-G1 (hatched). C, Cell death after 4 days of treatment monitored by Annexin V/propidium iodide (AV/PI) staining: early apoptosis (AV$^+$/PI$^-$/C0, white), late apoptosis (AV$^+$/PI$^+$, hatched), or necrosis (AV$^-$/PI$^+$, black). Results are the mean of three to four independent experiments. Error bars, SD. D, Membrane calreticulin exposure (ecto-CRT) after 3-day AsiDNA treatment: nontreated (gray), treated with AsiDNA 1.6 $\mu$mol/L (dashed line), 4.8 (dotted line), and 16 $\mu$mol/L AsiDNA (plain line). One representative experiment out of three is shown.
resting T cells (Fig. 1A). This increase was associated with a similar increase in AsiDNA uptake (Fig. 3A and C). The activation of DNA-PK, revealed by the extent of H2AX phosphorylation, was also higher in activated than resting cells (Fig. 3B). However, this increase was not associated with an increase of sensitivity to AsiDNA, as resting and activated T cells were similarly resistant to AsiDNA treatment (Fig. 1A). The resistance to AsiDNA may reflect the incapacity of the cells to trigger DNA-PK activation, one of the first steps required for AsiDNA/DbaI efficacy (14). However, there was no correlation between the level of phosphorylated H2AX and intracellular AsiDNA levels (Fig. 3D). Moreover, neither AsiDNA cell uptake (Supplementary Fig. S3A and S3B) nor activation of H2AX phosphorylation (Supplementary Fig. S3C) are predictive biomarkers for sensitivity to AsiDNA, contrary to the micronuclei frequency (Supplementary Fig. S3D) that have been recently reported on breast cancer cell lines (18).

Synergistic effects of AsiDNA in combination with chemotherapies

Genotoxic chemotherapies are often used to treat leukemia and lymphomas. They consist mostly of alkylating agents, such as cyclophosphamide (4-HC), which represents the spearhead of this drug class, drugs targeting DNA topoisomerase II, such as the nonintercalating poison etoposide, and mitotic poisons, such as vincristine. We analyzed the effect of combining AsiDNA with these drugs or irradiation (a treatment inducing a wide range of DNA damage) on the various blood cancer cell lines already used in this study. We first determined the EC50 for each monotherapy, using the same protocol used for AsiDNA (Fig. 4A). The cell lines displayed different sensitivities to conventional chemo- and radiotherapy (Fig. 4B). All chemotherapies demonstrated toxicity for normal hematologic cells after 4 days, except for etoposides, for which toxicity was only detectable after 7 days of treatment. AsiDNA was not toxic at the concentrations tested even after 14 days of culture (Figs. 5A and B and C).

Various concentrations of AsiDNA were added to the cultures 24 hours prior to the chemotherapy/radiotherapy to monitor its effect on conventional therapy efficacy. We determined the EC50 of conventional treatments with and without AsiDNA and analyzed the DRI; combination index and isobolograms of the various combinations (Fig. 4; Supplementary Figs. S4–S7; Table 1). All cell lines were sensitized by AsiDNA to at least one specific treatment (Fig. 4B), whereas nonmalignant cells did not even show sensitization to the highly toxic 4-HC, confirming the specificity of AsiDNA for cancer cells. AsiDNA strongly synergized with etoposide, 4-HC, and radiotherapy treatments, which mainly act by directly inducing DNA damage, and to a much lesser extent with vincristine, which is a mitotic spindle poison (Fig. 4; Supplementary Figs. S4–S7; Table 1).

AsiDNA does not affect primary immune cell functions

Most immune cells can be activated or repressed by small molecules. AsiDNA molecules have a DNA moiety that could...
activate immune cells due to their recognition by Toll-like receptors (TLR). AsiDNA does not contain any CpG sequence, which is known to be the major dinucleotide site detected by the TLRs. A molecule containing seven CpG in the DNA sequence (CpG-Dbait) was used as a positive control of immune stimulation. We assessed the ability of these molecules to trigger pDCs and CD4⁺ T-cell activation. Although pDCs display high levels of surface TLR-9 and are highly sensitive to the CpG response, AsiDNA had no effect on pDC. In contrast, CpG-Dbait triggered upregulation of the dendritic cell-surface activation markers CD80 and CD86 between 1 and 3 days after treatment, which decayed by the fourth day (Fig. 5C; Supplementary Fig. S8). IFNα secretion by the pDCs (assayed by ELISA) peaked after two days of Dbait-CpG treatment (Supplementary Fig. S8) but no significant variation after AsiDNA treatment (data not shown). We analyzed the effect of AsiDNA on resting CD4⁺ T cells (Fig. 5A) and their ex vivo activated and cycling counterparts (Fig. 5B). Treatment of resting T cells with AsiDNA or CpG-Dbait neither induced or reduced the surface expression of CD69 or CD25 activation markers (Fig. 5A). No effects were observed for AsiDNA treatments on activated T cells, whereas CpG-Dbait–treated cells showed a slight increase in the level of activation markers at the higher doses. These findings are concordant with our previous experiments on bone marrow stem cell differentiation by the GM-CFU assay that showed no effects on their differentiation at any concentration tested (Fig. 1C).

Discussion

Dbait is an innovative inhibitor of DSB and SSB repair (14, 15). The molecules were developed and tested to increase sensitivity of solid tumors to radiation (16) and chemotherapy (32, 33). However, no tests have yet been performed in blood cells. Here, we tested the effects of AsiDNA, the form of Dbait molecules used in the clinic (19), on the treatment of malignant blood cells and the toxicity of combined treatment to human blood cells. All tests were performed on human cells, as mouse blood cells have been shown to contain 50-fold less DNA-PK, one of the main targets of Dbait, than human blood cells (34).
All the cells were able to take up AsiDNA, although with different efficiencies. The sensitivity to AsiDNA did not correlate with uptake of the molecule or H2AX phosphorylation. This discrepancy probably reflects the numerous parameters involved in cell death induced by AsiDNA treatment. The fact that all the cell lines were sensitized to at least one treatment indicates that AsiDNA uptake and activity in malignant cells is not the limiting factor. Normal cells are fully resistant to the sensitizing effect of AsiDNA, although they incorporate AsiDNA and activate H2AX phosphorylation. These results suggest that specific alterations in malignant cells are required for sensitivity to AsiDNA. The possibility that P53 proficiency could protect cells from AsiDNA was excluded as in the three p53-proficient malignant cells lines (IM-9, MOLT-4, MT-4, and 174-CEM), AsiDNA show a synergistic effect in combination with at least one chemotherapy treatment. In contrast, AsiDNA alone or in combination with other treatments had no significant activity either in normal resting or activated cells, eliminating proliferation as a major condition for

Figure 5.
AsiDNA does not affect primary immune cell functions. Activation and treatment with AsiDNA or CpG-D bait molecules of CD4+ T cells. A-C, Resting CD4+ T cells (A), activated CD4+ T cells (B), and pDCs (C). Left, one representative flow cytometry analysis; right, mean of at least three independent experiments on different donors. Results are expressed as the percentage of cells for each compartment. A and B, CD69+/CD25+ (hatched), CD69+/CD25- (gray), CD69- /CD25+ (black), and CD69- /CD25- (white). C, CD86+/CD80+ (hatched), CD86+/CD80- (gray), CD86- /CD80+ (black), and CD86- /CD80- (white). Isotype, unactivated, and activated cell controls are shown. pDC-activated controls were treated by CpG oligonucleotides. Error bars, SD.
responsiveness to AsiDNA. Jdey and colleagues recently demonstrated in breast cancer cell lines that genetic instability revealed by transcriptional defects in DNA repair pathways and cell-cycle control, micronuclei formation, and large-scale chromosome rearrangements predicts sensitivity to AsiDNA. Our results indicate that genetic instability could also be a biomarker of AsiDNA sensitivity in hematologic malignancies. Indeed, micronuclei frequency was found to correlate with the sensitivity of the hematologic cell lines to AsiDNA (Supplementary Fig. S3D). Moreover, the most resistant cell line to AsiDNA, THP1, is considered to be the most physiologic model of the monocyte phagocyte lineage and can differentiate into macrophages or moDCs in response to cytokine treatment.

White blood cells are highly prone to enter apoptosis when exposed to DNA-damaging agents. However, we only observed a moderate increase (from 2 to 5-fold) of late apoptotic cells. Even p53-proficient IM-9 cells did not show higher levels of apoptosis than other p53-deficient cells, further eliminating a potential role of apoptotic cell death in the sensitivity of cancer cells to AsiDNA. The death induced by AsiDNA was delayed, detectable only at late stages, stochastic, and asynchronous, as we did not observe a large drop in the number of viable cells at any time.

AsiDNA sensitized cells to etoposide, 4-HC, and radiotherapy, which act by inducing chromosomal DNA damage, but not to the mitotic spindle poison vincristine. This result confirms that AsiDNA sensitizes liquid tumors to treatments by inhibiting the repair of DNA damage induced by the associated treatments, as it does in solid tumors. One of the most striking results was the high sensitization of the lymphoma models to radiotherapy. Radiotherapy is often used for the treatment of cancer, such as non-Hodgkin lymphoma, Hodgkin lymphoma, and all types of leukemia, and the addition of AsiDNA could increase the efficacy of such treatment. We previously demonstrated in a first-in-man clinical trial that AsiDNA does not increase local toxicity of the irradiation (19). Treating lymphomas by combining treatment with AsiDNA and radiotherapy is a potentially promising method.

Overall, the addition of AsiDNA to radiotherapy and DNA-damaging chemotherapy increased cytotoxicity of these DNA-damaging drugs in most malignant hematologic cell lines. Given the lack of toxicity of this combination in normal cells, the combination of AsiDNA and DNA damage–inducing agents should be considered for the treatment of such pathologies, in particular, at recurrence when resistance to treatments appears.

Disclosure of Potential Conflicts of Interest
M. Dutreix is a consultant at and reports receiving a commercial research grant from Onxeo. No potential conflicts of interest were disclosed by the other authors.

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References

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