The novel DNA methylation inhibitor zebularine
is effective against the development of
murine T-cell lymphoma

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ABSTRACT

Gene silencing by CpG island promoter hypermethylation has awakened the interest for DNA demethylating agents as chemotherapy drugs. Zebularine \[1-(\beta-D-\text{ribofuranosil})-1,2\text{-dihydropyrimidin-2-one}\] has been recently described as a new DNA methylation inhibitor. Here we have studied its effects in a mouse model of radiation-induced lymphomagenesis using Nuclear Magnetic Resonance (NMR) and Positron Emission Tomography (PET). All control animals presented large thymic T-lymphomas and died between 4-5.5 months. In contrast, 40% (12 of 30) of zebularine-treated animals were still alive after one year (Kaplan-Meier $p<0.0001$). NMR and PET imaging showed that surviving animals presented a thymus structure/volume similar to normal mice of the same age. Most important, zebularine demonstrated a complete lack of toxicity in non-irradiated control mice. DNA hypomethylation induced by zebularine occurred in association with depletion in extractable DNA methyltransferase 1 protein. Thus, our data support the role of zebularine as a DNA demethylating agent with antitumoral activity and little toxicity.
Introduction

Hypermethylation-associated silencing of tumor suppressor genes has been shown to occur in lymphoid/hematopoietic malignancies, disrupting many cellular pathways.\(^1\)-\(^3\) Unlike genetic alterations in cancer, hypermethylation changes are potentially reversible by pharmacological inhibition of DNA methylation.\(^4\) The cytidine analogs 5-aza-2'-deoxycytidine and 5-azacytidine are capable of reactivating tumor suppressor genes that were silenced by hypermethylation.\(^4\) These compounds act through DNA methyltransferase (DNMT) inhibition.\(^4\) Thus far, it is in hematological malignancies where DNA demethylating agents have had their greatest success.\(^4\)-\(^6\) For high-risk myelodysplastic syndrome (MDS) using 5-aza-2-deoxycytidine, it has been reported overall response rates of 49-54\% with 23-50\% complete responses\(^7\)-\(^9\) with a lasting effect on the platelet count.\(^10\) In myelogenous leukemia (CML), 5-aza-2-deoxycytidine achieved objective responses in 55\% of patients\(^11\), including imatinib-refractory patients\(^12\). For 5-azacytidine, the studies have demonstrated significant complete and partial remissions in MDS patients\(^13\),\(^14\) and improved quality of life.\(^15\) In 2004 the FDA approved 5-azacytidine for the treatment of all MDS subtypes.

Zebularine \([1-(\beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one]\) is a new cytidine analog that form a covalent complex with DNA methyltransferases,\(^16\) depletes human DNMT1,\(^17\),\(^18\) reactivates hypermethylated genes in yeast and solid tumor cells,\(^19\) has antitumoral effects in mouse xenografts,\(^19\) and shows a preferential effect on cancer cells.\(^18\) To gain insight into the effects of zebularine, we used a mouse model of radiation induced T-cell lymphoma.\(^20\) Here, we show that zebularine is effective against the development of thymic lymphoma inducing longer overall survival, and NMR-PET images and a pathological histology that closely resembles the normal thymus. These therapeutic changes occur in a context of lack of toxicity, global genomic hypomethylation and a depletion of the DNA methyltransferase 1 (DNMT1).

Study design

Murine models and drug treatments

C57BL/6J four-week mice were irradiated by gamma rays \((1,75 \, \gamma)\) per week during four consecutive weeks.\(^20\) About 90\% of these mice develop, with average latency of 4.5 months, thymic T-cell lymphomas.\(^20\) These thymus-dependent T-cell malignancies are formed by blasts that are positive for CD7, CD2, IgH\(^G\) and TCR\(\beta\)\(^R\) and negative for MPO and VpreB.\(^20\) Two groups of 30 mice (each one with 15 males and 15
females) were generated: the drug-group receiving zebularine dissolved in Phosphate Buffer Saline (PBS) and the control-group receiving only PBS. Intraperitoneal (IP) inoculation was used. For toxicity determination, two groups of 20 non-irradiated mice were also IP inoculated with zebularine or PBS. The histopathology analysis used tissue samples stained with hematoxylin and eosin (H&E). For the mouse xenograft model of T-cell acute lymphoblastic leukemia, 10^6 cells of the cell line MOLT-4 were injected subcutaneously to ten six-week-old female athymic nude mice nu/nu (Harlam Sprague Dawley, Indianapolis, IN).

**Nuclear Magnetic Resonance and Positron Emission Tomography**

Images were acquired on a Bruker Biospec spectrometer (Bruker, Ettlingen, Germany)/Magnex 4.7 T 40 cm bore horizontal magnet MRI system. Images of the mouse thymus taken from a 3D data set were acquired with standard gradient coil capable of a 200 µs rise time and 300 mT/m maximum gradient strength. We analyzed the mice for PET F-18 FDG whole body scans. F-18 FDG dose injected intravenously was 9.8 µCi. In all mice torso PET acquisition was performed using the UGM PENN PET 240H Camera. A transmission scan was acquired after injection F-18 FDG, with Cs-137.

**Determination of 5-methylcytosine (5mC) DNA content, CpG island methylation status and western blot of Dnmt1.**

The 5-methylcytosine DNA content was determined by High Performance Capillary Electrophoresis (HPCE)\(^{21,22}\) using a P/ACE MDQ system (Beckman-Coulter). Three replicate analyses were performed. Western blot analysis was developed using the Dnmt1 antibody (Abcam, Cambridge, United Kingdom), as previously described.\(^{22}\) The CpG island methylation status of the p16\(^{\text{INK4a}}\), p15\(^{\text{INK4b}}\), MGMT, MLT-1, RASSF1A and E-cadherin genes was analyzed by bisulfite genomic sequencing and methylation-specific PCR, as previously described.\(^{22}\)

**Results and discussion**

We used a mouse model of radiation-induced lymphomagenesis (Figure 1A) in which thymic T-cell lymphomas kill all mice in a period of 4-5.5 months.\(^{20}\) We
performed a complete and exhaustive non-invasive imaging follow-up by NMR and PET of zebularine-treated mice compared to a control group treated with the buffer PBS. A 8 mg/mouse/dose (400 mg/Kg), pH=7 dissolved in PBS (Phosphate Buffer Saline) of zebularine was given with the following experimental procedure: 200 μl/mouse/dose once a day, every day during seventy-eight consecutive days.

All the PBS-treated control animals presented large thymic lymphomas and died, as expected, between 4-5.5 months after irradiation (Figure 1B). However, 40% (12 of 30) of zebularine-treated irradiated animals were still alive after one year (Kaplan-Meier p<0.0001) (Figure 1B). The lymphoma relapse in the remaining mice always occurred after completion of the treatment, and in these cases the contribution of methylation-independent events to our lymphomagenesis model, such as homozygous deletions at the p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b} loci, could also be relevant.\textsuperscript{23} NMR and PET imaging results confirmed that surviving animals presented a thymus similar in structure and size to normal mice of the same age, and with a 5-8 fold lower volume than thymic lymphomas arising in the irradiated PBS-treated animals (Figure 1C and 1D). Furthermore, a high rate of metabolic activity was observed in the thymic lymphomas, whilst the thymus of zebularine-treated animals demonstrated a low metabolic activity, as in the thymus of normal mice (Figure 1D). Most importantly, zebularine demonstrated a complete lack of toxicity in non-irradiated control mice, showing the same thymic features by NMR and PET and overall survival time than those non-irradiated control mice that received the buffer PBS (Figure 1B and 1C).

All mice receiving zebularine, from the control group and the one year survival animals from the irradiated-lymphoma group, were sacrificed and careful pathological studies were developed. We failed to identify any sign of toxicity in both groups for any organ or tissue. We did not observe any presence of chromosomal abnormalities after zebularine treatment using an standard G-band karyotype and a Spectral Karyotype Analysis (SKY) (Supplemental Figures). In the case of the irradiated animals, the thymus had a phenotype similar to that observed in mice of similar age without any evidence of T-cell lymphoma (Figure 2A). Only in 2 of 12 (16%) cases we found abnormal lymphocyte hyperplasia in the thymus.

Our model of lymphomagenesis also demonstrated that the growth inhibitory effects of zebularine occurred in parallel with its DNA demethylating action. The thymus of one year survival irradiated-mice receiving zebularine had a 30% reduction of 5-methylcytosine genomic content in comparison with the radiation-induced thymic
lymphomas receiving only PBS: 2.25% ±0.02 vs 3.25 ±0.1 (Figure 2B). Moreover, we observed demethylation of the hypermethylated CpG islands of the p16^{INK4a}, MGMT, MLT-1 and E-cadherin genes in association with the restoration of their gene expression (Supplemental Figures). Furthermore, this DNA hypomethylating effect was associated with a depletion of the levels of extractable DNMT1 protein (Figure 2C), suggesting the trapping of this enzyme to zebularine-incorporated DNA.

Finally, we tested the efficacy of zebularine in a human experimental model equivalent to our irradiated-mice developing thymic T-cell lymphomas. Nude mice were subcutaneously injected with 10^6 cells from the T-cell acute lymphoblastic leukemia cell line (MOLT-4) and treated with zebularine or PBS. All mice were killed 16 days after injection and the tumors were dissected and weighed. Whilst the buffer-treated MOLT-4 xenografts formed tumors rapidly, the zebularine-treated group demonstrated extremely low tumorigenicity (Figure 2D). At the time of sacrifice, the lymphomas were fourteen times larger in PBS-treated mice, 650 ±117 mg, than in zebularine-treated mice, 47 ±37.6 mg (Figure 2D), supporting the efficacy of the drug in a human genetic background of T-cell lymphoma. The growth inhibitory effects of zebularine occurred in parallel with its DNA demethylating action demonstrated by a reduction in the 5-methylcytosine genomic content, the demethylation of the hypermethylated CpG islands of the RASSF1A and p15^{INK4b} genes, and the depletion of DNMT1 protein levels (Supplemental Figures).

In summary, recent important observations place zebularine as an important candidate agent for the epigenetic therapy of cancer. However, before clinical trials are undertaken, it is necessary to test the lack of toxicity and the antitumoral effect of zebularine in a well-defined mouse cancer model. We provide here this missing step demonstrating that a long-term administration schedule of zebularine inhibits the growth of thymic lymphomas in mice, in association with its DNA hypomethylating action without evident side effects for normal cells or the well-being of the animals.
References


Figure Legends

**Figure 1.** Effect of zebularine on a mouse model of gamma-induced thymic lymphoma determined by overall survival, NMR and PET. (A) Schematic diagram of the gamma-induced mouse model of lymphomagenesis, zebularine treatment and NMR and PET imaging follow-up. (B) Up, Kaplan-Meier survival curve; γ: gamma irradiation, Tb: start of zebularine treatment, Te: end of zebularine treatment. Dark and grey straight lines show non-irradiated control mice treated with PBS and zebulatine (Zeb), respectively; filled diamond line shows irradiated and PBS treated animals; and fill square line shows irradiated and zebularine treated mice. Below, Table summarizing the total number of animals used in the study (n) and the surviving animals (alive) (C) Follow-up of thymus and thymic lymphoma sizes by NMR using a Bruker Biospec spectrometer/Magnex 4.7 Teslas 40 cm bore horizontal magnet MRI system. Image analysis and tumor volume measurements were performed with the public domain software ImageJ (NIH, http://rsb.info.nih.gov/ij). A marked volume reduction is the thymic mass is observed in zebularine-treated mice. (D) Metabolic activity in FDG-PET imaging (Positron Emission Tomography scanning with (18F)fluorodeoxyglucose); PET image shows a significant "hot spot" (yellow colour) confined in the thymic lymphoma, that it is lost in the zebularine-treated mice.

**Figure 2.** Effect of zebularine on histology, lymphoma xenografts and DNA methylation parameters. (A) Hematoxylin and eosin (H&E) stained 4 μm section thymus samples. The pathological findings in the thymus of the zebularine-treated mice resemble the structure of the normal thymus. (B) Analysis of global DNA methylation by High Performance Capillary Electrophoresis (HPCE). The irradiated thymus of the zebularine treated mice shows DNA hypomethylation compared with the lymphomas appearing in the irradiated mice that only receive PBS. (C) Western blot analysis of DNMT1 protein levels in normal thymus, lymphomas appearing in the irradiated mice than only receive PBS and in irradiated thymus of the zebularine-treated mice. (D) Up, Female athymic nude mice 16 days after injection of 10⁶ MOLT-4 cells. Note the large tumor on the left, corresponding to a PBS-treated mouse, and the small tumor on the opposite figure, corresponding to zebularine-treated mouse. Tumor detail in mm and weight in mg. Below, effect of zebularine treatment on the in vivo growth of MOLT-4 cells. Tumor size was monitored over time, and size is shown in cubic milimeters.
Tumoral weight data at 16 days from PBS and zebularine treated MOLT-4 xenografts presented as means ± SD.
Figure 1

A. Whole body gamma-irradiation leads to I.P. Zebularine inoculation. NMR and PET follow-up reveals initial symptoms and last control death. Surviving animals exhibit thymic lymphoma incidence.

B. Graph showing % tumor progression over time (1-13 months).

C. Bar chart comparing thymus volume (mm³) in γ-irradiated and non-irradiated mice with and without Zebularine treatment:
- Thymic symptoms: 187mm³, 19mm³, 13mm³, 29mm³, 20mm³, 20mm³
- Zebularine treated: 154mm³, 21mm³, 10mm³, 19mm³, 18mm³, 19mm³
- Non-irradiated PBS-treated: 167mm³, 25mm³, 19mm³, 29mm³, 20mm³, 20mm³

D. Imaging of lymphoma progression:
- Thymic lymphoma before and after treatments.
- Zebularine treated: 19mm³, 19mm³, 20mm³, 20mm³
- PBS-treated: 21mm³, 20mm³, 20mm³, 20mm³
Figure 2

A

Normal thymus

Thymic lymphoma

Zebularine treated

B

% 5-methylcytosine DNA

3.5

3

2.5

2

1.5

1

0.5

0

Thymic lymphoma

Zebularine treated thymus

C

DNMT1

Actin

Normal

Thymic lymphoma

Zebularine treated thymus

D

PBS

ZEB

751 mg

26 mg
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