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Ceranib-2 has Cytotoxic Effects on Human Bronchial Epithelial Cells *in vitro*

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Abstract
Previous studies demonstrated that ceranib-2 as a ceramidase inhibitor has potent cytotoxic activity and induces apoptosis in different cancer cells. However, knowledge about the possible effects of ceranib-2 on healthy cells is limited. For this purpose, the morphological, cytotoxic and apoptotic effects of ceranib-2 (1 to 100 μM) on human bronchial epithelial (BEAS-2B) cells were investigated. In the morphological examination after ceranib-2 application for 24 and 48 h, the number of survived cells was decreased and apoptotic changes were observed due to increase in concentration and treatment time. Ceranib-2 decreased cell viability and the IC_{50} values were determined as 48 and 4 μM for 24 and 48 h, respectively. C2-ceramide was also used as positive control and found less toxic with IC_{50} values of 57 and 43 μM for 24 and 48 h, respectively. Moreover, we evaluated apoptotic/necrotic DNA fragmentation rates after 24 h application of ceranib-2 concentrations (10, 25 and 50 μM) and the apoptotic effect was higher than the necrotic effect compared to control by Cell Death Detection Elisa Plus Kit.
Consequently, we determined ceranib-2 had a more toxic effect on healthy human bronchial epithelial cells compared to ceramide analogues and this effect increased due to time. The findings of our study indicated the importance of investigating potential anticancer agents on healthy cells.
Keywords: BEAS-2B, ceranib-2, healthy cells, cytotoxicity, apoptosis

1. Introduction

In classical cancer chemotherapy, it is aimed to suppress the proliferation of cancer cells with chemotherapeutic drugs applied in the treatment. It is known that these drugs cause various side effects especially on dividing healthy cells in the treatment process (Eder & Skarin, 2010). Therefore, the search for chemotherapeutic agents that do not harm healthy cells but can selectively target cancer cells still continues and it is extremely important to evaluate the cytotoxicity of novel agents on healthy cells (Liu et al., 2015).

Acid ceramidase (AC) is the enzyme which hydrolyses the bioactive sphingolipid ceramide with pro-apoptotic properties (Mahdy et al., 2009). In recent studies, the effects of acid ceramidase (AC) inhibitors on proliferation, apoptosis and cell cycle arrest have been demonstrated in many cancer types such as prostate, lung, melanoma, breast, ovarian, colon and glioblastoma (Govindarajah et al., 2019). All these mechanisms have been linked to ceramide accumulation in cancer cells after AC inhibitor treatment in vitro. Besides, cancer cells with increased AC activity had been found to be more resistant to chemotherapeutic drugs (Zeidan et al., 2008). It was reported that the elevated level of AC in prostate cancer cells causes the resistance to drugs that stimulate apoptosis (doxorubicin, etoposide, cisplatin, ceramide analogue) while suppression of the enzyme decreases this resistance (Saad et al., 2007). In another study, drug resistance occurred in parallel with the increased AC activity in hepatoma cells (Morales et al., 2007). Inhibition of AC activity reduced cell survival in CD133+ glioblastoma stem-like cells (GSCs) and decreased invasiveness of melanoma cells [8,9].

Ceranib-2 is a novel AC inhibitor that firstly investigated in human ovarian cancer (SKOV3) and have been found to cause cellular ceramide accumulation which leads to cancer cell death (Draper et al., 2011). Furthermore, ceranib-2 suppressed the proliferation and triggered apoptosis in different cancer cells, for example, prostate cancer (DU145 and LNCaP), breast cancer (MCF-7 and MDA MB-231), human colorectal adenocarcinoma (CaCo-2), and glioma (T98G and U87MG) (Kus et al., 2015; Vethakanraj et al., 2015; Vejselova et al., 2016; Vethakanraj et al., 2018; Baspinar et al., 2017; Kuş et al., 2018). Also, hypoxia-inducible factor-1α expression was inhibited after ceranib-2 treatment which ended up with apoptosis and reduced cell viability in HepG-2 cells (Erkasap, 2019). Moreover, ceranib-2 induced the expressions of pro-apoptotic genes, caused apoptosis and had a synergistic effect when combined with carboplatin in addition to its antiproliferative effect in non-small cell lung cancer cell lines (A549 and H460) (Yildiz-Ozer et al., 2018).

However, studies on the possible effects of ceranib-2 on healthy cells are very limited. In a study, normal human astrocytes (NHAs) were applied with 1 to 50 μM ceranib-2 concentrations and cell viability was decreased after 24 h treatment but IC50 value was not found within the concentration range (Kuş et al., 2018). Vejselova et al. treated mouse embryonic fibroblast cells (NIH/3T3) with 0.1 to 20 μM ceranib-2 concentrations and found the IC50 value as 5 μM by MTT assay after 24 h treatment (Vejselova et al, 2014). There is only one study in the literature about the effects of long-term ceranib-2 therapy. After treatment with 10 to 100 μM ceranib-2, eryptosis was induced and percentage of erythrocyte hemolysis was increased for 48 h (Signoretto et al., 2016).

Human bronchial epithelial cells (BEAS-2B) has been used in many studies to compare the cytotoxic effects of new potential chemotherapeutic agents with cancer cells (Min et al., 2018; Ruberte et al., 2018). In our previous research on ceranib-2, we determined the cytotoxicity of ceranib-2 on BEAS-2B cells by MTT assay just for 24 h and found out ceranib-2 was less toxic in healthy cells compared to lung cancer cells within 24 h (Yildiz-Ozer et al., 2018). In this study, we aimed to investigate further and research the possible morphological and cytotoxic effects of 1 to100 μM of ceranib-2 in BEAS-2B cell line for 48 h, including repeat of 24 h treatment. C2-ceramide, a ceramide analogue, was

used as positive control in our study for comparison of the cytotoxicity with ceranib-2. Also, we evaluated the DNA fragmentation rates caused by apoptosis and necrosis. Furthermore, same treatment concentrations of c2-ceramide as a ceramide analogue were used to compare the cytotoxicity with ceranib-2.

2. Materials and Methods

2.1. Cell Culture and Preparation of Treatment Concentrations

Human bronchial epithelial cell line (BEAS-2B, ATCC® CRL 9609™) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% penicilin-streptomycine solution. The cells were seeded in culture medium containing flasks and incubated in a humidified atmosphere with 5% CO_2 at 37 ^{0}C. After confluence was achieved, cells were trypsinized and subcultured.

Ceranib-2 (Cayman Chemical) and c2-ceramide (Cayman Chemical) stock solutions were prepared as 10 mM by dissolving in dimethyl sulfoxide (DMSO). Prior to the treatment, both stock solutions were diluted in culture medium to obtain each working concentrations (1 to 100 µM).

2.2. Detection of changes in the cell morphology

When confluency was achieved, BEAS-2B cells were trypsinized, centrifuged in medium, stained with trypan blue and cell number was determined with Cedex Cell Analyzer (Cedex XS, Innovatis). Then, cells were seeded in 96-wellplate as $1x10^4$ cells per well and incubated for 24 h. Ceranib-2 concentrations (1-100 µM) were added and cells were treated in two different time periods. Only culture medium was added to control group. Morphological changes caused by ceranib-2 were observed at the end of the treatment by an inverted light microscope (Nikon Eclipse, TC100) and images were taken with a camera attachment.

2.3. Cytotoxicity assay

BEAS-2B cells were seeded in 96-wellplate ($1x10^4$ / well) and incubated for 24 h. After incubation, ceranib-2 and c2-ceramide concentrations (1-100 µM) were added to the wells. The wells containing only culture medium were used as control group. To evaluate the solvent toxicity, 75 and 100 µM DMSO concentrations were also prepared with culture medium and used as solvent control groups. The cytotoxic effects after 24 and 48 h treatment were determined by MTT assay. The absorbance values were detected with a microplate reader (BioTek, Powerwave XS) at 550 nm wavelength. The cell viabilities were calculated with the following formula:

$$\text{Cell viability (\%)} = (\text{Absorbance}_{\text{treated}}/\text{Absorbance}_{\text{control}}) \times 100$$

2.4. Determination of apoptotic and necrotic DNA fragmentation rates

Selected concentrations of ceranib-2 (10, 25 and 50 µM) by MTT results were used for 24 h treatment of BEAS-2B cells. After treatment, DNA fragmentation (mono and oligo-nucleosomes) caused by apoptosis and necrosis were determined with Cell Death Detection ELISA Plus kit (Roche). In order to detect mono and oligo-nucleosomes, cell lysate for apoptosis and supernatant for necrosis were used after the treatment. We performed the kit according to manufacturer's protocol. Finally, enrichment factors of concentration groups compared to the control were calculated with the following formula and represented as fold changes in DNA fragmentation rates:

$$\text{Enrichment factor (EF)} = \text{Absorbance}_{\text{treated}}/\text{Absorbance}_{\text{control}}$$

2.5. Analyze of cytotoxicity and cell death

One-way analysis of variance (ANOVA) and then, Tukey's multiple comparison test was used to analyze of cytotoxicity assay data. Apoptotic and necrotic DNA fragmentation data were evaluated by unpaired t-test. All experiments were performed at least 3 times. The data was presented as the mean ± SEM and results were considered significant for $p<0.05$.

3. Results

3.1. The decrease in cell number and apoptosis-like morphological changes

After the treatment of BEAS-2B with various ceranib-2 concentrations for 24 and 48 h, we detected morphological changes under an inverted microscope. Cells number decreased compared to the control group and the effect increased with prolonged treatment time (Fig. 1). Cells showed apoptosis-like morphological changes such as; shrinkage and rounding Also, we observed apoptotic bodies, blebbing on cell membrane surfaces and loss of cellular adhesion to the surface (Fig. 2). Cell viability images were same for 5, 10 and 25 µM concentrations in 24 h application. In addition, 75 and 100 µM ceranib-2 treated wells had almost no cells left. Therefore, to represent only specific changes we choose control, 1, 25 and 50 µM concentration results for 24 h (Fig. 1 a-d). For the same reasons, we presented control, 1, 5 and 10 µM ceranib-2 results for morphological changes after 48 h treatment (Fig. 1 e-h).

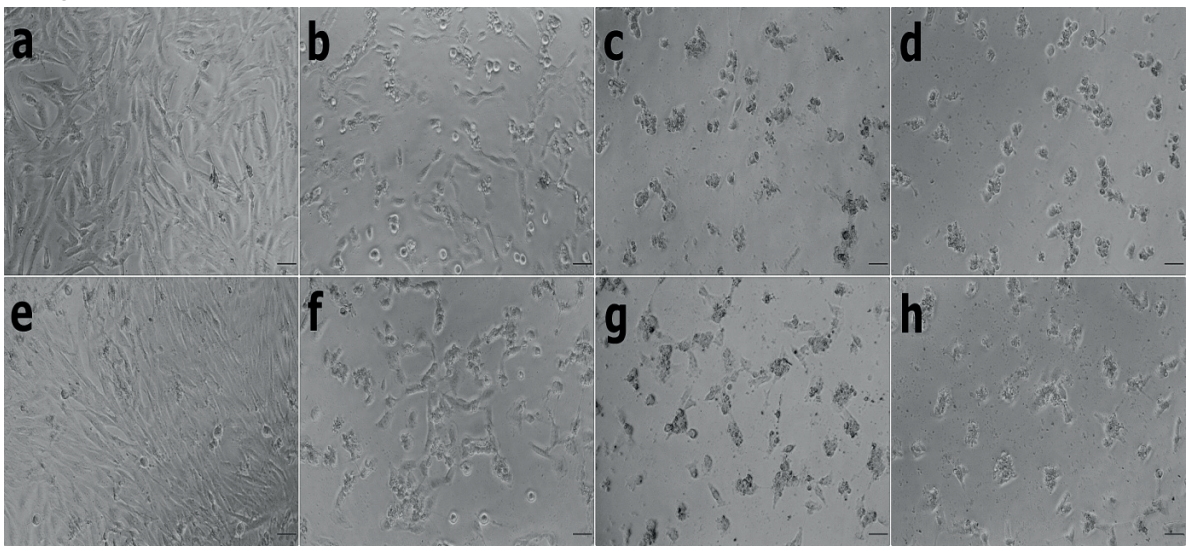


Fig. 1 Morphological changes in BEAS-2B cells after ceranib-2 treatment (a-d: 24 h images of control, 1, 25 and 50 µM ceranib-2; e-h: 48 h images of control, 1, 5, 10 µM ceranib-2) (Scale bar: 50 µm).

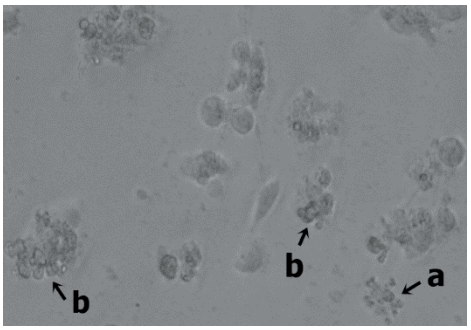


Fig. 2 Apoptotic bodies (a) and blebbing (b) were marked on the morphology image of 25 mm ceranib-2 treated cells for 24 h.

3.2. Reduction in cell viability and proliferation

BEAS-2B cell viability was evaluated by MTT assay after ceranib-2 and c2-ceramide treatments. Ceranib-2 caused significant decrease in cell viability in all applied ceranib-2 concentrations and the effect was increased ($p<0.001$) (Fig. 3). The IC_{50} values were determined as 48 and 4 µM for 24 and 48 h, respectively. The inhibitory effect of c2-ceramide treatment on cell proliferation occurred at 25 µM and higher concentrations for both time durations ($p<0.001$) (Fig. 3). Lower concentrations had no significant effect on cell viability. The IC_{50} values for 24 and 48 h were calculated as 57 and 43 µM, respectively. The solvent control group caused no significant decrease on cell viability in all experiments ($p>0.05$).

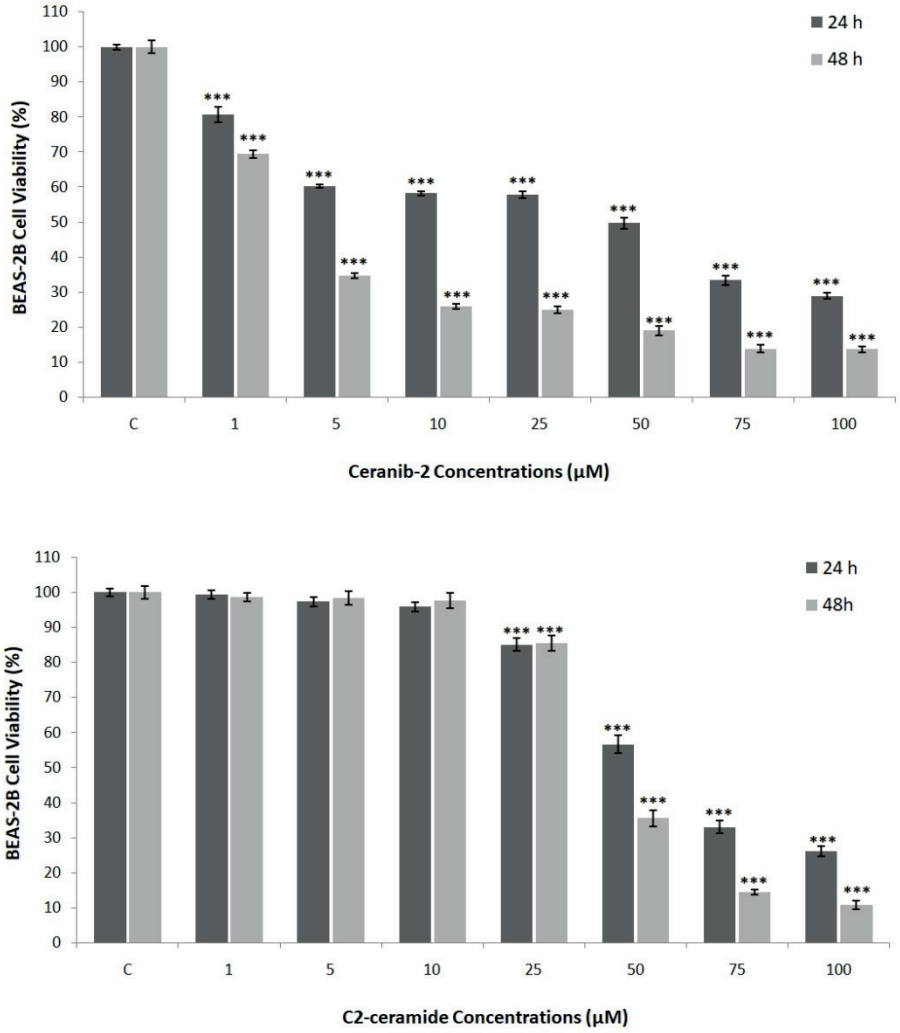


Fig. 3 BEAS-2B cells viability (%) after ceranib-2 and c2-ceramide treatment for 24 and 48 h (C: control, ***:$p<0.001$).

3.3. Comparison of apoptotic and necrotic cell death rates caused by ceranib-2

DNA fragmentation rates were determined quantitatively after treatment with 10, 25 and 50 µM ceranib-2 concentrations for 24 h. The enrichment factors of all concentrations were given as fold changes compared to the control group (EFK=1). According to the results, apoptosis was increased by 3.3, 4.2 and 5.2 fold ($p<0.05$) while necrosis was found to be 2.3, 2.4 and 3.4 fold increased ($p<0.05$), respectively for treatment concentrations (Fig. 3). It was determined that the ceranib-2 triggered apoptosis more than necrosis in BEAS-2B cell line within 24 h treatment.

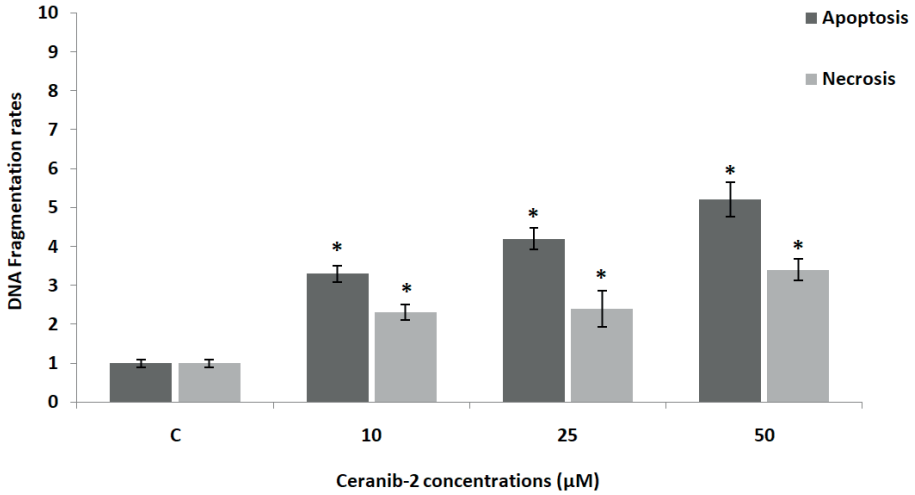


Fig. 3 DNA fragmentation fold change rates after ceranib-2 treatment of BEAS-2B cells. The enrichment factor of non-treated group was considered as 1 (EFK:1).

4. Discussion

Our present study showed that ceranib-2 treatment in BEAS-2B cells decreased cell number and proliferation in a concentration and time dependent manner. Ceranib-2 caused several morphological changes such as cell shrinkage, rounding and membrane blebbing which are known as hallmarks of apoptosis. Similar effects were occurred in NIH/3T3 cells in addition to condensed nuclei, cytoskeleton fragmentation and dysfunctional mitochondria after treatment with 5 µM ceranib-2 for 24 h (Kuş et al., 2018). All these results indicated that apoptotic cell death was induced in healthy cells.

According to MTT results, BEAS-2B line was susceptible to ceranib-2 and viability was reduced significantly in short treatment duration, but the cytotoxic effect dramatically increased with prolongation in time. The half-maximal inhibitory concentration (IC_{50}) for ceranib-2 was decreased from 48 to 4 µM within 24 h. This situation suggests that ceranib-2 should be used with caution in long-term treatment. The toxicity was higher in NIH/3T3 cells and IC_{50} was found 5 µM for 24h (Vejselova et al., 2014). Supporting our results, erythrocyte hemolysis was increased and eryptosis was induced after exposure to ceranib-2 for 48 h (Signoretto et al., 2016). Since ceranib-2 causes ceramide accumulation in cells, c2-ceramide as a ceramide analogue was used in our study for comparison of their cytotoxicity. As a result, the cytotoxicity of ceranib-2 was found higher than c2-ceramide for both 24 and 48 h treatments. This result might be as a result of ceramide abundance caused oxidative stress in cells but further investigation is needed (Maceyka et al., 2007; Signoretto et al., 2016).

We performed DNA fragmentation analysis to determine the cell death mechanism after 24 h ceranib-2 application in BEAS-2B cells. Ceranib-2 treatment was found to trigger apoptotic cell death more than necrosis and DNA fragmentation rate was increased depending on the dose increase. Taken together these data and our morphology images, ceramide abundance caused by ceranib-2 lead to apoptotic cell death in BEAS-2B cells.

The present study reveals ceranib-2 has antiproliferative and apoptotic effects on healthy human bronchial epithelial cells. Moreover, the cytotoxic effect of ceranib-2 increased as the prolongation of treatment. Therefore, ceranib-2 in long term treatment should be done carefully. These findings highlight the importance and suggest further investigation for the possible effects of potential chemotherapeutics on healthy cells.

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