Zebularine induces prolonged apoptosis effects via the caspase-3/PARP pathway in head and neck cancer cells

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Received October 2, 2013; Accepted November 18, 2013

DOI: 10.3892/ijo.2014.2386

Abstract. Zebularine, a potent DNA methyltransferase inhibitor, is potentially able to influence gene regulation and thereby alters cell behavior. This study illustrates the effect of zebularine on human squamous cell carcinoma (SCC-9 and SCC-25) in vitro. The results indicated that zebularine significantly (P<0.05) reduced viability and DNA synthesis of treated cancer cells, by induction of cell cycle arrest at G2/M phase and apoptosis in both tested cell lines. This effect was confirmed to be mediated through p21/CHK1- and caspase 3/PARP-dependent pathways, respectively. However, no methylation was observed in the promoter region of the upregulated p21 and CHK1 genes. This may indicate that the alteration of p21 and CHK1 following zebularine administration was not due to inhibition of methylation of their promoter. Interestingly, it was observed that zebularine continued to influence cell viability for a week following its withdrawal. This may indicate feasibility of novel drug administration strategies, in which, daily administration of the drug replaced by weekly use, leading to improved therapeutic process and cost-effectiveness of the treatment in head and neck cancer.

Introduction

Head and neck cancer includes a large group of epithelial malignancies, originated from the oral, nasal, larynx and pharynx areas, and is mostly described as squamous cell carcinoma. This type of cancer is frequently common in regions with high consumption of tobacco and alcohol, e.g., Western and Southern Europe, Southern Africa and South-Central Asia (1). More than 400,000 deaths from this disease occur each year; 80% of them in developing countries (2). Numerous gene-expression profiling studies of head and neck cancer have revealed a variety of gene-expression alterations among normal squamous cells and cancerous tumor cells, in metastatic and non-metastatic lesions, and among both young and older patients. In most tested cases, gene-expression changes were correlated with cell cycle regulation and cell proliferation (3). Recent reports on cancer and the carcinogenesis pathway refer to the disease as a polyepigenetic process, apart from its polygenetic properties (4). In addition, DNA hypermethylation of tumor-suppressor genes, which are responsible for gene silencing, was reported to be the main recurrent epigenetic event detected in cancer cells (5). Currently, a number of DNA methylation inhibitors are undergoing clinical trials for treatment of a wide variety of cancer types, including head and neck. Zebularine was originally synthesized as a cytidine deaminase inhibitor (6), however, a growing number of studies have reported its behavior as a cytidine analogue, behavior that is responsible for its inhibition of DNA methylation. Zebularine application to cancer cells elicited gene response that was correlated with cancer-related antigens (7) and was involved in apoptosis induction (8), which suggests that zebularine might exhibit antitumor potential in cancer cells. Unlike its analogues, 5-Aza-CR and 5-Aza-CdR, which are cytotoxic to treated cells, zebularine is more tolerated by the cells and exhibits only reduced toxicity when administered. Other studies reported cell cycle arrest and apoptosis induced by zebularine in numerous types of cancers, e.g. breast cancer (9), bladder cancer (10), gastric cancer (11), cervical cancer (12) and oral squamous carcinoma (13-15). However, to date, only limited data have been published regarding the course of action of zebularine in head and neck cancer cells. In the present study we addressed the hypothesis that zebularine plays an important role in induction of apoptosis in head and neck cancer cells; the aim was to evaluate our hypothesis regarding whether and how zebularine is capable of inhibiting proliferation and inducing apoptosis in head and neck cancer cells.

Materials and methods

Cell lines and culture. Head and neck cancer cell lines SCC-9 and SCC-25 (obtained from ATTC, Bethesda, MD, USA) were grown in 25 or 75-cm² sterile flasks and maintained in a humidified atmosphere of 5% CO₂ at 37°C in MEM: F12 supplemented with 5% fetal bovine serum (Biological
Industries, Beit-Haemek, Israel), and 2.5 mM L-glutamine, hydrocortisone at 400 ng/ml, penicillin at 100 U/ml, and streptomycin at 100 µg/ml (Biological Industries). Cells were seeded at a density of 50-60% for initiation of each experiment and after 24 h the medium was changed to media containing various concentrations of zebularine, for another 24 to 96 h.

**Cell proliferation assays.** The cells were treated with 50-1,000 µM of zebularin for 24 to 96 h. Cell viability was assayed with the XTT Cell Proliferation Kit (Biological Industries), according to the manufacturer’s protocol. Results were calculated as percentages of the proliferation in the vehicle control.

**DNA synthesis.** BrdU was detected immunochemically with a 5-Bromo-2′-deoxy-uridine Labeling and Detection Kit (Roche, Mannheim, Germany), which assesses BrdU levels incorporated into newly synthesized DNA. Zebularine-treated cells were incubated with 10 µM BrdU for 4 h, and incorporated BrdU was detected with the monoclonal anti-BrdU-POD and Fab fragments according to the manufacturer’s protocol.

**Flow cytometry.** Cells were plated at a density of 60% in 10-cm culture dishes and after 24 h were treated with 350 µM zebularin for 24 to 120 h, after which they were trypsinized and washed with PBS and then fixed with 70% EtOH for 1 h. They were then incubated with 0.1% NP-40 for 5 min at 4°C, washed once more with PBS, and incubated with RNase at 100 µg/ml and PI at 50 µg/ml (Sigma-Aldrich Ltd, Rehovot, Israel) for 20 min. Finally, the cells were analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

**Cytotoxicity assay.** A Cytotoxicity Detection Kit (Roche) was used to quantify cytotoxicity/cytolysis levels following zebularine treatment. This method is based on measurement of LDH activity released from damaged cells using 96-well plate. Cells were cultured overnight in a 96 well-plate and then treated with zebularine at 50-1,000 µg. Twenty-four hours post-treatment LDH activity levels were measured at a wavelength of 492 nm.

**TUNEL assay for apoptosis detection.** Apoptotic cells were detected with an In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s protocol. In brief, cells were grown overnight in an 8-chamber slide and then were treated with zebularine; after a further 96 h they were fixed and permeabilized, and were exposed to TUNEL reaction mixture for 60 min at 37°C in the dark. Samples were analyzed under a fluorescence microscope (515-565 nm).

**Methyl-specific PCR (MSP).** Genomic DNA was extracted from cells with the High Pure PCR Template Preparation kit (Roche), and the DNA was subjected to bisulfite treatment with the EpiTect Bisulfite kit (Qiagen, Valencia, CA, USA). The methylation status of p21 and CHK1 genes were determined by MSP assay with the EpiTect MSP kit (Qiagen) according to the manufacturer’s protocol, supplemented with specific primers for methylated and unmethylated forms, in accordance with previous reports (16,17). The amplified samples underwent electrophoresis on 2% agarose gel and were visualized with an XRS Molecular Imager (Bio-Rad Laboratories Ltd, Rishon Le Zion, Israel).

**Real-time PCR.** The real-time PCR method was used for gene-expression quantification experiments. Relative quantitation of target genes was compared with that of an internal standard gene (β-actin). In summary, 1 µl of suspension containing 50 µg of cDNA was mixed with SYBR Master mix (Applied Biosystems, Foster City, CA, USA) and with the following primers: p21, forward 5′-GGCAGACACACGTGACAGATT-3′; CHK1, forward 5′-CCCGCAGGTTTCTTT-3′; and reverse 5′-GGCGGGAAAAGCTGATCC-3′. Results were calculated as RQ and analyzed with Step-One software (Applied Biosystems).

**Western blot analysis.** Treated cells were collected by using trypsin, washed once and lysed with Loui’s glycerol lysis buffer on ice for 10 min, and the protein fraction was separated by centrifugation for 15 min at 1,500 rpm. Protein samples were electrophoresed on non-denaturing 10% sodium dodecyl sulfate-polyacrylamide gels at 120 V for 1.5 h. Then, protein samples were transferred by semi-dry transfer to a 0.45-micrometer-pore-size nitrocellulose membrane. The membrane was blocked with 5% BSA for 1 h at room temperature, following incubation with primary antibodies: monoclonal rabbit anti-caspase 3 (Abcam, Cambridge, UK); polyclonal rabbit anti-PARP (Cell Signaling, Danvers, MA, USA); and polyclonal rabbit anti-p21, monoclonal mouse anti-chk1, and monoclonal mouse anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was then washed three times with TBST, each for 10 min, incubated with secondary antibody conjugated to horseradish peroxidase (Jackson Immune Research Laboratories and DAKO, Glostrup, Denmark) for 1 h at room temperature, and washed three times. Antigen/antibody complex was detected with the ECL System (Biological Industries). Western blot analysis results were quantified by means of Quantity One software (Bio-Rad Laboratories Ltd). β-actin levels (as standard protein occurring naturally in these cells) were taken as a reference.

**Statistical analysis.** Statistical analyses were performed with SPSS software, and means were compared by the two-tailed Student’s t-test, one-way analysis of variance (ANOVA), or their non-parametric counterparts, depending on the desired number of comparisons. Statistical significance levels were set at *P<0.05, **P<0.01* and ***P<0.001.

**Results**

**Zebularine inhibits human head and neck cancer cell growth in a time-dependent manner.** The effect of zebularine on viability of head and neck cancer cells was measured according to the XTT method. SCC-9, SCC-25 and normal fibroblast cells were treated with increasing concentrations of zebularine, from 50 up to 1,000 µM. Cell viability was measured at time points of 0 to 96 h following treatment. Overall, 24 h of zebularine treatment did not elicit significant changes in cell viability (Fig. 1) but at 48 h following treatment, reduced cell viability was observed in the cell lines SCC-9 and SCC-25 even with the smallest dose (50 µM). At this point, we detected, on average, a 20% reduction of cell viability. In SCC-9 cells, treatment with 200 µM zebularine for 96 h reduced viability to 50%, and...
Figure 1. Cell viability following zebularine treatment. Squamous cancer cells (A) SCC-9 and (B) SCC-25, and (C) normal fibroblast cells were seeded and treated after 24 h with increasing doses of zebularine; control cells were treated with 0.01% DMSO. Cell viability was detected by XTT. Presented data are means ± SD of three experiments, each conducted in five replicates, and are expressed as percentages of the respective control. Statistical significance was determined by two-tailed Student's t-test. Significance is indicated as *P<0.05, **P<0.01, ***P<0.001.
the reduced state stabilized at 70% on average for zebularine concentrations of 200-1,000 µM, indicating that the main controlling factor was the duration of treatment rather than the zebularine concentration (Fig. 1A). The SCC-25 cells were highly sensitive to zebularine treatment (Fig. 1B): cell viability was reduced almost to 20% at 96 h post-treatment; treatment for 72 h reduced cell viability by more than 40%. Interestingly, exposure of normal fibroblast cells to zebularine had a minor effect on their viability compared with its effect on cancer cells. The constant effect of zebularine on these cells resulted in 80% cell viability for all concentrations, regardless of treatment duration (Fig. 1C). For both SCC-9 and SCC-25, IC\(_{50}\) was determined as 350 µM for 72 h of treatment; in contrast, an IC\(_{50}\) value for the fibroblast cells was indeterminable in these conditions. Furthermore, toxicity levels of zebularine in the cells were measured with LDH activity methods (data not shown): the results revealed low levels of toxicity, which resembled previously published data, and further strengthened the perception that zebularine is not toxic to human cells, even at the highest tested concentration of 1,000 µM.

**DNA synthesis levels remain depressed after zebularine withdrawal.** It is known that zebularine becomes incorporated into DNA, and binds DNMT via a covalent link. However, the duration of its continued influence on cell proliferation, following its removal was previously not addressed. In order to examine the continuing influence of zebularine subsequent to drug withdrawal, DNA synthesis levels were evaluated by BrdU: SCC-9 and SCC-25 cells were treated for 48 h with four concentrations of zebularine, 50, 200, 500 and 1,000 µM, and DNA synthesis was measured on days 3, 5 and 7 following zebularine emission. In both cell lines, zebularine was able to influence newly synthesized DNA: DNA synthesis was reduced to less than 50% of that in the vehicle control, and this effect was observed even 7 days after drug removal (Fig. 2). Moreover, the reducing effect on DNA synthesis saturated at 50 µM for SCC-9 cells and at 200 µM for SCC-25 cell lines, and this effect was constant for all tested times.

**Zebularine induces sub-G1-phase accumulation in both SCC-9 and SCC-25 cell lines.** Subsequent to establishing that zebularine reduced cell viability and DNA synthesis, the exact influence of the drug on proliferation in the cell cycle was analyzed by flow cytometry. Zebularine influenced the cell cycle of treated SCC-9 by causing cell cycle arrest at G2/M after 72 h of treatment, whereas G2/M of the control was 28.5% and rose to 41% after 72 h of zebularine treatment. Moreover, extended periods of treatment; 96 and 120 h, caused cells to exit from the cell cycle towards the sub-G1 phase, indicating cell death. Sub-G1 levels at 96 and 120 h post-treatment were 32 and 30%, respectively, whereas control sub-G1 was approximately 5% (Fig. 3A and C). The SCC-25 cells reacted more strongly to zebularine than the SCC-9 cells in which zebularine increased accumulation of cells in sub-G, intensifying from 3.7% in the control up to approximately 40, 53.9, and 50% after 72, 96 and 120 h, respectively. Furthermore, the percentages of cells in the S and G2/M phases were unchanged as a result of the treatment, whereas numbers of cells in the G1 phase fell from about 54 to 20%, on average, for all treatment times. It is noteworthy that the effect of zebularine did not increase in a time-dependent manner, demonstrating that drug saturation was already reached at 72 h of treatment (Fig. 3B and D).

**Zebularine regulates upregulation of p21 and CHK1.** A total of 48 cell cycle related genes were analyzed following zebularine treatment (data not shown), in which 2 cell cycle-checkpoint regulated genes namely p21 and CHK1 exhibited upregulated levels (RQ>2). Real-time PCR was used to ratify the expression levels of p21 and CHK1 mRNA in the cell lines (Fig. 4): p21 mRNA expression levels showed no significant change in SCC-9, but in SCC-25 cells, p21 expression levels increased...
Figure 3. Cell cycle analysis of SCC-9 and SCC-25 following zebularine treatment. Cells were treated with 350 µM zebularine and subjected to cell cycle analysis by flow cytometry as described in Materials and methods. (A and B) Representative histograms of SCC-9 and SCC-25 FACS results, respectively. (C and D) Summarized FACS results following zebularine treatment of SCC-9 and SCC-25, respectively, for 72-120 h. Presented data are mean ± SD of four experiments, each conducted in duplicate, and are expressed as percentages from total 10,000 analyzed cells. Statistical significance was determined by two-tailed Student's t-test (treatment vs. control for each phase) and significance is indicated as *P<0.05; **P<0.01.
Figures 4 and 5. mRNA expression levels of p21 and CHK1 in zebularine-treated cells. Total RNA was extracted from treated cells after 24-120 h, and converted to cDNA, of which 1-µg aliquots were used in a SYBR-Green mix, with a p21 or CHK1 primer set. β-actin primers were used as an amplification control. Results were detected with Step-One real-time PCR. Presented data are mean ± SD of three independent experiments and are presented as RQ (relative quantity) after equalization, as percentages of the respective controls. Statistical significance was examined with Student’s t-test (treatment vs. control) and significance is presented as: *P<0.05; **P<0.01.

Figure 5. Cell cycle and apoptosis-related protein levels following zebularine treatment. Cells from each cell line (SCC-9, SCC-25) were treated with 350 µM zebularine, and total protein was extracted after various treatment durations. Proteins, in 60-µg aliquots, were separated on SDS-PAGE and transferred to nitrocellulose membranes. Caspase 3, PARP, p21 and CHK1 were detected by using primary antibodies. For equalization of protein amounts, mouse anti-human β-actin monoclonal antibody was used. Figure shown is representative of three experiments. Results were analyzed with Quantity-One software (Applied Biosystems).

significantly (P<0.05), starting at 48 h of treatment. For CHK1, significant increase (P<0.05) was detected in both SCC-9 and SCC-25 cells at 96 and 48 h of treatment, respectively. Longer treatment times elicited no significant change in the CHK1 expression level.

Upregulation of p21 protein following zebularine treatment was observed in the two cell lines, for SCC-9 extreme upregulation of the protein was observed at 96 h of treatment and in SCC-25 cells, p21 protein showed upregulation at 48 h of treatment. CHK1 protein exhibited basal levels in the control SCC-9 cells, and its expression level was slight increases after 72 and 96 h. The protein levels of CHK1 increased at 24 h and vaguely at 72 h of zebularine treatment in SCC-25 cells (Fig. 5).
after 72 h of treatment, the active form (85 kDa) showed high levels in SCC-9 cells, 10 times more than in the controls, whereas in SCC-25 cells there were only 4 times more, at most, after the same time interval.

The TUNEL assay results demonstrated the presence of apoptotic cells among both SCC-9 and SCC-25 cells (Fig. 6). Expectedly, the fibroblast cells did not show any signs of apoptosis following treatment with zebularine, a finding that supported the XTT results.

Zebularine regulates p21 and CHK1 independently of promoter methylation. Effects of aberrant methylation of the p21 and CHK1 genes in the cell lines were tested following their regulation. In the case of p21, one region of approximately 300 bp in the promoter region was analyzed, whereas for CHK1, two regions in the promoter area, each of 300 bp, were analyzed. The results of p21 promoter methylation analysis revealed merely amplification of the unmethylated region in both cell lines following 72 h of zebularine treatment (Fig. 7A). However, the two sections of the CHK1 promoter showed differing results (Fig. 7B and C). In one section, both methylated and unmethylated primers elicited amplification in treated cells as well as in control cells (Fig. 7B), and the ratio between the bands was unchanged following zebularine treatment. In the second section (Fig. 7C), only the unmethylated primers were capable of amplifying bands, indicating that methylation caused no modification in that segment. Since the effects of the CHK1 promoter in the first segment were ambiguous with respect to methylation status, a more sensitive method, bisulfite genomic sequencing (BGS), was used for methylation analysis. Methylation patterns were similar for both SCC-9 and SCC-25 cells, in which none of the tested CG positions were methylated, either in the control or the treated cells (data not shown).

Discussion

The present results indicate that zebularine reduced cell viability of human head and neck cancer cells in vitro. This effect was found to be mediated through induction of apoptosis, as confirmed by TUNEL assay and activation of pre-apoptotic genes. Interestingly, zebularine had no effect on normal human fibroblast cells. Thus, the overall effect of zebularine was reduction of cell viability in a time-dependent rather than dose-dependent manner, as reported recently also by You and Park (12). The present findings match those of previous studies of the effects of zebularine on numerous cancer types, e.g., breast, bladder, cervical, and head and neck cancers (9,10,12–15). A study by Cheng et al (18) also supported these results in which they showed that continuous treatment with zebularine substantially retarded the growth of human cancer cell lines, with little effect on growth of normal human fibroblasts.

Current studies concerning zebularine and other methyltransferase inhibitors are focusing mainly on combinations of these inhibitors with established chemotherapeutic drugs in order to sensitize treated tumors. However, remethylation and resilencing of tumor-suppressor genes is a common
problem with methylation-inhibitor drugs, and it potentially can cause complications in clinical application, which requires continuous drug administration. To date, the full duration of zebularine influence on cancer cell proliferation following its emission was not approached, but in the present study, we were able to show that despite withdrawal of zebularine from the medium, cells exhibited continuous reduction of DNA synthesis several days after its removal. This finding indicates that zebularine should be preferred to existing analogous drugs, and these results could promote the development of new administration and management strategies in the treatment of head and neck cancer, with daily administration of zebularine being replaced by weekly application.

The cell cycle findings that SCC-9 cells were arrested in G2/M following zebularine treatment, whereas SCC-25 cells were arrested at the sub-G1 phase, show that even cells from the same origin (in this case, tongue carcinoma) respond differently to zebularine administration. The results of Suzuki et al (13-15) concerning the effect of zebularine on HSC-3 cells originating from tongue squamous cell carcinoma also showed increased percentages of cells arrested at the G2/M phase of the cell cycle for 48 h following each of the tested doses; 120 and 220 µM. The disparity between the results of Suzuki et al and our present findings are not considered to indicate any conflict, because our cell cycle assay used different treatment durations from theirs; 72-120 h and 48 h, respectively. Moreover, the two assays were conducted with differing tongue cancer cell lines, which could account for the differences in the FACS results. Another study indicated that zebularine induced S phase arrest of the cell cycle in lung cancer cells (19). Therefore, it is possible to postulate that the effect of zebularine on cell cycle progression is tissue specific.

We postulated that apoptosis resulting from zebularine treatment occurs in a caspase 3- and PARP-cleavage-dependent manner in head and neck cancer cell lines. This notion is reinforced by a previous study on breast cancer (9), in which zebularine was able to increase activated levels of caspase 3, with enhanced cleavage of PARP, in MDA-MB-231 cell lines. Nonetheless, the MCF-7 cells, which are known for their lack of caspase 3 expression, did not show significant upregulation of PARP cleavage, but were able to undergo apoptosis via a different path.

Interestingly, no methylation was observed in the promoter region of the upregulated p21 and CHK1 genes. However, previous studies concerning methylation of p21 promoter reported discrepancies among results, in some of the tested cells and tumors methylation and silencing of the p21 gene were observed, whereas in other studies no methylation was observed in the promoter region of p21. In squamous cells originating from lung cancer, aberrant methylation of p21 was reported, especially for NSCLC, but not in MPM cells, and consequently p21 protein was frequently lost from NSCLC and SCLC (20). However, we found no reports of methylation of p21 in oral squamous cells. For the CHK1 gene, a report from Tort et al (17) showed low levels of CHK1 expression in human lymphoid neoplasm, which was suspected to be methylated but, nonetheless, methylation analysis revealed no methylation at the CpG positions of the CHK1 promoter. In conclusion, we can state that alteration of p21 and CHK1 following zebularine administration was not due to inhibition of methylation of their promoter.

In summary, our results confirm that zebularine induced apoptosis in squamous cell carcinoma SCC-9 and SCC-25 cells, in a caspase 3- and PARP-dependent manner, and its anti-proliferative activity continued to be effective for several days following its withdrawal. Nevertheless, the observed upregulation of p21 and CHK1 were not due to inhibition of promoter methylation by zebularine. The issue of apoptotic pathway activated by zebularine need to be further studied in cancer cells. This may lead to improved strategies for combating drug resistance in head and neck cancer, by integrating zebularine into existing therapies.
References