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Cytotoxic and apoptotic effects of ceranib-2 offering potential for a new antineoplastic agent in the treatment of cancer cells

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Abstract: Fibrocarcinomas are malignant tumours that originate in mesenchymal cells. The tumours typically form in the presence of connective tissue and are of key consideration when assessing research priorities for future healthcare management. Ceramidase inhibitors, such as ceranib-2, have demonstrated capacity to interfere with cellular DNA functionality, initiating apoptosis in many cancer cell lines. The enzyme ceramidase can regulate cellular levels of sphingosine and sphingosine-1-phosphate by controlling hydrolysis of ceramide. The present study investigates the antigrowth effects of ceranib-2 on mouse embryonic fibroblast cells (NIH/3T3 normal cell line) and rat embryonic fibroblast cells (5RP7 cancer cell line). Research was conducted using colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, confocal microscopy, and transmission electron microscopy. The results indicate that in vitro fibrocarcinomas have the potential to undergo apoptotic death when influenced by the antigrowth behaviour of ceranib-2. This is true even of cells exposed only to minimal doses of ceranib-2. Indicators of apoptotic death, specifically presentation of a pyknotic nucleus, were observed by confocal micrographs in treated cells double-stained with acridine orange and Alexa Fluor-488 Phalloidin. It is theorised that ceranib-2 will function in accordance with the antiproliferative activity of other acid ceramidases, ultimately resulting in the molecule's classification as a new antitumour agent.

Key words: Ceramidase inhibitor, ceranib-2, cancer treatment, cytotoxicity, apoptosis

1. Introduction

Sphingolipids such as ceramide, ceramide-1-phosphate, sphingosine (SPH), and sphingosine-1-phosphate (S1P) offer structural integrity to cell membranes and also assist in the signalling of cellular processes. These cellular processes, including proliferation, apoptosis, inflammation, and cell cycle arrest, play a key role in the evaluation of the antitumour effects of different agents (Beckham et al., 2010). Ceramide and S1P are consequential second messengers derived from sphingolipid metabolites. They exert opposing effects on cell survival. Ceramide induces apoptosis (termed proapoptotic), whereas S1P supports cell survival (termed antiapoptotic) (Oskouian and Saba, 2010). As a result of these opposing effects, the balance between ceramide and S1P is considered a decisive factor in the final metabolic response of a cell (Proksch et al., 2011).

As a result of its growth inhibition effects, ceramide is the sphingolipid most commonly investigated in cancer studies and is often termed the 'tumour suppressor lipid' (Taha et al., 2006). The cellular actions of ceramide include modulation of cell apoptosis by inhibiting prosurvival kinases, activation of stress-activated kinases and phosphatases, modification of mitochondrial transmembrane potential, translocation of cytochrome c and apoptosis-inducing factor from mitochondria to cytoplasm, and activation of caspase-3 (Chen et al., 2008). Cancer chemotherapeutic agents such as etoposide, vincristine, daunorubicin, doxorubicin, fludarabine, paclitaxel, and irinotecan all exacerbate suppression of cellular growth by increasing levels of ceramide (Senchenkow et al., 2001; Reynolds et al., 2004).

Despite the significant body of work already completed to evaluate the relationship between ceramide metabolic activity and cell death, the precise molecular mechanisms of ceramide-mediated apoptotic signalling are not fully understood. Ceramidases decompose ceramide to SPH, which immediately generates S1P. S1P is the final product of sphingolipid catabolism and is generated under the influence of sphingosine kinase (SK) (Chalfant

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and Spiegel, 2005). S1P inhibits apoptotic pathways by influencing changes in mitochondrial membrane potential, including prevention of cytochrome c release. S1P induces proliferation, differentiation, and migration of various cells (Oskouian and Saba, 2010). In addition, increased levels of the S1P-generating enzyme SK-1 inhibit apoptosis, block activation of antitumour agents, and induce tumorigenesis (Nava et al., 2002; Sarkar et al., 2005; Ponnusamy et al., 2010). Research has shown that 89% of human colon cancers exhibit a higher expression of SK-1 in comparison with normal colon mucosa. Similarly, studies have found that the growth of prostate cancer cells can be restricted by inhibition of SK-1 (Akao et al., 2006). The view that inhibition of the SK-1 pathway may be adapted as a potential target in chemoprevention (Kawamori et al., 2009) is a theory currently gaining significant momentum in the scientific community.

Ceramidases serve as mediators of tumour cell growth by rapidly removing ceramide as found in fibrosarcomas (Strelow et al., 2000), prostate cancers, melanomas (Seelan et al., 2000; Proksch et al., 2011), hepatomas (Osawa et al., 2005), and cervical cancers (Xu et al., 2006). Furthermore, research has demonstrated that prostate cancer cells with an overexpression of acid ceramidase will resist death even under the influence of chemotherapeutic agents.

Available ceramidase inhibitor drugs with long-chain alkyl moieties have poor pharmacological properties and poor bioavailability. Limitations of current therapies result from the affinity for albumin binding, ultimately distorting the structural integrity and effectiveness of the chemicals (Draper et al., 2011). For this reason, small drug-like molecule inhibitors of ceramidases such as ceranib-2 are needed for cancer treatment. At present, no research has been conducted to examine the possible antitumour activities of ceranib-2 as a ceramidase inhibitor in 5RP7 and NIH/3T3 cells. The present study aims to address this gap in the available data. It is hypothesised that the inhibition of ceramidase enzymes, specifically through the application of ceranib-2, will serve as an important target for the chemotherapeutic intervention of cancer.

2. Materials and methods

2.1. Cell lines

In the present study rat embryo fibroblast cells transfected with the *H-Ras* oncogene known as *H-Ras* transformed 5RP7 cells are used as cancer cells. Because of the role of Ras in regulating cell proliferation, differentiation, morphology, and apoptosis, *H-Ras* transformed cells are used and termed as 5RP7 cells in this study. NIH/3T3 cells are used as normal cells in order to compare the effects of ceranib-2 on cancer cells with the effects on noncancer cells. NIH/3T3 cells are noncancer fibroblast cells of NIH/Swiss mouse embryos.

2.2. MTT assay

A stock solution of ceranib-2 (10 mM) in dimethyl sulphoxide was prepared and diluted with Dulbecco's Modified Eagle Medium (Sigma, Germany). 5RP7 and NIH/3T3 cells were plated into 96-well plates with 1×10^3 cells per well. The 96-well plates were treated with ceranib-2 at concentrations of 0.1, 0.3, 0.5, 1, 3, 5, 7, 10, and 20 μM for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. To each well, 20 μL of MTT solution (5 mg/mL) was added and wells were incubated for 2 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. The medium in each well was changed to 200 μL of dimethyl sulphoxide (Sigma) and mixed thoroughly for 10 min at room temperature. The plates were read on an ELISA reader (EL × 808, BioTek, USA) at a wavelength of 540 nm (n = 3).

2.3. Confocal microscopic analysis

5RP7 and NIH/3T3 cells with IC_{50} ceranib-2 inhibition concentrations were incubated for 24 h at 37 °C. After incubation, growth medium was removed, and the cells were washed with phosphate buffered saline (PBS, Invitrogen, USA) and fixed in 2% glutaraldehyde for 15 min at room temperature. Following fixation, the cells were washed in PBS and double-stained with Alexa Fluor-488 Phalloidin and acridine orange. The morphological changes of the stained cells were observed with a Leica ICS-SP5 II confocal microscope with the support of the supplied software (Leica Confocal Software Version 2.00, Leica, Germany).

2.4. Ultrastructural analysis

The ultrastructural changes in 5RP7 cells and NIH/3T3 fibroblasts were examined by transmission electron microscope (TEM). 5RP7 and NIH/3T3 cells treated with ceranib-2 inhibition concentration (IC_{50}) for 24 h (5RP7 IC_{50}: 3 μM, NIH/3T3 IC_{50}: 5 μM) were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The samples were postfixed in 2% osmium tetroxide and dehydrated in graded alcohol (70%, 90%, 96%, and absolute alcohol). The cells were then embedded in Epon 812 epoxy and sectioned on ultramicrotome (Leica EMUC6). The samples were thin-sectioned using a glass knife to a maximum thickness of 100 nm. The sections were stained with lead citrate and uranyl acetate and viewed by TEM (FEI Tecnai BioTWIN).

2.5. Statistical analysis

SPSS 11.5 for Windows was used to conduct one-way variance analysis for multiple comparisons.

3. Results and discussion

In several human tumours, the level of ceramide is lower than in normal tissue and is inversely correlated with the degree of malignant progression (Riboni et al., 2002;

Ogretmen et al., 2004). To address this relationship, various tumour-suppression signals act to stimulate the production of ceramide, which in turn promotes apoptosis of cancer cells. Current data suggest that the enzyme pathway controlling intracellular ceramide levels might offer potential new targets for antineoplastic therapy (Canals et al., 2011). The enzyme of paramount importance to the intracellular ceramide pathway is ceramidase.

Ceramidase, through the control of ceramide hydrolysis, regulates cellular levels of the structural cell membrane sphingolipids SPH and S1P. There are 5 different ceramidase enzymes responsible for the production of SPH; however, the precise biological mechanism of this process remains unknown. The metabolic pathway of S1P in mammalian cells starts when sphingomyelin is hydrolysed by sphingomyelinases to generate ceramide (Quintern et al., 1989; Tomiuk et al., 1998; Hofmann et al., 2000). Further hydrolysis converts ceramide to SPH via ceramidases (Koch et al., 1996), which in turn phosphorylates to S1P (Kohama et al., 1998; Liu et al., 2000).

The present study investigated the role of ceranib-2 as an acid ceramidase (AC) inhibitor in normal NIH/3T3 fibroblast cells and 5RP7 cell lines. Acid ceramidases are cysteine amidases that hydrolyse the proapoptotic lipid ceramide, which in turn modulates the ability of ceramide to influence the survival, growth, and death of tumour cells (Ogretmen et al., 2004; Canals et al., 2011). Several structural analogues of ceramide that inhibit AC activity in vitro have been recorded. The present study identified ceranib-2 as a highly potent inhibitor of AC and demonstrated that AC blockage plays an important role in the antiproliferation of cancer cells. The results obtained suggest that alteration of the chemical scaffold of ceranib-2 will allow for the production of new AC inhibitors to be applied synergistically with standard antitumour agents. Draper et al. (2011) support these findings, commenting that ceranib-2 toxicity and the resultant suppression of cell proliferation is a consequence of cellular ceramidase inhibition.

Cytotoxic effects of ceranib-2 in 5RP7 and NIH/3T3 cells were detected by MTT assay in the present study. Cell viability was found to decrease in proportion to the concentration of ceranib-2 applied (Table), with inhibition occurring more effectively at lower concentrations. 5RP7 cells demonstrated lower levels of viability than NIH/3T3 cells. The IC_{50} inhibition concentration of ceranib-2 for 5RP7 cells was 3 μM, while for NIH/3T3 cells it was 5 μM. From these data it can be concluded that ceranib-2 is more effective at supressing the growth of cancer cells than that of normal cells.

Morphological analysis of NIH/3T3 and 5RP7 cells treated with ceranib-2 (IC_{50} value for each cell line) was conducted through confocal microscopic evaluation. Morphological changes recorded include condensed nuclei, fragmented DNA and cytoskeleton, and various cell membrane misconfigurations. 5RP7 cells exposed to 3 μM ceranib-2 for 24 h presented signs of apoptosis in the form of fragmented nuclei (Figure 1A), chromatin condensations (Figure 1B), and cytoskeleton laceration (Figure 1C). NIH/3T3 cells treated with 5 μM ceranib-2 for 24 h revealed altered configurations (Figure 2A), including fragmented cytoskeleton (Figure 2B) and condensed nuclei (Figure 2C). The results of confocal microscopic observation indicate that ceranib-2 effectively alters cell morphology at low concentrations, with the recorded structural changes representing cellular apoptosis.

Table. MTT analysis results of 5RP7 cells and NIH/3T3 cells treated with different concentrations of ceranib-2 for 24 h. IC_{50} concentration of ceranib-2 was found to be 3 μM for 5RP7 cells and 5 μM for NIH/3T3 cells.

Applied concentrations (μM)	Viability of 5RP7 cells (%)	Viability of NIH/3T3 cells (%)
0.1	71.18	81.79
0.3	68.96	72.22
0.5	62.93	62.48
1	52.05	51.28
3	50.00	51.21
5	33.24	50.00
7	28.14	43.81
10	12.45	8.52
20	9.02	8.50

In ultrastructural analysis performed using a TEM, it was found that NIH/3T3 cells exposed to 5 μM ceranib-2 for 24 h exhibited slight blebbing and shrinkage of cell membranes (Figure 3A), as well as mitochondrial dysfunction (Figure 3B) and nuclei condensation (Figure 3C). TEM observation of 5RP7 cells treated with ceranib-2 revealed high levels of nuclear damage, including fragmentation, membrane shrinkage, and form irregularity (Figure 4A). 5RP7 cells also presented dilated Golgi organelles, lipid accumulation (Figure 4B), loose cristae, and extreme degradation of mitochondria (Figure 4C), as well as hole formation (Figure 4D). The morphological changes of the Golgi complex are considered a result of the Golgi ceramidase enzyme that is responsible for regulation of cell proliferation and survival through the control of the sphingolipids SPH and S1P (Xu et al., 2010).

Results of the present study show that ceranib-2 caused fragmentation of the Golgi complex that may occur as a

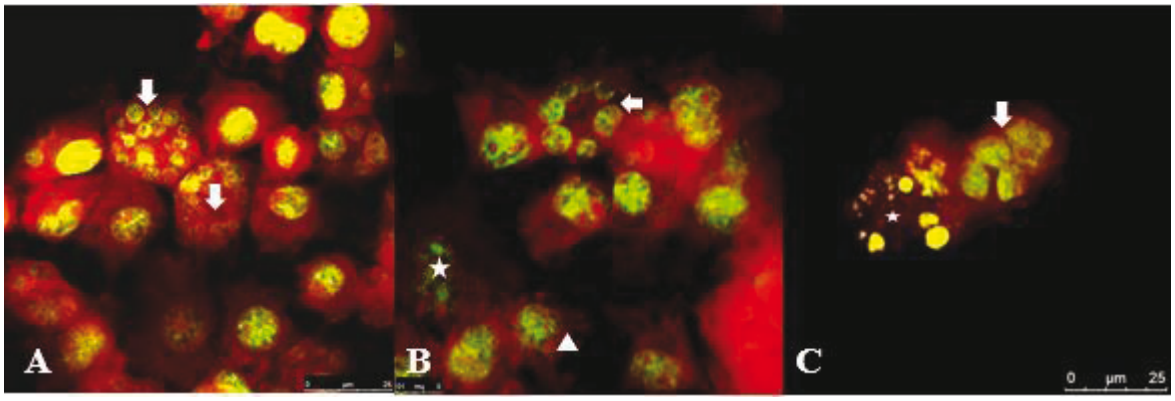


Figure 1. 5RP7 cells treated with IC_{50} inhibition concentration of ceranib-2 for 24 h, double-stained with acridine orange and Alexa Fluor 488-Phalloidin. **A)** Arrow: fragmented nuclei; **B)** arrow: fragmented nucleus, asterisk: chromatin condensation, triangle: fragmentation on cytoskeleton; **C)** arrow: fragmented and condensed nucleus, asterisk: laceration of cytoskeleton.

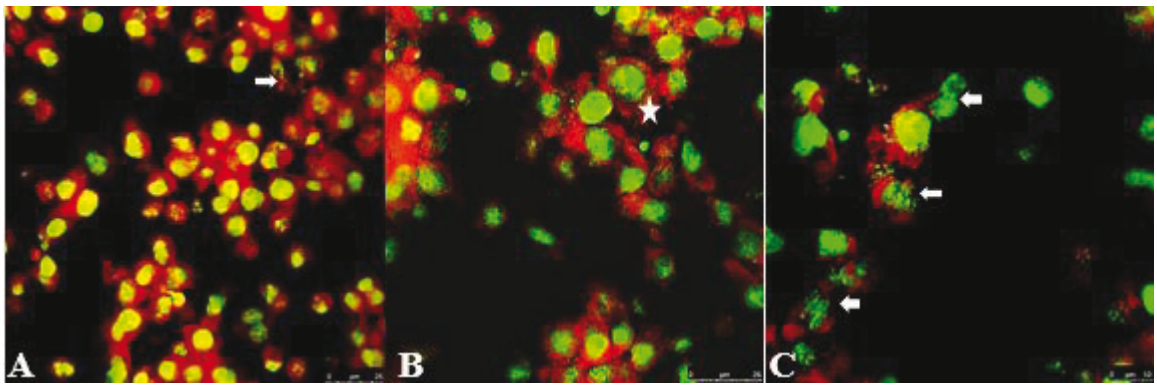


Figure 2. NIH/3T3 cells treated with IC_{50} inhibition concentration of ceranib-2 for 24 h, double-stained with acridine orange and Alexa Fluor 488-Phalloidin. **A)** Arrow: condensed nucleus; **B)** asterisk: fragmentation of cytoskeleton; **C)** arrow: fragmented and condensed nuclei and cytoskeleton.

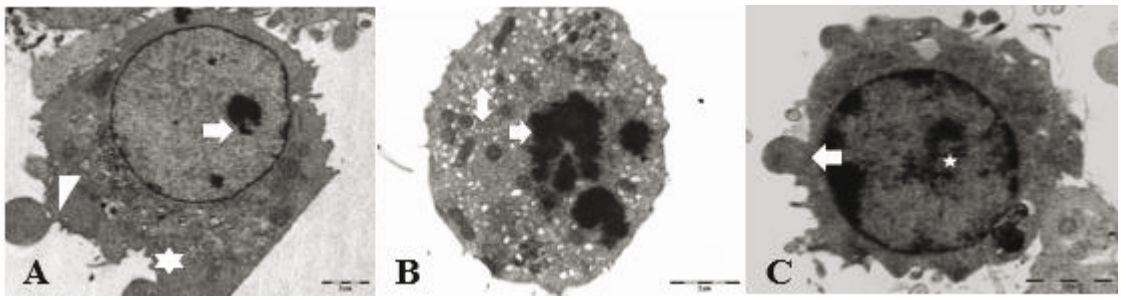


Figure 3. TEM micrographs of NIH/3T3 cells treated with IC_{50} inhibition concentration of ceranib-2 for 24 h (12,000×). **A)** Rectangle: blebbing on cell membrane, asterisk: shrinkage of cell membrane, arrow: condensation of the nucleus; **B)** arrow: nuclear condensation, double-headed arrow: mitochondrial dysfunction; **C)** arrow: blebbing on cell membrane, asterisk: condensation in nucleus.

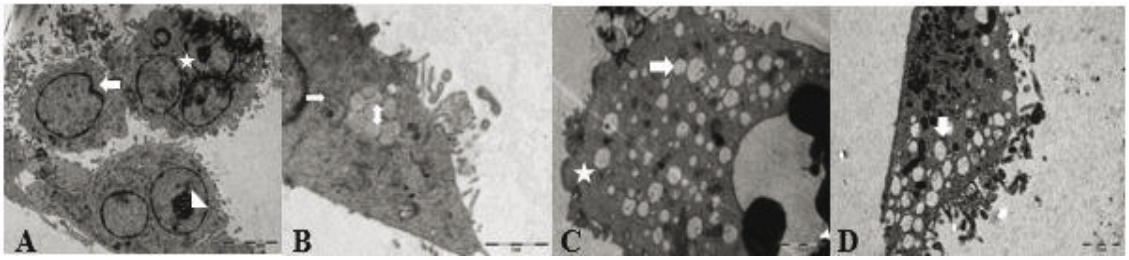


Figure 4. TEM micrographs of 5RP7 cells treated with IC_{50} inhibition concentration of ceranib-2 for 24 h. **A)** Triangle: chromatin condensation, asterisk: nuclear fragmentation, arrow: shrinkage of nuclear membrane (5000×); **B)** arrow: dilatation of Golgi vesicles, double-headed arrow: lipid accumulation (15,000×); **C)** arrow: loosening of cristae, degradation of mitochondria, asterisk: blebbing on cell membrane (15,000×); **D)** arrow: holes in the cell (10,000×).

result of accumulation of SPH. The relationship between ceranib-2 and SPH suggests that SPH is highly cytotoxic and may offer an explanation for its tight method of cellular regulation.

Cell-based assay results have revealed high levels of cytotoxicity in 5RP7 cells treated with ceranib-2, as well as reduced viability of NIH/3T3 cells. The present study also demonstrated the potential for morphological and ultrastructural changes in 5RP7 and NIH/3T3 cells resulting from the application of ceranib-2. Furthermore, morphological and ultrastructural analysis supports the theory that ceramidase inhibition by ceranib-2 controls the generation of S1P in the Golgi complex. With strong cytotoxic and apoptotic effects, ceranib-2 may serve as a template for the design of antitumour agents to be applied in chemotherapeutic practices.

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