BŞEÜ Fen Bilimleri Dergisi
6. Cilt - Prof. Dr. Fuat SEZGİN Bilim Yılı Özel Sayısı
204-212, 2019

BSEU Journal of Science
DOI: 10.35193/bseufbd.597942

e-ISSN: 2458-7575 (http://dergipark.gov.tr/bseufbd)

Araştırma Makalesi / Research Article

İnsan Prostat Kanseri Hücrelerinde D-e-MAPP kaynaklı Sitotoksisitenin Araştırılması

Investigation of D-e-MAPP-derived Cytotoxicity on Human Prostate Cancer Cells

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Geliş / Received: 29/07/2019 ***Revize / Revised: 28/08/2019*** ***Kabul / Accepted: 11/09/2019***

Öz- Sfingolipidler, birçok kanserde hücre ölümü, hayatta kalma ve ilaç direnci gibi biyolojik işlemlerde kritik rol oynamaktadır. Seramid ve dihidroseramid, proliferasyon ve ölüme bağlı olan sfingolipidlerdir. Son kanser araştırmaları, kanser-sfingolipid metabolizmasının netleştirilmesine odaklanmıştır. Son yıllarda, sfingolipid metabolizması ilişkisinde kilit bir molekül olarak seramid, antikanser aktivitesi için seramidaz inhibitör uygulaması ile hücre içi seviyesini arttırarak araştırılmıştır. Prostat kanseri, en sık görülen insan kanserleri arasındadır ve kansere bağlı en yaygın ikinci ölüm nedeni olarak rapor edilmektedir. Prostat kanseri, 65 yaşından büyüklerde yaygındır. Bu çalışmanın amacı, bir seramidaz inhibitörü olan (1S,2R)-D-erythro-2-(N-Myristoylamino)-1-phenyl-1-propanol'un (D-eritro-MAPP) insan prostat kanseri DU-145 hücreleri üzerindeki potansiyel sitotoksik aktivitesini MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) testi, akış sitometrisi, konfokal ve TEM mikroskopisi kullanarak araştırmaktır. MTT bulguları D-eritro-MAPP'nin DU-145 hücrelerinde düşük dozlarda toksisiteye neden olduğunu göstermiştir. Konfokal ve TEM mikroskopi bulguları, hücre iskeletinde delik oluşumunu, kromatin yoğunlaşmasını, morfolojik değişiklikler olarak at nalı şeklindeki hücre çekirdeklerini ve hücre zarı üzerindeki tomurcuklanmaları, çekirdeklerin parçalanmasını, kromatin yoğunlaşmasını ve ultra yapısal değişiklikler olarak krista kaybını göstermiştir. Akış sitometrisi bulguları, D-eritro-MAPP'in DU-145 hücrelerine 24 saatlik kısa süreli uygulamasında apoptozu tetiklediğini göstermiştir. Elde edilen sonuçlara göre, D-eritro-MAPP'in DU-145 hücrelerinin canlılığını doza bağlı bir şekilde azalttığı söylenebilir. Bulgularımız, D-eritro-MAPP'in DU-145 hücreleri üzerindeki anti-kanser ve sitotoksik potansiyelini ortaya koymuştur ve bu ajanın, in vitro ve in vivo çalışmalardan sonra kanser tedavisi için ilaç geliştirmede kullanılabileceği belirtilmiştir.

Anahtar Kelimeler- Sfingolipid, DU-145, Konfokal Mikroskopi, Akış Sitometrisi

***Abstract*- Sphingolipids play critical role in biological processes such cell death, survival and drug resistance in many cancers. Ceramide and dihydroceramide are proliferation and death associated sphingolipids. Recent cancer research are focused on clarifying cancer-sphingolipid metabolism. In last years, ceramide as a key molecule in sphingolipid metabolism relationship has been investigated for its anticancer activity via augmenting its intracellular level by ceramidase inhibitor application. Prostate cancer is among the most frequent human cancers and is reported as second most common cancer-related death cause. Prostate cancer is common at ages older than 65. The aim of this study was to investigate the potential cytotoxic activity of a ceramidase inhibitor (1S,2R)-D-erythro-2-(N-Myristoylamino)-1-phenyl-1-propanol (D-erythro-MAPP) on human prostate cancer DU-145 cells by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay, flow cytometry, confocal and TEM microscopy. MTT findings showed that D-erythro-MAPP caused toxicity in low doses on DU-145 cells. Confocal and TEM microscopy findings indicated hole formation in cytoskeleton, chromatin condensation, horseshoe-shaped cell nuclei as morphological changes and blebbings on cell membrane, fragmentation of nuclei, chromatin condensation and loss of cristae as ultrastructural changes, respectively. Flow cytometry findings showed that D-erythro-MAPP triggered apoptosis in short-term application of 24 hours on DU-145 cells. According to results it can be concluded that D-erythro-MAPP decreased viability of DU-145 cells in dose-dependent manner. Our findings stated the anti-cancer and cytotoxic potential of D-erythro-MAPP on DU-145 and this agent might be used in drug developing for cancer treatment after the further in vitro and in vivo studies.**

***Keywords*- Sphingolipid, DU-145, Confocal Microscopy, Flow Cytometry.**

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I. INTRODUCTION

Therapeutic development has been focused on targets of sphingolipid metabolism, recently. Bioactive sphingolipids analogues are reported to have a great potential for therapy of cancer disease as well as immune and metabolic disorders. These sphingolipids have been claimed to be main regulators for major characteristics of cancer disease and cancer cells. These bioactive molecules are a class of membrane lipids with structural roles as well as they have functional roles in the regulation of lipid bilayers and fluidity. Futhermore, sphingolipid members such ceramide, glycosylceramide, sphingosine and sphingosine-1-phosphate also are claimed to have various important biological functions as apoptosis, proliferation and migration. On the basis of their roles in that biological processes, sphingolipids are directly important for the development and progression of many cancer types. Thus, these molecules are emerged in regulation of the effectiveness of the anticancer therapeutic agents [1]. The biological roles of the different sphingolipids on cancer cells is dependent on the type of the molecule. Sphingosine and its bases are declared to target actins of cell skeleton, cell cycle, and apoptotic cell death whereas ceramide serve as regulator of cell growth, differentiation, senescence as well as cancer cell apoptosis [2,3].

In addition, ceramide as a major member of sphingolipids, mediates apoptosis, growth arrest, susceptibility to chemotherapeutic agents and aging of cancer cells [4]. Ceramide sources of cells derives from three pathways, by *de novo* biosynthesis, sphingomyelin degradation and salvage. *De novo synthesis* by serine and palmitate condensation [5].

De novo biosynthesis is critical for membrane structure, cell-cell and cell-matrix interactions [6]. Moreover, the biosynthesis and catabolism pathways include a wide range of intermediate metabolites with different biological roles. The relationships of the mentioned metabolites are shown in Figure 1 [5].

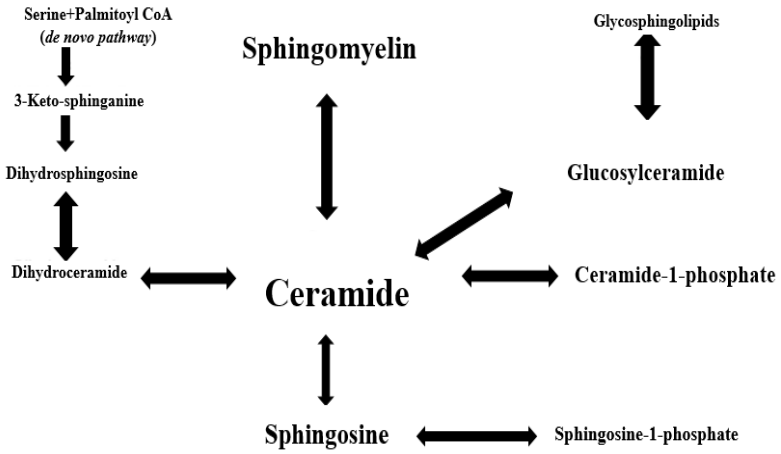

Figure 1: Sphingolipid metabolism pathways [7].

Ceramide pathway has been shown to have significant effect on drug resistance and cell survival [8,9]. In addition, recent cancer research results showed that ceramide has been related directly to cell death and survival [9]. The intracellular level of this molecule, is known to decrease by inhibiting the activity of ceramidase enzyme responsible for the conversion of ceramide to sphingosine. Sphingosine and sphingosine-1-phosphate are emerged molecules in inducing cell proliferation, survival and migration. Thus, the ceramide and sphingosine-1-phosphate ratio is critical for balance of apoptosis and proliferation. Briefly, increased intracellular ceramide levels lead to death, whereas increased sphingosine-1-phosphate levels lead to cell proliferation [10].

In last decades, cancer research emerged about how sphingolipid metabolism survives cells and/or cause cell death. The results of these studies mostly indicate ceramidases and their inhibition as major targets for cancer therapy. Ceramidase inhibitors such ceranib-2, D-e-MAPPD and D-e-MAPP have been started to be investigated for their drug developmental potential based on their anticancer properties. The cytotoxic and anticancer properties of D-erythro-MAPP on human prostate cancer cells does not investigated in details, yet.

Therefore, this study aimed in investigation of cytotoxic, antiproliferative and proapoptotic abilities of D-erythro-MAPP on human prostate cancer DU-145 cells.

II. EXPERIMENTAL STUDY

A. Materials

DU-145 human prostate cancer cells were obtained from the American Type Culture Collection. D-erythro-MAPP, foetal bovine serum, penicillin-streptomycin, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (USA), and Roswell Park Memorial Institute medium (RPMI-1640) was obtained from GIBCO (USA).

B. Cell Culture

Human DU-145 cells were cultured in RPMI medium containing 10% fetal bovine serum (v/v) and penicillin-streptomycin (100 units / mL) at 37°C under humidified incubator conditions with 5% CO_2. The medium of cell was changed with complete RPMI medium at each third day and cells were passaged thrice at week. Cell culture flasks with a confluence of 80% were used for the tests.

C. MTT Assay

The cytotoxicity of D-e-MAPP on DU-145 cells was tested by using MTT colorimetric assay. For this manner, D-e-MAPP stock solution (100 mM) was prepared in DMSO. The concentrations starting from 100 µM were prepared in 96-well plates by serial dilution with final concentration of DMSO of 0,01% per well and cells ($5x10^3$/well) were incubated in the same plates at 37°C for 24 hours. At the end of the incubation, 20 µL/well of MTT dye was added in each well and incubated for a further 4 hours. After incubation time, 100 µL of DMSO per well was added and absorbances were read in an ELISA reader at 570 nm wavelength (HTX Synergy, Bio-Tek, USA) [11]. The viability percentages were calculated from the absorbances obtained and the IC_{50} value was determined.

D. Evaluation of Morphological Changes by Confocal Microscopy

DU-145 cells were seeded on sterilized coverslips in six-well plates and exposed to IC_{50} value of D-e-MAPP for 24 hours at 37°C. Then, the cells were washed with PBS and stained with acridine orange and Alexa fluor p488 phalloidine. The preparations were determined by using Leica TCS-SP5 II laser scanning confocal microscope and morphological changes were imaged.

E. Evaluation of Structural Changes Using TEM Microscopy

In order to examine the fine structural changes by transmission electron microscope (TEM), DU-145 cells at a density of $1x10^6$ / mL were seeded in three replicates in cell culture flasks and exposed to D-e-MAPP IC_{50} concentration for 24 hours at at 37°C incubator conditions. Another 3 flask of DU-145 cells were incubated without treatment and used as control group. After the incubation, the cells were fixed with glutaraldehyde and in osmium tetraoxide. The fixed cells were dehydrated in the ethyl alcohol series (50%, 70%, 90%, 96% and absolute ethyl alcohol). Dehidrated cell samples were treated with propylene oxide, then embedded in resin. Resin embedded samples were polymerised at 60°C for 48 hours and 80-100 nm thin sections were obtained from the blocks. The sections were stained in lead citrate and uranyl acetate photographed under a transmission electron microscope (Biotwin FEI, USA).

F. Apoptosis Determination by Annexin V-PE Staining Technique in Flow Cytometry

This experiment was done according to user manual instructions of the used kit Muse® Annexin V and Dead Cell Assay Kit. For this manner DU-145 cells were seeded ($5x10^5$/well) in 6-well plates and applied with IC_{50} value of D-e-MAPP for 24 hours at 37 °C in a 5% CO_2 incubator conditions. After 24 hours, cell samples were collected and centrifuged twice with PBS at 1200rpm for 5 minutes. Washed cell samples were resuspended in PBS and 100 µL of prepared cell samples were transferred to eppendorf tubes. 100 µL of annexin was added to each tube and incubated for 15 minutes at room temperature in the dark. Following the incubation samples were analyzed on a cell analyzer (Muse ™ Cell Analyzer Merck, Millipore, Hayward, California, USA). For the evaluation this terms were taken in consideration;

Living cells: [Annexin V-PE (–) and Dead Cell Marker (–)]

Early apoptic cells: [Annexin V-PE (+) and Dead Cell Marker (–)]

Late apoptotic cells: [Annexin V-PE (+) and Dead Cell Marker (+)]

Necrotic cells: [Annexin V-PE (–) and Dead Cell Marker (+)]

G. Statistical Analysis

Statistical comparison of the groups was made by using one-way analysis of variance for multiple comparisons using GraphPad Prism 6.0 and $p < 0.0001$ and $p < 0.005$ were considered significant.

III. RESULTS

A. Cytotoxicity Results of D-e-MAPP in DU-145 Cells

D-e-MAPP was shown to reduce the proliferation of DU-145 cells significantly in a dose-dependent manner. The IC_{50} value of D-e-MAPP on DU-145 cells for 24 hours detected to be 36.9 μM.

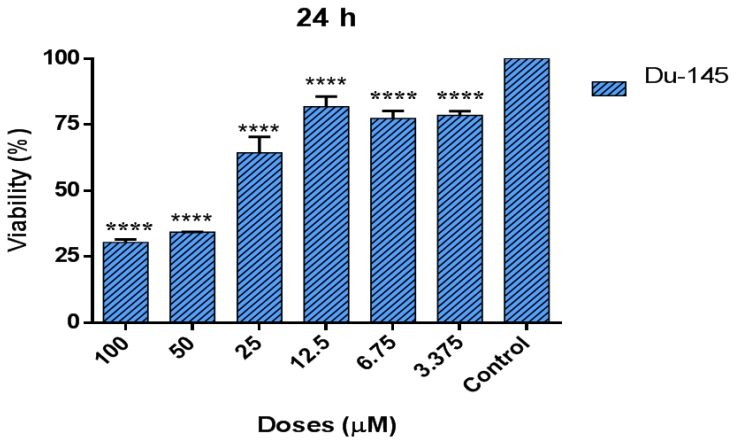

Figure 2: Viability percentages of DU-145 cells exposed to different concentrations of D-e-MAPP for 24 hours. (****; p<0,0001)

B. TEM Analysis Results

TEM findings of DU-145 cells showed that untreated cells were with unchanged cytoskeleton, nucleus and cell membrane (Figure 3). Ultrastructural changes detected in D-e-MAPP-treated DU-145 cells for 24 hours, cell skeleton, chromatin condensation, membrane blebbing, and disintegrated mitochondrion (Figure 4).

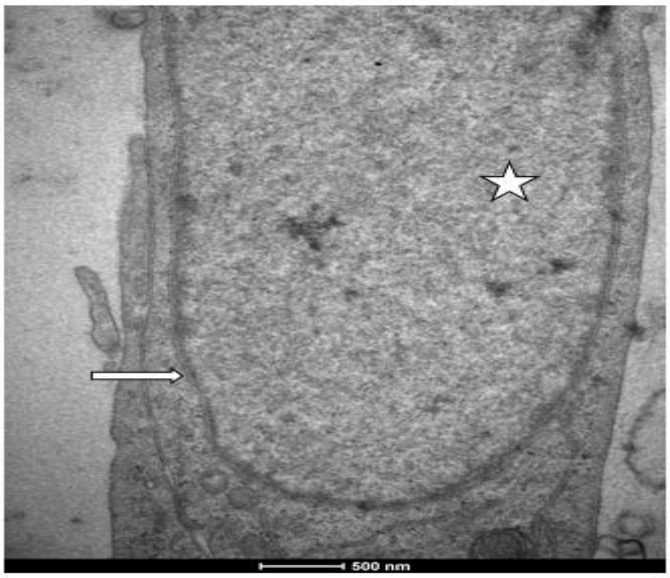

Figure 3: Du-145 cells control group without D-e-MAPP application; Asteriks-compact nucleus, Arrow-compact nuclear membrane.

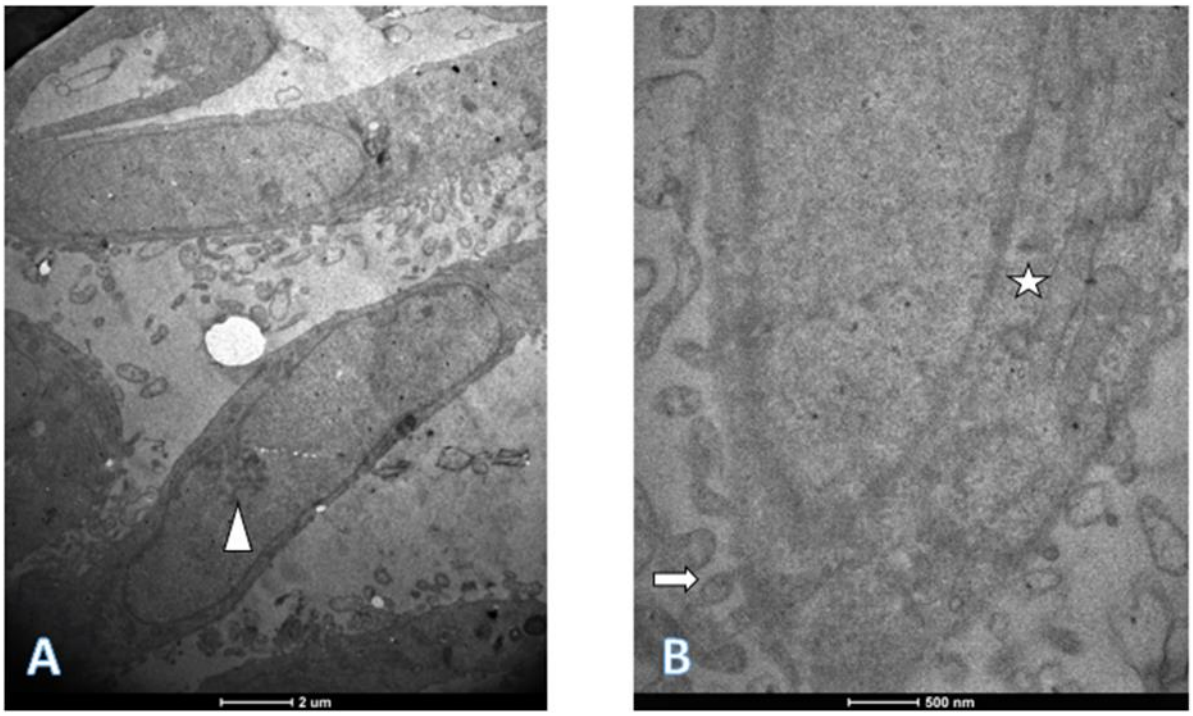

Figure 4: DU-145 cells exposed to IC_{50} value of D-e-MAPP for 24 hours; A) Arrowhead-Chromatin condensation B) Asteriks-loss of cristae, Arrow- membrane blebbing.

C. Confocal Microscopy Results

The morphology of DU-145 cells exposed to IC_{50} value of D-e-MAPP for 24 hours was found to change significantly compared to untreated cells. The nuclei of the control DU-145 cells and the cell skeleton had a compact structure (Figure 5). However, morphological changes were detected in DU-145 cells treated with D-e-MAPP. These changes were chromatin condensation, holes and fragmentation on the cell skeleton, shrunken cells (Figures 6 A and B).

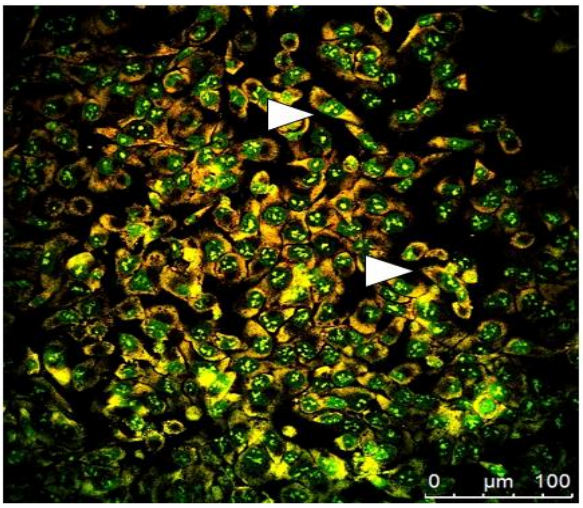

Figure 5: Du-145 cells control group without D-e-MAPP application; Arrow heads-normal cells.

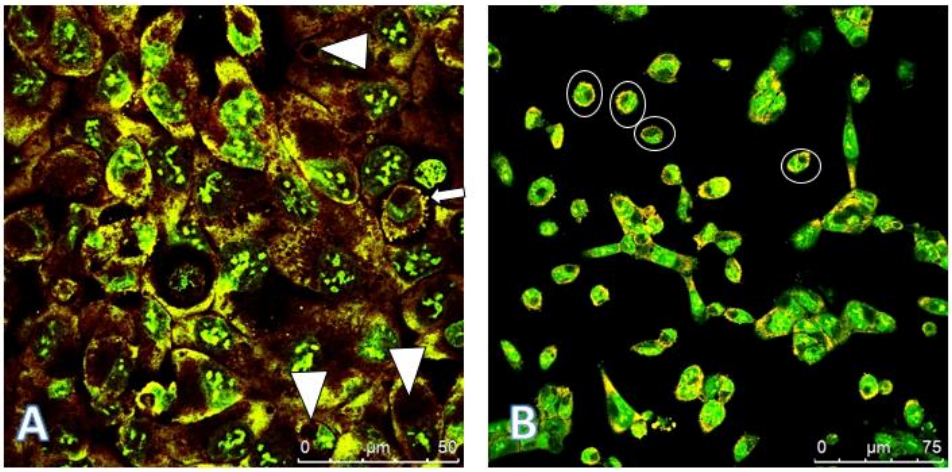

Figure 6: DU-145 cells treated with IC_{50} value of D-e-MAPP for 24 hours; A) Arrowhead-holes in the cell, Arrow-membrane blebbings B) Circle-Shrunken cells (circular cell shape).

D. Anneksin V-PE Results

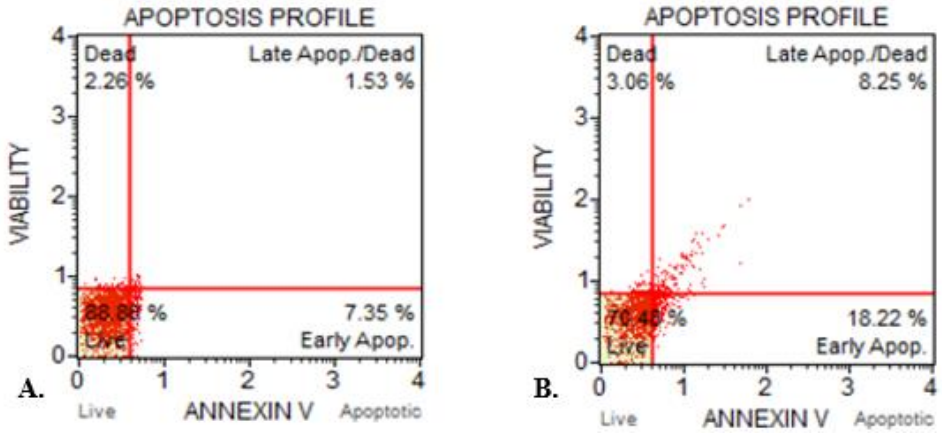

Figure 7: A. Untreated DU-145 cells stained with anexin V. (88% viable cells, 2.6% dead cells, 7.35% early apoptosis and 1.53% late apoptosis cells were determined total 12% dead cells.). B. D-e-MAPP applied DU-145 cells stained with anexin V. (70% viable cells, 3.6% dead cells, 18.22% early apoptosis and 8.25% late apoptosis cells, total dead cells of 29.52% were determined).

IV. DISCUSSION

Sphingolipids as bioactive molecules are shown to be involved in the survival, growth, and differentiation of cells, as well as in pathophysiological process such as inflammation and neuropathic pain [2, 12, 13, 14, 15]. Cell survival and proliferation can be augmented by sphingosine-1-phosphate, while ceramides exert proapoptotic activity both in normal and cancerous cells [16]. Intracellular ceramide levels can be increased by chemotherapeutics, DNA damage, via *de novo* synthesis, hydrolysis of sphingomyelin, or the salvage pathway. On the other hand, cytotoxicity of certain anticancer drugs depend on the same pathway [17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27]. Based on that roles of ceramide and sphingosine in cells these sphingolipids have become critical targets in cancer therapy [28]. Lastly, several cancer therapy startegies have been focused on ceramidases in order to augment the intracellular ceramide level via their inhibition in turn triggering apoptosis in cancer cells. This activity was shown with overexpression of ceramidases in murine L929 fibrosarcorma cells suppressed apoptotic cell death induced by tumor necrosis factor alpha (TNFα). In addition, inhibitors of the activity of this enzyme can lead to increased intracellular ceramide levels and promote apoptotic cell death. For example, a ceramidase inhibitor N-oleoylethanolamine, increased ceramide formation and enhanced apoptosis in L929 cells [29].

In a study, D-e-MAPP was reported to selectively inhibit ceramidases isolated from HL-60 human promyelocytic leukemia cells. The IC_{50} concentration and time-dependent growth suppression of the agent arrested cell cycle at the G0/G1 stage [30]. Choi et al. (2003) showed that the expression of neutral ceramidase in a high level of mesengial cells in a study. The researchers stated that the inhibition of neutral ceramidase by D-erythro-MAPP increases the level of ceramide in cultured mesengial cells and cells in certain regions of the mouse small intestine and consequently apoptotic cell death occurs [31]. In 2008, Zdzislaw et al. (2008) synthesized analogues of D-e-MAPP in their studies aimed to find more effective ceramidase inhibitors [32]. Our MTT results of the and their study results were found to be similar. The IC_{50} value of D-e-MAPP for 24 hours of administration was detected to be 36.9 µM in DU-145 cells on prostate cancer cells. When compared with other research findings the total apoptosis percentage of D-e-MAPP applied DU-145 cells for 24 hours were determined to be 29%. This finding indicate the apoptosis triggering effect of the agent on human prostate cancer cells. On the other hand, TEM and confocal microscopy findings are indicators of high cytotoxic potential of D-e-MAPP on DU-145 cells. The ultrastructural and morphological changes detected on D-e-MAPP applied cells such as chromatin condensation, membrane blebbings, fragmentation of the nuclei and cytoskeleton as well as disintegration of membranous organels like mithochrondria are clear structural signs of programmed cell death.

Taken all together, our finding underline the cytotoxic and proapoptotic activities of D-e-MAPP on DU-145 human prostate cancer cells and based on our findings we suggest this agent for further experimentations on drug designing for cancer therapy.

V. CONCLUSION

Recent cancer research have been focused on clearing the relation of ceramide metabolism and cancer treatment. Current cancer therapeutics has high level side effects on whole body of the patients. Thus, novel applications and agent to cure cancer disease are requried. From our results we can conclude that D-e-MAPP, as a ceramidase inhibitor has a valuable potential of anticancer activity via causing cytotoxicity and being antiproliferative and proapoptotic on human prostate cancer cells, DU-145. Consequently, D-e-MAPP is a strong candidate to be evaluated for its further anticancer activities on different cancer cell lines and *in vivo*.

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Conflıct of Interest: Authors claim no conflicts of interest for this study.