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Zebularine Significantly Sensitizes MEC1 cells to External Irradiation and Radiopharmaceutical Therapy When Administered Sequentially *in Vitro*

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Abstract

Zebularine is a cytidine analog incorporated into DNA during replication, inhibiting DNA methyltransferase 1 (DNMT1), resulting in demethylation and changes in gene expression. Such modification may improve radiosensitivity in resistant lymphoma cells. The hypothesis of this study was that zebularine and radiation would synergistically inhibit cell growth and viability. Human MEC1 malignant B cells were incubated with 0–200 μM zebularine for 48h. Media containing zebularine was removed, and the cells were irradiated with 0–2 Gy of either external beam irradiation or ¹⁷⁷Lu-DOTA-TATE, a radiolabeled somatostatin analogue. Concentration and viability were measured over 48–72h. The proportion of apoptotic cells was identified using an active Caspase 3/7 assay. Zebularine inhibited growth of cells in a dose-dependent manner during exposure. No residual growth inhibition occurred following removal of the drug. Zebularine and external irradiation inhibited cell proliferation in a dose-dependent, synergistic interaction, but the effect on viability was additive. Treatment with zebularine and ¹⁷⁷Lu-DOTA-TATE resulted in less inhibition of proliferation (P=0.0135), but a synergistic decrease in viability. Apoptotic fraction was much higher in cells irradiated with ¹⁷⁷Lu-DOTA-TATE than external irradiation. External irradiation arrests growth arrest rather than apoptosis. Apoptosis is the primary effect of radiopharmaceutical therapy on tumor cells. Treatment with the methylation inhibitor, zebularine, appears to synergistically augment these natural effects *in vitro*, which could be exploited clinically.

Keywords

lymphoma; demethylation; radiopharmaceuticals; somatostatin analogue; radiosensitivity

1. Introduction

Radiation has been used in conjunction with chemotherapy to treat non-Hodgkin lymphomas (nHL) (Williams *et al.*, 2004; Rodel *et al.*, 2005). For both relapsed-refractory lymphoma

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and in total body irradiation in preparation for hematopoietic stem cell transplant, radiation is used with the intention of eradicating residual disease (Kaminski *et al.*, 2000; Gyurkocza *et al.*, 2010). The quality of radiation, high dose-rate external beam and low dose-rate radiopharmaceutical radiation, can have differing effects on the biology of the disease. Sensitizing the lymphoma cells could result in better control or eradication of the disease. Lymphomas have been shown to have many genes epigenetically dysregulated, often by hypermethylation, that serve in progenitor-cell pathways (Rahmatpanah *et al.*, 2006; Choi *et al.*, 2010). The use of demethylating drugs to disrupt pathway hypermethylation and sensitize lymphomas to radiation has not been explored.

The demethylating agent, zebularine, is a cytidine analog that is incorporated into DNA during replication. It has a preferential inhibiting effect on DNA methyltransferase 1 (DNMT1) through formation of a covalent bond with the enzyme at the 6-position on the pyrimidine ring resulting in irreversible binding (Cheng *et al.*, 2003, 2004). This bond results in the metabolic destruction of the enzyme, and demethylation of the DNA sequence. Zebularine is an acid-stable ribonucleoside with previously demonstrated parenteral and oral bioavailability (Holleran *et al.*, 2005). This makes it an attractive compound for clinical use, as all other cytidine analogues in current use must be administered parenterally to have activity (Holleran *et al.*, 2005).

¹⁷⁷Lu-DOTA-TATE has shown promise as a therapy agent in human somatostatin receptor (SSTR) expressing tumors (Valkema *et al.*, 2006; van Essen M. *et al.*, 2006). As nHL expresses SSTR2 receptors in ~80% of cases, this radiopharmaceutical compound offers potentially effective, selectively targeted radiotherapy (Weiner and Thakur, 2005). The efficacy of radiopharmaceutical therapy has been demonstrated by the currently approved products, Zevalin® and Bexxar® (Oriuchi *et al.*, 2005; Wiseman *et al.*, 2000). However, their ability to interact with chemotherapy is hampered by bone marrow dose limitations. The favorable plasma kinetics of a radiolabeled peptide may deliver a similar lethal radioactive payload to the malignant cells with minimal marrow effect, allowing it to be combined with chemotherapy in close temporal proximity. The question of optimal radionuclide between the currently available radioimmunotherapy drugs is a source of debate (Cheson, 2005). Lutetium-177 offers potential advantages over both current drugs. Compounds labeled with ¹⁷⁷Lu have resulted in improved tumor responses in solid tumors over other radionuclides at similar tumor-absorbed doses in mouse xenografts (Mohsin *et al.*, 2005). Combination with demethylation therapy may enhance the efficacy of the radiolabeled peptide, resulting in better tumor control and patient outcome. There has been no recorded examination of this question in nHL.

We have examined the interactive effects of the demethylating agent zebularine and two qualities of radiation, high dose-rate external irradiation and low dose-rate radiopharmaceutical therapy. The experiments followed the effect of external irradiation alone, ¹⁷⁷Lu-DOTA-TATE therapy alone, and each following treatment with increasing doses of zebularine on the MEC1 nHL cell line (Stacchini *et al.*, 1999). The overall hypothesis tested was that zebularine therapy prior to radiation with external beam or the radiopharmaceutical compound would sensitize the MEC1 cells to the effects of the radiation.

2. Materials and Methods

2.1 Cell Culture

Zebularine treatment: 10 mL of MEC1 cells (purchased from the ATCC) were incubated at 4 X 10⁵ cells/mL in four T25 Corning flasks (Corning Inc., Corning, NY) in RPMI medium altered to contain 1mM sodium pyruvate, 10 mM HEPES, 2 mM L-glutamine, 0.4 mg/mL

gentamicin supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ air atmosphere. To these were added sufficient zebularine (Sigma-Aldrich, St. Louis, MO) diluted in PBS to yield final concentrations of 0, 50, 100 and 200 µM. Cells were incubated for 48 h, removed by centrifugation, washed, and resuspended in complete RPMI medium. Zebularine was stored at -20 °C after reconstitution.

2.2 Proliferation and viability assays

Because MEC1 cells would not grow in low enough concentrations to perform a traditional clonogenic assay, a proliferation and viability assay was used. MEC1 cells were diluted to 4 X 10⁵ cells/mL and incubated in 1 mL complete RPMI medium in 24-well plates (Corning Inc., Corning, NY). The concentration of 4 X 10⁵ cells/mL was selected because the MEC1 cells grew reliably in an exponential fashion at that concentration. Experiments were limited to 72 h because the growth rate in control cells tapered off markedly when concentrations exceeded 3 X 10⁶ cells/mL. Cells were treated with 0–200 µM zebularine for 48 h prior to irradiation in replicates of 3 (Dote *et al.*, 2005; Luong *et al.*, 2006; Kelly *et al.*, 2003; Holleran *et al.*, 2005). Cells were counted daily on a Bright Line hemacytometer (Hausser Scientific, Horsham, PA). Cells were diluted with Trypan Blue vital stain and the viable fraction recorded at each counting. Dilution was appropriate to yield a minimum 100 cell count. Three replicates allow detection of a 25% difference with a power of 0.80 and α of 0.05. Experiments were restricted to a maximum of 72 h to keep all populations, including controls, within an exponential phase. For the external-beam experiments, viability also was determined after 48 h using a WST-1 assay (Roche, USA) measuring a control of 0 Gy radiation and no zebularine, as well as 2 Gy radiation, 200 µM zebularine, and the combination of 2 Gy radiation and 200 µM zebularine. Inhibition of the DNMT1 target was confirmed by Western blot assay using the rabbit anti-DNMT1 antibody (Cell Signaling, Danvers, MA). Briefly, Mec-1 cells were treated with 0–200 mM zebularine for 48 h. After harvesting the cells, protein was extracted and processed for gel electrophoresis followed by Western blotting. The membrane was probed with DNMT-1 antibody followed by an anti-rabbit secondary antibody. Detection of protein bands was done using enhanced Supersignal, West Dura chemiluminescence reagent.

2.3 Irradiation

MEC1 cells were irradiated with a Nordion Gammacell 40 Exactor ¹³⁷Cs irradiator for 31 s, 61 s, or 122 s. For these experiments, dose to the cells delivered by the MDS Nordion Gammacell 40 Exactor ¹³⁷Cs irradiator was calculated by a rate of 0.984 Gy/min (98.4 rad/min). This irradiator delivers an extremely homogeneous dose to the contents of the chamber. Exposure times were calculated to deliver 0.5, 1 and 2 Gy, doses that were expected to be sub-lethal (Wiseman *et al.*, 2000).

2.4 Radiolabeling

Radiolabeling involved adding 37 MBq (1 mCi) ¹⁷⁷LuCl₃ to 0.2 M NH₄-acetate, pH 5.0, and 1 µg DOTA-TATE (Lewis *et al.*, 2000; deJong M. *et al.*, 2005). The ¹⁷⁷Lu-labeled peptide was incubated at 95°C for 25 min. Radiometal incorporation and radiochemical purity (typically >98%) were determined by radio-thin layer chromatography. The radiolabeled peptide was diluted with normal saline for HPLC injection with no further processing. Reversed-phase HPLC used a Waters 626 chromatograph equipped with a manual Rheodyne injector, 2487 dual wavelength UV detector, busSAT/IN analog-digital interface and Empower Pro software (Build No. 1154). A Phenomenex (Torrance, CA) Jupiter C18 column (5 µm, 4.6 × 250 mm) was used for purification and analysis of radiolabeled compounds. A gradient of 0–50% solvent B (solvent A, 0.1% TFA/H₂O; solvent B, 0.1% TFA/acetonitrile) over 30 min at a flow rate of 1.0 mL/min was used. UV

detection was accomplished at 280 nm, and radioactivity detection was accomplished using a NaI(Tl) radioactivity monitor (Canberra Meriden, CT).

2.5 Cell uptake studies

The methods for cell uptake were based on previously described techniques (Smith *et al.*, 2003; Balkin *et al.*, 2011). Three aliquots of 1.5×10^6 MEC1 cells/mL, suspended in 2 mL of serum-free RPMI Medium containing 5% CO₂ (Invitrogen Corporation, Carlsbad, CA), were incubated in an orbital shaking water bath at 37°C. To each was added 0.037 MBq/mL (1 μ Ci/mL) of ¹⁷⁷Lu-DOTA-TATE. After 15 min, 30 min, 1 h, and 2 h of incubation, 200 μ L aliquots were removed, 800 μ L of media was added, and the samples were centrifuged at 10,000 rpm for 1 min, after which 900 μ L of supernatant was removed. To the pellet was added 900 μ L of media and the centrifugation was repeated. The 900 μ L wash was removed. Radioactivity in each sample (supernatant, wash, and cell pellet) was counted on the gamma counter. Uptake was calculated by dividing the pellet-associated radioactivity by the total radioactivity >1% supernatant activity remaining with the pellet. Cell viability, as determined by trypan blue exclusion and hemacytometry, was >90% after 4 h of incubation.

2.6 Cell efflux studies

Three aliquots of 1.5×10^6 MEC1 cells/mL, suspended in 2 mL of serum-free RPMI Medium containing 5% CO₂ (Invitrogen Corporation, Carlsbad, CA), were incubated in an orbital shaking water bath at 37°C. To each was added 0.037 MBq/mL (1 μ Ci/mL) of ¹⁷⁷Lu-DOTA-TATE. After a 2 h incubation, supernatant was removed, the cells were washed and resuspended in the original volume of medium before 200 μ L aliquots were removed at 15 min, 30 min, 1 h, 2 h and 4 h; 800 μ L media was added, and the samples were centrifuged at 10,000 rpm for 1 min, after which 900 μ L of supernatant was removed. To the pellet was added 900 μ L of media and recentrifuged. The 900 μ L wash was removed. Radioactivity in each sample (supernatant, wash, and cell pellet) was counted in the gamma counter. Efflux was calculated by dividing the pellet-associated radioactivity by the total radioactivity less the 1% supernatant activity remaining with the pellet. Cell viability, as determined by trypan blue exclusion and hemacytometry, was >90% after 4 h of incubation.

2.7 Dosimetry

The dose delivered by the radiopharmaceutical preparation of ¹⁷⁷Lu-DOTA-TATE was calculated using an MCNP model (MCNP5). This is a Fortran-based program that uses multiple iterations of random modeled decay events to estimate the dose to nucleus or cytoplasm based on the location of the decay and the geometry of the total, closed system (Miller *et al.*, 2005). The assumptions for this model were that the MEC1 cells have an average radius of 4 μ m, a nuclear volume of ~40%, and a cell volume fraction of 0.15% of the total water matrix. Dosimetry curves for ¹⁷⁷Lu-DOTA-TATE were established using MEC1 cell uptake and efflux data determined in MEC1 cells. This was a two-phase calculation with an initial 2 h phase of homogeneous dosimetry during incubation, then an infinite decay dosimetry model after 2 h. An MCNP model (Miller *et al.*, 2005) will be calculated using the β^- of ¹⁷⁷Lu. Due to the planar arrangement of the cells, the γ of ¹⁷⁷Lu was ignored. The total dose to the cells was calculated according to the formulae in Table 1. Doses were delivered to the cell nuclei by activity both within and without the cell, in the water matrix, for the first 2 h, then primarily by internalized activity for the remainder of the 48 h study period. Briefly, the total number of decays was calculated integrating the radioactivity in Bq over the time-period of interest. Doses were derived from the sum of decays occurring within the entire system for the first 2 h, then only decay events within the cell over the succeeding 46 h were calculated using the uptake and efflux data. Internalized

radioactivity was estimated using the uptake and efflux curves generated in MEC1 cells for ^{177}Lu -DOTA-TATE, and an estimated nucleus:cytoplasm distribution of 1:2.

2.8 Radiopharmaceutical study

^{177}Lu -DOTA-TATE was peak-purified using reverse-phase high-performance liquid chromatography prior to treatment to insure 100% radiochemical purity. MEC1 cells were incubated with ^{177}Lu -DOTA-TATE for 2 h. The medium was transferred to a microcentrifuge tube, cells were pelleted and washed in complete RPMI culture medium to remove unbound agent. Cells were resuspended at 4×10^5 cells/mL, and plated in triplicate in 24-well culture plates. Doses delivered by the radiopharmaceutical preparation of ^{177}Lu -DOTA-TATE were calculated as described above. Activities used were 0.103 MBq/well (2.78 μCi /well) for 0.5 Gy, 0.206 MBq/well (5.55 μCi /well) for 1 Gy, and 0.411 MBq/well (11.1 μCi /well) for 2 Gy.

2.9 Apoptosis assay

MEC1 cells (0.4×10^4 cells) were incubated in 0.1 mL complete RPMI medium in Corning 96 well plates (Corning Inc., Corning, NY). Cells were treated with 0–200 μM zebularine for 48 h prior to irradiation. Following zebularine treatment, the cells were exposed to 1 and 2 Gy external beam irradiation. In parallel, to control for the individual effect of each treatment on apoptosis, the cells were treated with zebularine alone or exposed to radiation only. Untreated control cells were processed simultaneously without any exposure to zebularine or radiation. Apoptosis assay was performed using Apo3/7 HTS assay kit according to the manufacturer's (Cell Technology, Mountain View, CA) instructions. Briefly, to the wells containing cells in 50 μl media, the apo HTS assay reagent containing quenched (z-DEVD)-R110 dye substrate (50 μl) was added and incubated for 1 h at 37°C. When R110 is cleaved by active caspase 3/7, the free dye excites at 488 nm and emission at 520 nm was read in a fluorescent plate reader. Apoptosis fluorescent intensities were normalized to the control sample intensity as well as cells per well at the time of evaluation and reported as the fold of the control.

2.10 Statistical analysis

Treatment groups were compared by repeated-measure ANOVA using SAS 9.0 software (SAS, Cary, NC). A mixed model was used with Fisher's least significant difference used to separate means. Growth inhibition by zebularine was assessed by linear regression analysis. Significance was set at $P = 0.05$.

3 Results

3.1 Growth inhibition and apoptosis by zebularine

MEC1 cells plated at 4×10^5 cells/mL per well in 24-well plates were treated for 48 h with 0 μM (control), 50, 100 and 200 μM zebularine. Zebularine decreased cell proliferation in a dose-dependent manner over 48 h ($P < 0.001$, $r^2 = 0.819$) (Figure 1). The IC_{20} and IC_{50} values of zebularine for MEC1 cells were calculated as 75 and 195 μM . No statistically significant alteration in cell viability was observed among concentrations of zebularine as measured by Trypan blue exclusion. Although zebularine decreased MEC1 proliferation in a dose-dependent manner during the period of exposure, once the drug was removed, there was no residual effect on proliferation. Following removal from zebularine, washing with complete medium, and plating at 4×10^5 cells/mL per well in 24-well plates, all treatment groups grew at the same rate. The target of zebularine, DNMT1, was reduced by 85–91% by treatment with zebularine for 48 h, as assessed by Western blot (Figure 2).

3.2 Growth inhibition and apoptosis by external beam irradiation

MEC1 cells received 0–2 Gy of radiation as a single dose from the external irradiator. The radiation reduced the proliferative potential of the cells in a dose-dependent manner to a maximum decrease of 37% at a dose of 2 Gy on day 2 ($P=0.0026$) (Figure 3). Cell doubling time increased from 24 h with no radiation to 39 h following 2 Gy of radiation. Cell viability was decreased in the combined zebularine and radiation groups; however, the differences were not statistically significant ($P=0.0961$ by Trypan blue and 0.487 by WST-1 assay) (Figure 4). Apoptotic fraction was significantly increased from baseline in the combined zebularine and radiation groups ($P < 0.001$) (Figure 5). The sensitive detection of early apoptotic, but still viable, cells using the caspase 3/7 activity assay likely explains this discordance. The control cells grew to a concentration by day 3 that resulted in a lag phase and decreased proliferation rate and viability.

3.3 Interaction between zebularine and external beam irradiation

After 48 h treatment with 0–200 μM zebularine, MEC1 cells were pelleted, washed to remove zebularine, and plated at 4×10^5 cells/mL per well in 24-well plates. Cells received 0–2 Gy radiation as a single dose from the external irradiator. At each dose of radiation, a zebularine dose-dependent decrease in cell proliferation was observed, with the most dramatic effect in the cells treated with 2 Gy radiation (Figure 6). The interaction between zebularine dose and radiation dose on cell proliferation was highly statistically significant ($P<0.0001$), confirming a synergistic interaction. A decrease in cell viability over time was also observed, with a significant effect of zebularine ($P=0.002$) and radiation ($P=0.0006$), but no interaction ($P=0.4976$), suggesting an additive, not a synergistic effect in this instance. This was similar when evaluated by WST-1 assay ($P<0.0001$ for a decrease in viability, but no interaction ($P=0.092$)). Apoptotic fraction of cells was significantly increased from control in a treatment-and dose-dependent manner ($P < 0.001$) with a maximum apoptotic fraction approximately 7-fold the control for any combination of zebularine and radiation (Figure 5).

3.4 Uptake and efflux of ^{177}Lu -DOTA-TATE by MEC1 cells

An uptake study of ^{177}Lu -DOTA-TATE was performed with untreated MEC1 cells and MEC1 cells treated with 50, 100 and 200 μM zebularine (Table 2). Similar to previously-reported results, uptake peaked at ~2% at 2 h (Jia *et al.*, 2006; Balkin *et al.*, 2011). Overall, the concentration of zebularine resulted in a statistically significant difference in uptake ($P=0.02$). An efflux study was done with untreated cells and cells treated with 200 μM zebularine (Table 2). Efflux was marked, 60% of the radioactivity leaving the cells in 15 min, but the residual remained relatively stable over the 4 h window, with no statistically significant effect of zebularine concentration ($P=0.28$).

3.5 Interaction between zebularine and external beam irradiation

After treatment with 0–200 μM zebularine, MEC1 cells plated at 4×10^5 cells/mL per well in 24 well plates received ^{177}Lu -DOTA-TATE calculated to give 0–2 Gy of radiation. There was a dose-dependent decrease in proliferation with increasing radiation dose observed without pretreatment with zebularine; however, the differences were not statistically significant ($P=0.609$) (Figure 7). For MEC1 cells treated with zebularine followed by ^{177}Lu -DOTA-TATE, there was a significant, interactive dose-response of decreased cell proliferation ($P=0.0135$). The maximal decrease in proliferation, 34%, was seen in cells treated with the highest doses of each compound (Figure 7). This decrease in proliferative potential is significantly lower than that seen with single-dose, external beam irradiation and zebularine ($P=0.0135$). In contrast, cell viability was significantly decreased by zebularine and ^{177}Lu -DOTA-TATE in a dose-dependent manner as measured by Trypan blue uptake,

with a highly significant interaction between zebularine and targeted radiation ($P < 0.001$). No difference was observed between the 50 μM zebularine and controls, but treatment with 100 and 200 μM zebularine caused a substantial and statistically significant decrease in cell viability (Figure 8). Apoptotic fraction was significantly increased from control by both treatments in a dose-dependent manner ($P < 0.001$). The maximum apoptotic fraction was >14-fold the control for the highest dose groups (Figure 5). This decrease was larger than that seen with the combination of 200 μM zebularine and external beam irradiation ($P = 0.005$) (Figure 7).

Discussion

The chronic lymphocytic leukemia (CLL) line, MEC1, was relatively resistant to zebularine treatment compared to other previously reported tumor cell lines. (Balch *et al.*, 2005; Stresemann *et al.*, 2006) The calculated IC_{20} and IC_{50} values were 75 and 195 μM , respectively, contrasting with IC_{50} values of 152 μM for A2780/CP cisplatin-resistant ovarian cancer cells, 92 μM for A2780 ovarian cancer cells, and 64.7 μM for Hey ovarian cancer cells (Balch *et al.*, 2005). The IC_{20} values for human ALL cell lines TK6 and Jurkat are only 20 and 10 μM (Stresemann *et al.*, 2006). This is consistent with the biology of CLL, in which appropriate apoptotic function is lost. After removal of the MEC1 cells from zebularine, the proliferative potential was identical, regardless of dose. This is consistent with the observation that demethylation is transient, and hypermethylation patterns re-establish if therapy is discontinued, (Egger *et al.*, 2007). However, we did not directly test this hypothesis.

External irradiation in the range of 0 to 2 Gy resulted in a predictable dose-dependent decrease in cell proliferative capability with no significant effect on cell viability as measured by Trypan blue exclusion and WST-1 assay. This radiation is of similar quality to that administered to patients in preparation for hematopoietic stem cell transplantation. The MEC1 doubling time observed in our laboratory was shorter (24 h) than the 40 h reported in the article describing the cell line (Stacchini *et al.*, 1999). Proliferative potential and cell viability assays were selected as a result of the growth properties of the MEC1 cells (Balch *et al.*, 2005). The noted difference in the effects of external irradiation and radiopharmaceutical treatment highlight the strength of these assays in addressing the question of the interaction of zebularine therapy and treatment with different qualities of radiation. The therapeutic effect of zebularine may be predicated on the epigenetic profile of the cells being treated.

High dose-rate external irradiation following zebularine exposure resulted in a highly statistically significant reduction in proliferative potential when zebularine exposure was followed by radiation. Given that MEC1 cells had normal proliferative potential following zebularine exposure alone, this interaction with radiation demonstrates synergy. Decreased clonogenic potential has been demonstrated following radiation after prior treatment with zebularine in solid tumor cell lines (Dote *et al.*, 2005). Cells treated with zebularine and then irradiated show suppressed DNA repair activity characterized by increased double-strand DNA break lesions as measured by γH2AX foci (Dote *et al.*, 2005; Kim *et al.*, 2012). Decrease in viability by the combination of the highest dose of zebularine and external irradiation was much less than the decrease in proliferative potential. This may be a function of the low apoptogenic effect of external beam (Macklis *et al.*, 1994). The primary effect of external irradiation at high dose-rate is typically cell-cycle arrest and mitotic catastrophe. The end-result following the double-strand breaks would not most typically be apoptosis. The significantly lower apoptotic fraction for cells treated with external irradiation than with the radiopharmaceutical supports this result.

In contrast, low dose-rate irradiation with radiopharmaceuticals is thought to cause apoptosis more frequently than mitotic catastrophe (Macklis *et al.*, 1994). This phenomenon is supported by the finding in the present study that the viability of the MEC1 cells as measured by Trypan blue exclusion was synergistically affected by treatment with zebularine followed by ^{177}Lu -DOTA-TATE, while the interactive effect on proliferative potential was far less dramatic. This result is similar to that previously reported comparing external irradiation to ^{90}Y irradiation added to the cell suspension of nHL cell lines (Macklis *et al.*, 1994). By measurement of DNA fragmentation, apoptosis was significantly more common after ^{90}Y treatment than external irradiation (Macklis *et al.*, 1994). Addition of caffeine, an apoptosis enhancer, markedly increases this effect (Macklis *et al.*, 1994). In contrast, cell cycle arrest was more prominent in the externally irradiated cells (Macklis *et al.*, 1994), as observed in the present study. The quality of radiation differs greatly between the single dose over 2 min or less from external irradiation and the low dose-rate of the ^{177}Lu with a half-life of 6.7d. The difference in outcome between these 2 experiments suggests that the mechanism of interaction between the types of radiation and zebularine is different. If the effect of zebularine was unimechanistic, the radiation dose from ^{177}Lu should have a similar, but less dramatic, effect on proliferative potential relative to the external irradiation. Instead, to have a much larger impact on viability and apoptotic fraction suggests that the interaction between the zebularine and ^{177}Lu -DOTA-TATE involves the apoptotic mechanism. It cannot be ignored that this radiopharmaceutical compound has two potential mechanisms of cellular response, the radioactive payload, and the effect of somatostatin agonism. Somatostatin has been shown to inhibit activated lymphocyte proliferation *in vitro* at concentrations between 10^{-11} M to 10^{-9} M (Lattuada *et al.*, 2007). In the present work, the concentration of TATE was 2 orders of magnitude lower at 10^{-13} M, making a pharmacologic effect of the ligand extremely unlikely. Nevertheless, the interactive effect observed was between the zebularine and ^{177}Lu -DOTA-TATE as a compound. If the ^{177}Lu -DOTA-TATE has any effect directly on the cancer cells, this would be expected to occur *in vivo* as well. Human patients in imaging studies of related compounds have experienced no beneficial therapeutic effect. The effect of the radiometal is the critical ingredient for therapy.

The interaction being followed is due to an effect of zebularine independent of its DNMT inhibition effect. Other nucleoside analogues have been used for radiosensitization (LeBlanc *et al.*, 2004). Zebularine has a ribose base, allowing it to be incorporated into RNA, which could contribute to radiosensitization. However, the fact that zebularine treatment preceded radiation and is no longer present in the repair phase makes this explanation unlikely. Indeed, if zebularine radiosensitizes *in vivo* as it appears to do *in vitro*, it may be a useful radiosensitizer. The compound has a preferential effect on cancer cells compared to normal cells *in vitro* (Cheng *et al.*, 2004), which seems to be mediated by cytidine kinase levels, which are higher in cancer cells than normal cells, and are required to activate zebularine (Cheng *et al.*, 2004). If this is the case, the compound could serve to radiosensitize tumor tissue while having minimal effect on normal tissue, thereby improving the therapeutic ratio.

We have also found a specific, receptor-mediated uptake of ^{177}Lu -DOTA-TATE in MEC1 xenografts implanted in SCID mice (unpublished data). Thus, this radiopharmaceutical may have potential for therapeutic applications in this mouse model. However, treatment of MEC1-bearing mice with ^{177}Lu -DOTA-TATE showed only a modest 7-day growth inhibition starting at day 4 post-injection, after which the tumor grafts began to grow exponentially. It is possible that treatment of MEC1-bearing mice with zebularine could augment ^{177}Lu -DOTA-TATE therapy in preclinical models of CLL.

Zebularine significantly sensitizes MEC1 cells to radiation effects when administered prior to radiation exposure. Because it does not sensitize normal cells *in vitro*, zebularine could

have very good radiosensitizing properties for clinical application to the therapy of lymphomas. The drug can be administered sequentially, not simultaneously, with external beam or radiopharmaceutical radiation to have the desired effect to eliminate resistant or residual disease with minimal apparent impact on normal cells. This could be applied in the treatment of relapsed, resistant disease, or potentiate total-body irradiation for elimination of minimal residual disease prior to hematopoietic stem cell transplantation. The radiation doses used in these investigations were significantly sub-lethal compared to those used in the clinic, offering the potential that escalation of the dose could result in clinically meaningful advances with application of this approach.

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Abbreviations

DNMT1	DNA methyltransferase 1
nHL	non-Hodgkin lymphoma
SSTR	somatostatin receptor
¹⁷⁷Lu-DOTA-TATE	lutetium-177-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid-Tyr3-octreotate

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Growth Inhibition by Zebularine

Figure 1.

Proportion of MEC1 growth is inhibited by zebularine in a dose-dependent and linear manner ($P < 0.001$, $r = 0.904$, $r^2 = 0.819$). No change in cell viability was observed over the concentrations. $IC_{20} = 75 \mu M$. $IC_{50} = 195 \mu M$.

Figure 2. Western blot of DNMT1 protein (~240 kD) with GAPDH protein as a loading control. The lane on the left is the control lane. DNMT 1 was significantly reduced by treatment with zebularine at all concentrations from 50 μ M to 200 μ M.

Cell Proliferation w/Radiation

Figure 3. Cell proliferation after external irradiation (in 10^4 cells/mL over time). Proliferation over a three day period was decreased significantly by increasing dose of radiation ($P=0.0026$).

Figure 4. Cell viability measured by WST-1 assay of MEC1 cells relative to controls following treatment with 2 Gy external irradiation, 200 μ M zebularine, or the combination of the two. Zebularine and the combination of zebularine and external irradiation decreased cell viability compared to control ($P < 0.0001$), but in an additive, not synergistic, manner.

Figure 5.

Apoptotic proportion scaled relative to cell population and control apoptosis. Cells were pre-treated with zebularine at 100 μ M and 200 μ M (Zeb 100 or Zeb 200) and ^{177}Lu -DOTA-TATE (Lu-177) or external beam radiation (EBR). Relative apoptotic proportion was increased from baseline for combined zebularine and both forms of radiation ($P < 0.001$). At high doses of zebularine and radiation, relative apoptotic proportion was much greater for the radiopharmaceutical-treated cells ($P = 0.005$).

Figure 6.

Cell proliferation after 2Gy radiation presented by zebularine dose including cells exposed to zebularine without radiation (in 10^4 cells/mL over time). Note the progressive, dose-dependent decrease in proliferation observed at increasing concentrations of zebularine treatment up to approximately 50% for the cells treated with 200 μ M zebularine. Contrast this with the lack of zebularine dose-effect in cells without radiation exposure.

Cell Proliferation: Zebularine + Lu-177-DOTA-TATE **Figure 7.**

Cell proliferation of MEC1 cells at increasing doses of zebularine treated with 2Gy radiation by ^{177}Lu -DOTA-TATE (in 10^4 cells/mL over time). In the analysis, the effect of day interacted with dose, suggesting less direct impact of the radiopharmaceutical on cell proliferation than was observed following irradiation by the external source.

Figure 8.

Cell viability over time for MEC1 cells treated with 2Gy radiation from ^{177}Lu -DOTA-TATE or external beam radiation at a 200 μM dose of zebularine (in 10^4 cells/mL over time). The maximal decrease using ^{177}Lu -DOTA-TATE was over 16% relative to controls, compared to only 6% decrease in cells receiving external beam irradiation, a >2-fold difference ($P = 0.005$).

Table 1Calculation of cell dose from 2.78 μ Ci ^{177}Lu -DOTA-TATE

Calculation of the total number of decays	$Decays = \frac{Act_o(Bq)}{\lambda(sec)} (1 - e^{-\lambda t})$
Decay events over 2 h	$=(2.78 * 37 * 10^3 / 0.00433) * (1 - (e^{(-0.00433 * 2)}))$
Decay events over 72 h	$=(2.78 * 37 * 10^3 / 0.00433) * (1 - (e^{(-0.00433 * 72)}))$
Percent uptake	Estimated over 72 h by multiplying uptake by percent efflux
Decays per cell	Total number of decay events divided by the total number of cells in the system
Total dose	Dose is calculated by multiplying each location factor (Table 4.1) by the number of decays expected to occur in that location over the 72 h study period

Radioactivity localization	Location of Lu-177	Dose to Nucleus (Gy/decay)	Dose to cytoplasm (Gy/decay)
	Nucleus	0.0091	0.0041
	Cytoplasm	0.0041	0.0053
	Water matrix outside of the cell	0.0009	0.001

Table 2

Uptake and efflux study data. Each time-point is expressed as a percentage of cell-associated radioactivity with the standard error of the mean provided. The differences in uptake as a function of concentration of zebularine were statistically significant over the time-period ($P=0.02$).

Percent Uptake					
Zebularine	30min	SEM	60min	SEM	120min
0	1.0863	0.0988	1.3352	0.0718	2.0322
50	1.0095	0.0811	1.2257	0.1760	2.0155
100	0.8416	0.0570	1.1517	0.1030	1.5871
200	0.5544	0.1190	0.7127	0.0840	1.0454
Efflux- Percent Cell Retention					
Zebularine	15min	SEM	30min	SEM	60min
0	39.1889	3.5970	37.3892	2.4620	31.9767
200	39.5105	2.7420	42.0717	1.7660	36.1122
Zebularine	120min	SEM	240min	SEM	
0	29.3233	1.5050	24.7351	1.2510	
200	33.1307	1.2960	27.3712	0.8270	