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# Efficacy of ceramidase inhibition on human renal cell carcinoma: a cell culture study

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**Abstract. – OBJECTIVE: Cancer-preventative medicines like curcumin, resveratrol, and nonsteroidal anti-inflammatory medications all have their effects modulated by ceramide. According to research, these medications raise ceramide levels in cancer cells, leading to programmed cell death. Recently, cancer research has been involved in sphingolipid metabolism. The critical molecule here is ceramide. We aimed to investigate if the inhibition of ceramidases induces death in the human renal cell carcinoma cell line.**

**MATERIALS AND METHODS: Human kidney carcinoma A-498 (ATCC® HTB-44™) cells were used as test cells. Ceranib-2, fetal bovine serum (FBS), penicillin/streptomycin, dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide and Dulbecco's Modified Eagle Medium High Glucose, caspase 3/7, annexin-V, Bcl-2 activation dual detection, and MitoPotential kits were used. 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay, annexin-V analysis, caspase 3/7 analysis, Bcl-2 activation analysis, and measurement of mitochondrial membrane potential were performed.**

**RESULTS: MTT colorimetric assay results for 24 hours indicated that the viability of human renal cell carcinoma cells decreased compared to the control group with an increase in the applied concentration of the ceramidase inhibitor-ceranib-2. The growth inhibition by ceranib-2 for 24 hours did not decrease the viability under 50%; thus, it could not be possible to calculate the $IC_{50}$ value for the short-term application of ceranib-2 for 24 hours to A-498 cells. A statistically significant decrease in cell viability was recorded at doses of 100, 50, 25, and 12.2 μM of ceranib-2, and no significant decrease was detected at the lower doses of ceranib-2. The highest inhibition caused by ceranib-2 on human renal cell carcinoma cells A-498 was detected at an application time of 72 hours. This inhibition was statistically significant for all applied doses of ceranib-2 on A-498 cells compared to untreated cells. Annexin-V technique that detects the translocation of phosphatidylserine to the outer membrane of apoptotic cells indicated that after the application of ceranib-2, apoptosis was triggered on A-498 cells with a total apoptotic profile of 12.12% compared to the untreated cells that were used as controls. Compared to untreated A-498 cells, a rise in percentage to 16.25% of cells with activated caspases 3/7 was recorded after applying $IC_{50}$ concentration of ceranib-2 on A-498 cells for 48 hours.**

**CONCLUSIONS: The results of our study indicated that the application of ceramidase inhibitor, ceranib-2 on human renal cell carcinoma A-498 cells cause cytotoxicity, antiproliferative, growth inhibitory, and apoptotic efficacies in a dose and time-dependent manner probably *via* inhibiting the acid ceramidases that hydrolyze ceramides that induce cell death. For further conclusions, more mechanical, pharmacokinetic, and pharmaceutic, as well as *in vitro* and *in vivo* anti-cancer activity investigations are required.**

*Key Words:*
Apoptosis, Cell culture, Cytotoxicity, Ceramidase Inhibition, Human renal cell carcinoma.

## Introduction

Renal cell carcinoma (RCC) is a stealthy neoplasm that accounts for around 2% of all cancer diagnoses and deaths globally and is expected to become even more significant in the future. According to the most recent statistics[1-3], kidney cancer ranks fifteenth among all cancers worldwide. More than 400,000 new instances of kidney cancer were diagnosed in 2020, accounting for 2.2% of all cancer diagnoses. About 75% of all kidney cancers are renal clear cell carcinomas, also known as epithelial neoplasms. The incidence of kidney and renal pelvis cancers has more than doubled in the United States between 1975 and 2002. This alarming trend has been observed throughout the industrialized world in recent decades[3-5].

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There were an expected 431,288 new kidney carcinoma cases worldwide in 2020[1]. Histologically, renal cell carcinoma (RCC) accounts for the vast majority (90%) of RCC cases, mainly containing clear cell RCC (ccRCC; 70%), papillary RCC (pRCC; 10-15%), and chromophobe RCC (5%)[1,2]. Most of the epidemiologic data pertains to RCC in general. The remaining subtypes have an incidence of less than 1% combined and are, therefore, outside the scope of this review. Histologic subtypes have been demonstrated to vary in clinical presentation, prognosis, and genetic predisposition, but further descriptive research is constrained by the need for granularity in available epidemiologic data. Geographic location, gender, and age all affect the wide range of KC incidence and fatality rates. Obesity, hypertension, smoking, and chronic or end-stage kidney disease are all associated with an increased risk of KC, and their effects can be mitigated or eliminated. A better knowledge of the germline and somatic mutations that predispose patients to KC development has resulted from recent improvements in the genetic characterization of these malignancies, opening the door to identifying therapeutic targets that may improve outcomes for these at-risk patients[3-5].

Cancer is a complex disease to treat, often resulting in death. Sphingolipids affect the signaling functions of cancer cells, hence regulating their growth, proliferation, migration, invasion, and metastasis[6]. Ceramide, sphingosine, and sphingosine-1-phosphate are sphingolipid compounds important in regulating cell growth and death[7]. Sphingolipids like ceramide and sphingosine mediate cell death, aging, and cell cycle arrest after being triggered by chemo, radiation, or oxidative stress[8,9].

DNA damage, stress, hypoxia, and the production of apoptotic molecules all lead to the accumulation of ceramides in the cell[10,11]. Ceramide acts as a negative regulator of cell proliferation, which allows it to promote apoptosis. However, sphingosine-1-phosphate (S1P) controls processes, including cell proliferation, survival, and apoptosis inhibition, and is involved in invasion and angiogenesis[12]. An inhibitor of ceramide-dependent apoptosis is sphingosine-1-phosphate. Thus, the ratio of ceramide to S1P in cells is strongly correlated with whether or not those cells will survive. Therefore, the mechanism of apoptosis relies heavily on regulating the ceramidase enzyme and controlling intracellular ceramide, sphingosine, and S1P ratios[12]. The hydrolysis of ceramide by ceramidase enzymes results in the production of sphingosine, a critical lipid mediator. These features have led to these enzymes, which play a crucial role in sphingolipid metabolism, being identified as potential novel targets in cancer treatment. It has been suggested that inhibitors of these enzymes have great potential as new anti-cancer medicines[13]. Cancer-preventative medicines like curcumin, resveratrol, and nonsteroidal anti-inflammatory medications all have their effects modulated by ceramide. According to research, these medications raise ceramide levels in cancer cells, leading to programmed cell death[13,14].

Newly discovered ceranib-2 is a powerful ceramidase inhibitor that inhibits cancer cell proliferation and slows tumor growth despite being weakly water-soluble. However, its utility as an anticancer drug is not well known yet. Studies[4,5,10] are being conducted to determine its cellular absorption and water solubility.

Recently, cancer research has been involved in sphingolipid metabolism. The critical molecule here is ceramide. We aimed to investigate if the inhibition of ceramidases induces death in the human renal cell carcinoma cell line.

## Materials and Methods

### Materials

Human kidney carcinoma A-498 (ATCC® HTB-44™) cells were obtained from Yeditepe University Faculty of Engineering Department of Genetics and Bioengineering (Istanbul, Turkey). Ceranib-2, fetal bovine serum (FBS), penicillin/streptomycin, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide (MTT), trypsin/ETDA and Dulbecco's Modified Eagle Medium High Glucose (DMEM) were purchased from Sigma-Aldrich (St. Louis, USA). Caspase 3/7, annexin-V, Bcl-2 activation dual detection, and MitoPotential kits were obtained from (Merck, Millipore, USA).

### Cell Culture

A-498 human renal cell carcinoma cells were cultured in freshly prepared High Glucose Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum (10%) and penicillin/streptomycin (1%) in a humidified $CO_2$ (5%) incubator[11]. Cells were passaged twice weekly and used for experimentations at a confluency of 85% and passage number 18.

### MTT Colorimetric Assay

A-498 cells were inoculated in flat bottom 96 well plates (Corning, NY, USA) in triplicates ($3x10^5$ cells/well) and were incubated for 24 hours. A stock solution (100 mM) of ceranib-2 was prepared in DMSO. Concentrations ceranib-2 ranging from 3.13 to 100 µM were prepared by dilution in a fresh culture medium and applied to the wells. Plates were incubated for 24, 48, and 72 hours under the same conditions. After the incubation, 20 µL/well of MTT solution [5 mg/mL in phosphate-buffered saline (PBS)] was added and further incubated for 3 hours. After this period, liquids were discarded, and 200 µL of DMSO was added to wells to dissolve the formazan crystals. Plates were read on an ELISA reader (BioTec HTX Synergy, VT, USA) at a wavelength of 560 nm[6]. Viability percentages were calculated from the obtained absorbances as mean SD values on an Excel. The values of inhibition concentration 50 ($IC_{50}$) were determined for all application times from the calculated viabilities.

### Annexin-V Analysis

Annexin-V analysis was used to test the translocation of phosphatidylserine on A-498 cells treated with ceranib-2. For this manner, A-498 cells were seeded in six-well plates ($5x10^5$ cells/well) and applied with $IC_{50}$ value of ceranib-2 for 48 hours at 37°C and 5% $CO_2$ standard incubator conditions. The treated and untreated cells were collected by trypsinization and were washed with PBS. Washed cell samples were resuspended in PBS, and 100 µL was transferred to the Eppendorf tube (Eppendorf, Hamburg, Germany). 100 µL of annexin-V solution was added to all samples and incubated for 20 minutes in the dark at room temperature and were analyzed with Muse™ Cell Analyzer (Merck, Millipore, Hayward, CA, USA) according to the user guide of the annexin-V kit (Merck, Millipore, Hayward, CA, USA).

### Caspase 3/7 Analysis

The activated caspases on A-498 cells by application of ceranib-2 were determined with Caspase 3/7 staining technique. Briefly, A-498 cells exposed to $IC_{50}$ value of ceranib-2 for 48 hours in six-well plates (Corning, NY, USA) and untreated A-498 cells cultured at a density of $5x10^5$ per well were trypsinized and washed with phosphate-buffered saline (PBS) by centrifugation at 1,200 rpm for 5 minutes. Caspase 3/7 working solution and 7-ADD solution were prepared according to the kit's user manual and added to wells. All samples were prepared in line with the user manual of the manufacturer of the caspase 3/7 kit (Merck, Millipore, Hayward, CA, USA). At the end of this period, all samples were read on a cell analyzer (Muse Cell Analyzer™).

### Bcl-2 Activation Analysis

The samples were prepared based on the manufacturer protocol of the Bcl-2 activation kit (Merck, Millipore, Hayward, CA, USA). For this purpose, human renal cell carcinoma A-498 cells were treated with $IC_{50}$ value for 48 hours, and untreated A-498 cells were trypsinized and centrifuged at 1,200 rpm for 5 minutes and washed in PBS. After that, samples and fixed with fixation buffer on ice for 5 minutes. The fixed samples were washed in PBS, permeabilized on ice using a permeabilization buffer for 5 minutes, and washed in PBS again. A-498 cells ($2x10^5$) were transferred to tubes and incubated with 10 µL of the antibody cocktail and 90 µL assay buffer at room temperature in the dark for 30 minutes. Following the incubation, cells were collected with centrifugation at 1,200 rpm for 5 minutes and washed. The washed cells were resuspended in assay buffer (200 µL) and were analyzed with Muse™ Cell Analyzer (Merck, Millipore, Hayward, CA, USA).

### Measurement of Mitochondrial Membrane Potential

The mitochondrial membrane potential of A-498 cells was determined following the Muse™ MitoPotential Kit manufacturer instructions. For this purpose, firstly, the Muse™ MitoPotential working solution was prepared by diluting MitoPotential Dye (1:1000 in 1X Test Buffer). A-498 cells incubated with ceranib-2 ($IC_{50}$ value) and untreated cells cultured in the same culture conditions were harvested by trypsinization, resuspended with fresh complete DMEM high glucose, and used for experimentation. 95 µL of Muse™ MitoPotential working solution was added to 100 µL of cell suspension, and cells were incubated at 37°C for 20 minutes. Then, 5 µL of Muse™ 7-AAD solution was added, and cells were incubated again for 5 minutes at room temperature. All test samples were prepared according to the user guide of the Mitopotential kit (Merck, Millipore, Hayward, CA, USA). After mixing, they were analyzed with Muse™ Cell Analyzer.

### Statistical Analysis

Statistical significances of the data were analyzed on GraphPad prism 8 package programs

(GraphPad Software, La Jolla, CA, USA) by One Way ANOVA analysis and Tukey post-test. $p<0.5$ values were considered statistically significant.

## Results

### MTT Colorimetric Assay Results

MTT colorimetric assay results for 24 hours indicated that the viability of human renal cell carcinoma cells decreased compared to the control group with an increase in the applied concentration of the ceramidase inhibitor-ceranib-2. The growth inhibition by ceranib-2 for 24 hours did not decrease the viability under 50%; thus, it could not be possible to calculate the $IC_{50}$ value for the short-term application of ceranib-2 for 24 hours to A-498 cells. A statistically significant decrease in cell viability was recorded at doses of 100, 50, 25, and 12.2 μM of ceranib-2, and no significant decrease was detected at the lower doses of ceranib-2 (Figure 1).

As shown in Figure 2, the same concentrations of ceranib-2 at the application time of 48 hours highly reduced the viability of A-498 cells in a dose-dependent compared to the untreated cells manner, and the $IC_{50}$ concentration was detected to be 73 μM for this application time. The highest reduction in cell viability was obtained at concentrations of 100, 50, and 25 with a level of significance of $p<0.5$. Growth inhibition for an application time of 48 hours occurred dose- and time-dependent (Figure 2).

The highest inhibition caused by ceranib-2 on human renal cell carcinoma cells A-498 was

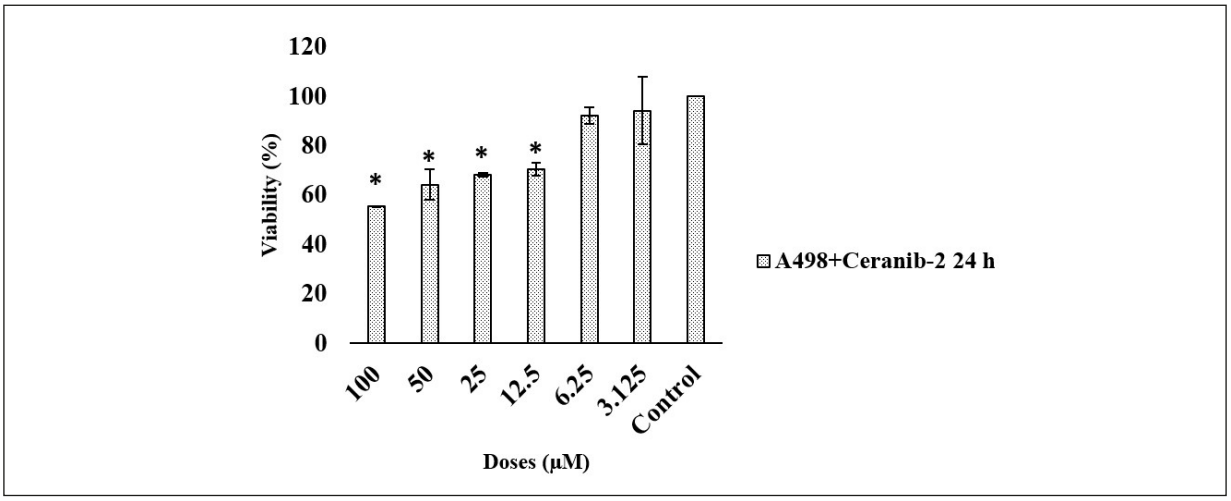


**Figure 1.** Viability percentages of A-498 cells treated with ceranib-2 for 24 hours (*$p<0.05$).

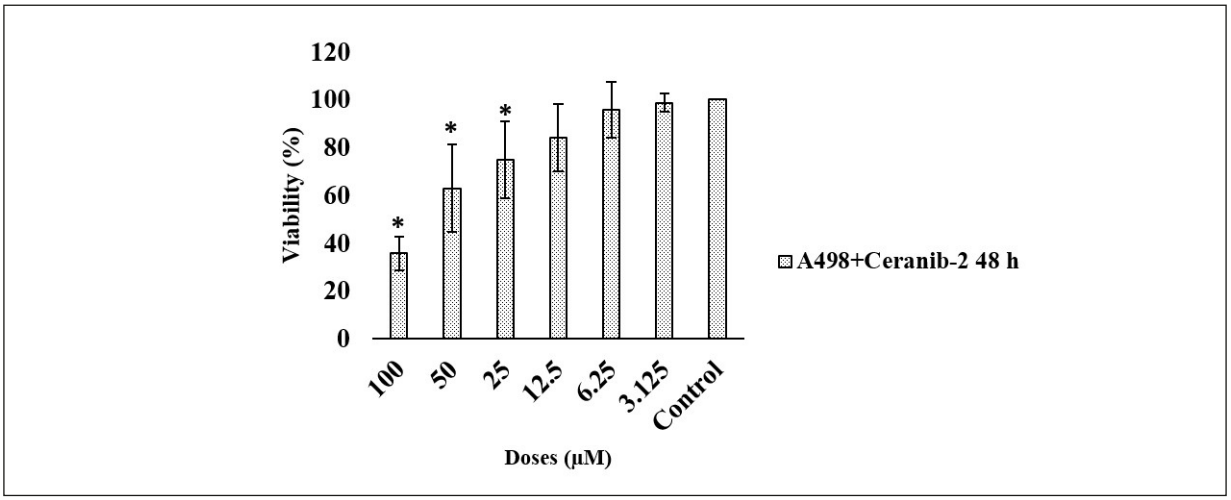


**Figure 2.** Viability percentages of A-498 cells treated with ceranib-2 for 48 hours. (*$p<0.05$); ($IC_{50}$ value was detected to be 73 μM for 48 hours).

detected at an application time of 72 hours. This inhibition was statistically significant for all applied doses of ceranib-2 on A-498 cells compared to untreated cells. The $IC_{50}$ value of ceranib-2 was detected to be reduced to 31 µM, almost twice the lower dose for 48 hours. Based on the MTT results for 24, 48, and 72 hours, it can be concluded that ceranib-2 caused cytotoxicity and antiproliferative efficiency in a dose and time-dependent manner on human renal cell carcinoma cells (Figure 3).

### *Annexin-V Analysis Results*

Annexin-V technique that detects the translocation of phosphatidylserine to the outer membrane of apoptotic cells[7] indicated that after the application of ceranib-2, apoptosis was triggered on A-498 cells with a total apoptotic profile of 12.12% compared to the untreated cells that were used as controls. 11.59% of the ceranib-2 treated cells were in the early apoptotic stage, whereas 0.27% were detected to be apoptotic or dead cells (Figure 4). This study result indicated that the application of ceranib-2 for 48 hours at a value of $IC_{50}$ dose induced programmed cell death probably by causing an increase in the cellular ceramides[8,15].

### *Caspase 3/7 Activation Analysis Results*

Caspases are cysteine proteases that lead to inducing apoptosis in response to various pro-apoptotic signals. Apoptosis was induced by the application of ceranib-2 on human renal cell carcinoma cells, and a part of this apoptotic cell population underwent apoptosis by activating the cascade of caspases[16] (Figure 5). Compared to untreated A-498 cells, a rise in percentage to 16.25% of cells with activated caspases 3/7 was

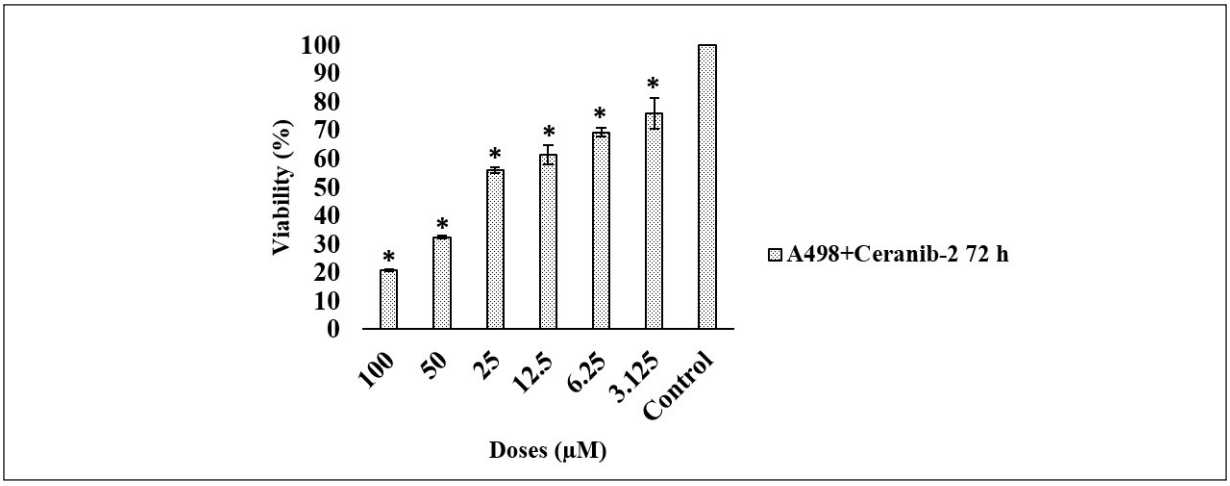


**Figure 3.** Viability percentages of A-498 cells treated with ceranib-2 for 72 hours (*$p$<0.05); ($IC_{50}$ value was detected to be 31 µM for 72 hours).

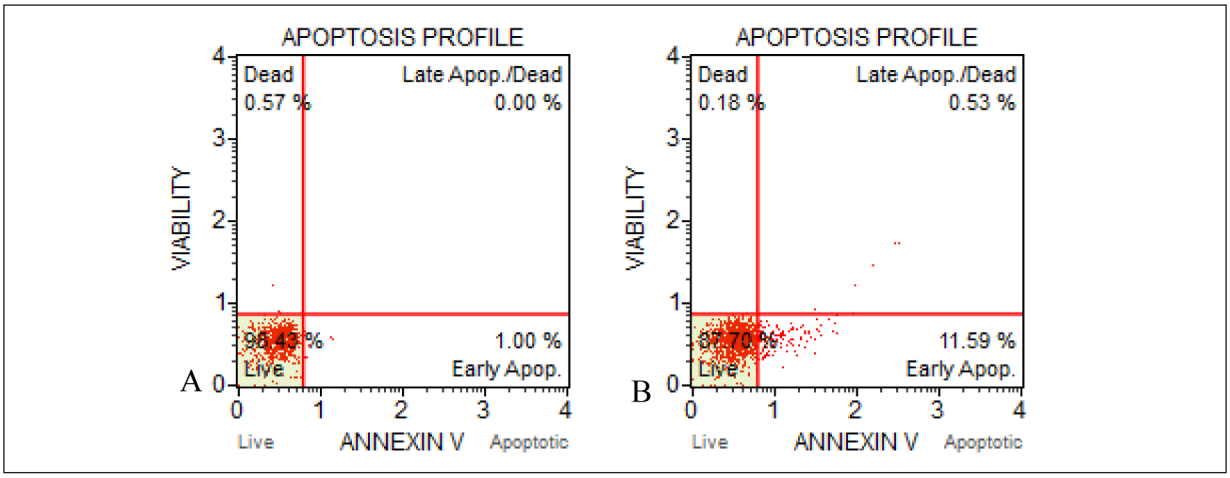


**Figure 4.** Apoptotic profiles of A-498 cells treated with $IC_{50}$ value of ceranib-2 for 48 hours.

recorded after applying $IC_{50}$ concentration of ceranib-2 on A-498 cells for 48 hours.

### *Bcl-2 Activation Analysis Results*

The percentage of non-expressing cells slightly decreased by ceranib-2 application. Also, a slight increase was recorded in the percentage of inactivated Bcl-2 in a test group of A-498 cells. The percentage of activated Bcl-2 in both control and cediranib-2-treated A-498 cells remains unchanged (Figure 6).

### *Mitochondrial Membrane Potential Measurement Results*

Mitopotential kit revealed that the percentage of cells with intact mitochondrial membranes was 19.15% after ceranib-2 application, and 79.9% were cells with depolarized mitochondrial membranes (Figure 7). The depolarization of the inner mitochondrial membrane has been considered a dysfunction in mitochondria[17] as a sign of apoptosis and drug toxicity.

## Discussion

Cancer rates are rising globally, affecting every organ. The etiological variables and environmental elements are under constant investigation, and efforts are being made to halt this growth rate. In addition, an increasing number of researchers are studying cancer treatment to prevent and treat the disease. Recent years have seen an increase in the involvement of sphingolipid metabolism in cancer research. Ceramide is the essential molecule in this metabolism. Ceramides are involved in regulating aging, as well as apoptosis and growth suppression. Ceramidases are responsible for the

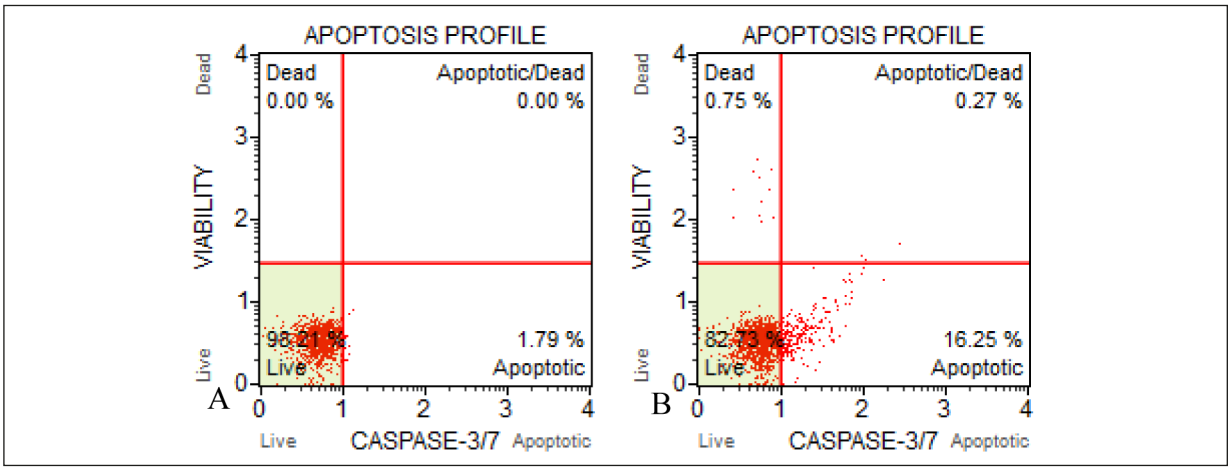


**Figure 5.** Caspase 3/7 activation percentages of A-498 cells exposed to ceranib-2 for 48 hours.

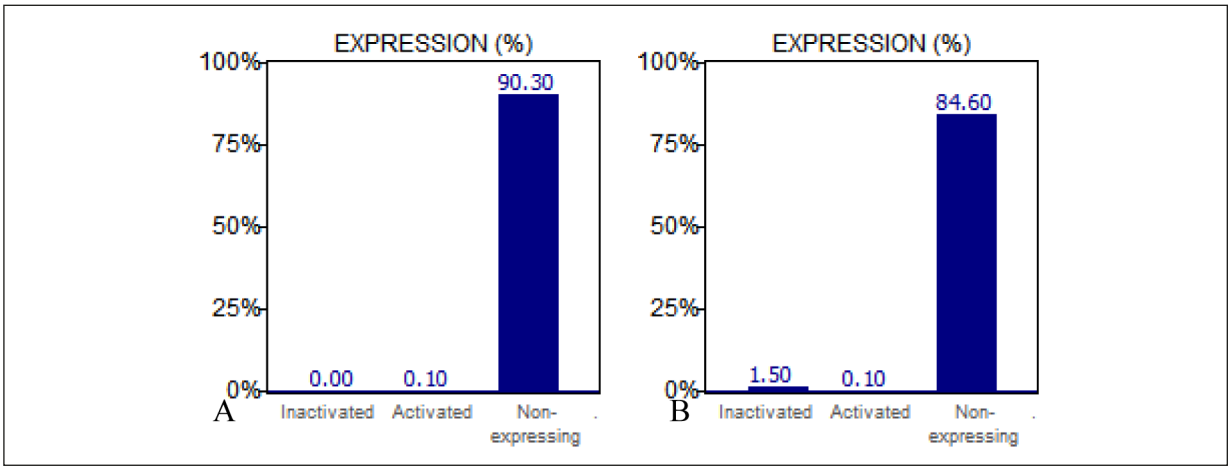


**Figure 6.** Bcl-2 expression levels of A-498 cells treated with ceranib-2 $IC_{50}$ value for 48 hours

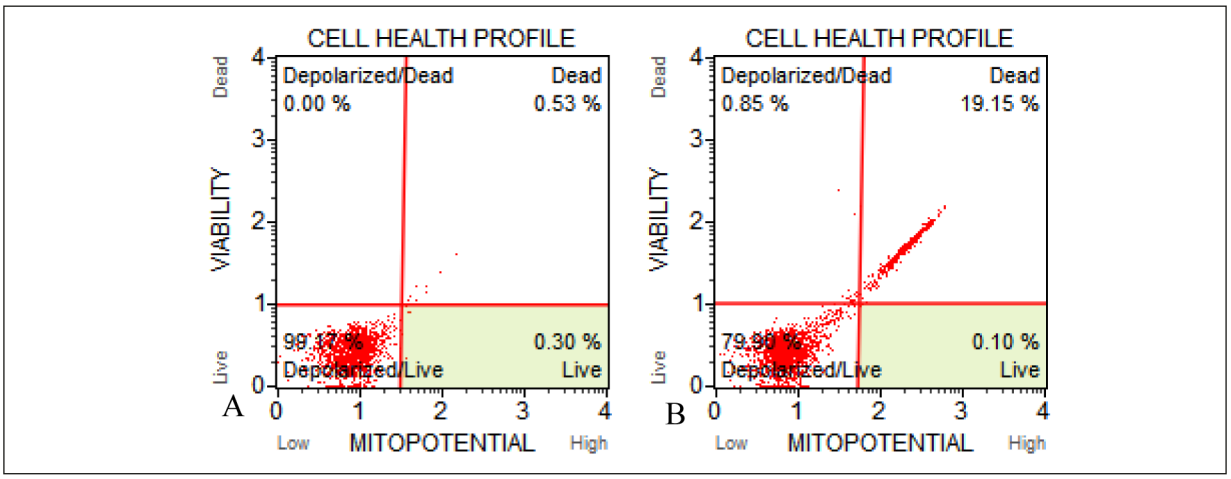


**Figure 7.** Mitochondrial membrane potentials of A-498 cells treated with $IC_{50}$ concentration of ceranib-2 for 48 hours.

breakdown of ceramide, which lowers the amount of ceramide found in cells and ultimately leads to cell death. The literature demonstrates that the inhibition of ceramidases can be used as new targets for cancer treatment.

The $IC_{50}$ value of ceranib-2, an acid ceramidase inhibitor was detected to be reduced to 31μM in application time of 72 hours, almost twice the lower dose for 48 hours. Based on the MTT results for 24, 48, and 72 hours, it can be concluded that ceranib-2 caused cytotoxicity and antiproliferative efficiency in a dose and time-dependent manner on human renal cell carcinoma cells (Figure 3).

Alterations in membrane potential have been demonstrated to be connected with programmed cell death or apoptosis, as stated by Vejselova et al[15]. According to Hearps et al[16] research, one of the defining markers of apoptotic cells is the failure of the mitochondria. This dysfunction is defined by increased mitochondrial membrane permeability, which facilitates the release of cytochrome C, which can then trigger apoptosis. Additionally, this malfunction is characterized by the death of cells. It has been demonstrated that the potential of the mitochondrial inner transmembrane drops during the early stages of apoptosis. This is connected with the death of mitochondria. In the study, it was shown that the percentage of dead cells that still had their mitochondrial membranes intact after the administration of ceranib-2 was 19.15%, but 79.9% of the same cell group consisted of live cells that had depolarized mitochondrial membranes (Figure 7). This was discovered after the application of ceranib-2. According to Friedrich[17], depolarization of the potential of the inner mitochondrial membrane has been interpreted as an indication of a dysfunction in the mitochondria. This depolarization has been significant for the investigation of apoptosis as well as the toxicity of medications. According to Lee et al[18], the cytotoxic, antiproliferative, growth-inhibitory, and apoptotic effects of the ceramidase inhibitor known as ceranib-2 were dose and time-dependent. These effects were observed in human renal cell carcinoma A-498 cells. This was probably brought on by the inhibition of acid ceramidases, which are enzymes that hydrolyze ceramides and are known to cause apoptosis. Additional research into the mechanistic, pharmacokinetic, and pharmaceutic elements of the anti-cancer effect and *in vitro* and *in vivo* testing is required before any more conclusions can be drawn.

When it comes to cancer treatment, having a solid understanding of the apoptotic signaling pathways is essential. Proteins such as Bcl-2 can regulate the apoptotic process in cells. The Bcl-2 protein family is the protein that governs the permeability of the mitochondrial membrane. They are responsible for regulating apoptosis. Anti-apoptotic proteins, such as Bcl-2, are members of the Bcl-2 family, which also contains pro-apoptotic proteins. Bcl-2 is an anti-apoptotic protein that binds to pro-apoptotic proteins, such as Bax and inhibits their ability to insert themselves into the mitochondrial membrane. If an apoptotic signal is detected, Bcl-2 will release Bax, which will then combine with other proteins to form a complex on the mitochondrial membrane[16,17]. This complex will then release cytochrome C into the cytoplasm. Caspases are activated when cytochrome C is released, ultimately leading to the cell's demise. There is evidence that phosphorylation of

Bcl-2 by JNK plays a role in its regulation[7]. It is reported that phosphorylation of Bcl-2 at the serine 70 positions was critical for both the process of apoptosis and autophagy[7]. The administration of ceranib-2 resulted in a marginal reduction in the proportion of cells that did not express their genes. In addition, the percentage of A-498 test cells found to have inactive Bcl-2 showed a slight rise during the experiment. The percentage of activated Bcl-2 in A-498 cells treated with either control or ceranib-2 does not vary (Figure 6).

The use of various targeted therapies, according to recent evidence[7,8] appears to have improved the long-term survival rate of patients whose KIRC has progressed to an advanced stage. New therapeutic targets for cancer treatment have been found in recent years. The modulation of apoptosis and immunology are examples of these newly discovered therapeutic targets. In addition, a large body of information implies that apoptosis can affect anti-cancer immunity by interacting with immune cells, such as NK cells and CD8+ T cells[9-11]. This evidence was gathered over several years. The results of several investigations have led researchers to this conclusion. Apoptosis affects cDC1s and NK cells[12], which causes the immune response to become less effective against malignancies. Research done in the past[13,14] suggests that apoptosis and the immune system may have complementary impacts on cancer treatment.

Recent evidence[7,8] suggests that using various targeted medicines has improved the long-term survival of patients whose KIRC has progressed to an advanced stage. In recent years, new therapeutic targets for cancer treatment have been discovered. These new therapeutic targets include the regulation of apoptosis and immunology.

In addition, a vast body of evidence suggests that apoptosis can modulate anti-cancer immunity by interacting with immune cells, such as NK cells and CD8+ T cells[9-11]. This conclusion was reached based on the findings of the literature. Apoptosis weakens the immune response against tumors by affecting cDC1s and NK cells[12]. Previous research[15,16] has indicated that apoptosis and the immune system may have synergistic effects concerning cancer treatment. As a result, in the current study, we conducted a comprehensive analysis of signaling pathways that trigger apoptosis to examine the correlation between ceramidase inhibition and apoptosis[17,18]. It is also reported that BIIB021 inhibited the chaperone activity of HSP90, resulting in anti-proliferating effects in cervical cancer cells *via* the induction of the intrinsic apoptotic pathways. It is promising as cervical cancer is the fourth most common cancer type among women worldwide and may also be applicable to renal cell Ca[19].

## Conclusions

The results of our study indicated that the application of ceramidase inhibitor, ceranib-2 on human renal cell carcinoma A-498 cells cause cytotoxicity, antiproliferative, growth inhibitory, and apoptotic efficacies in a dose and time-dependent manner probably *via* inhibiting the acid ceramidases that hydrolyse ceramides that induce cell death[18]. For further conclusions, more mechanistical, pharmocokinetic, and pharmaceutic, as well as *in vitro* and *in vivo* anti-cancer activity investigations are required.

### Conflict of Interest
The authors declare no conflict of interest.

### Ethics Approval
Not applicable.

### Informed Consent
Not applicable.

### Funding
This study received no funding.

### Authors' Contributions
All authors contributed equally to planning, writing, and editing.
The cell culture study was performed by Canan Vejselova Sezer and Hatice Mehtap Kutlu.

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