Araştırma Makalesi/Original Article
Makale Dili "İngilizce"/Article Language "English"

Türk Hijyen ve Deneysel Biyoloji Dergisi

Arachidonoylcyclopropylamide (ACPA) reduces viability and induces endoplasmic reticulum stress in glioma cells

Araşidonilsiklopropilamid (ACPA) glioma hücrelerinin canlılığını azaltır ve endoplazma retikulumu stresini indükler

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ABSTRACT

Objective: The endocannabinoid system (ECB) is present throughout the human body and has been extensively studied, particularly in the nervous system. Cannabinoid 1 (CB1) ligands and receptors are primarily found in the central nervous system and its cells. Neuroglioma is an aggressive and life-threatening cancer that accounts for approximately 50% of primary central nervous system (CNS) tumors. The treatment protocol for gliomas typically includes surgery, radiation therapy or chemotherapy. In this study, we evaluated the antiproliferative effects of Arachidonoylcyclopropylamide (ACPA), a CB1 receptor agonist, on H4 human neuroglioma cell line.

Methods: In this study, we investigated the antiproliferative, pro-apoptotic, antimigratory and organelle-stress-related effects of ACPA on H4 neuroglioma cells. xCELLigence cell impedance assay (n=6) was performed on untreated H4 cells exposed to ACPA at half the maximum inhibitory concentration (IC50) dose. Annexin V/PI labeling was performed by

ÖZET

Amaç: Endokannabinoid sistem (ECB) insan vücudunun tamamında mevcuttur ve özellikle sinir sisteminde kapsamlı olarak incelenmiştir. Kannabinoid 1 (CB1) ligandları ve reseptörleri öncelikle merkezi sinir sisteminde ve hücrelerinde bulunmaktadır. Nöroglioma, primer merkezi sinir sistemi (MSS) tümörlerinin yaklaşık %50'sini oluşturan agresif ve yaşamı tehdit eden bir kanserdir. Gliomaların tedavi protokolü tipik olarak cerrahi, radyasyon tedavisi veya kemoterapiyi içerir. Bu çalışmada, bir CB1 reseptör agonisti olan Arachidonoylcyclopropylamide'in (ACPA) H4 insan nöroglioma hücre hattı üzerindeki antiproliferatif etkileri değerlendirilmiştir.

Yöntem: Bu çalışmada, ACPA'nın H4 nöroglioma hücreleri üzerinde özellikle antiproliferatif, pro-apoptotik, antimigratuvar ve organel-stres ile ilgili etkileri araştırıldı. Maksimum inhibitör konsantrasyonun yarısı (IC50) dozunda ACPA'ya maruz kalan ve tedavi edilmeyen H4 hücreleri üzerinde xCELLigence hücre empedans deneyi (n=6) gerçekleştirildi. IC50 ACPA'ya maruz bırakılmış ve tedavi

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Geliş Tarihi / Received : 11.04.2025
Kabul Tarihi / Accepted : 25.06.2025

DOI ID : 10.5505/TurkHijyen.2025.75133

Karakoç E, Ayan Türeli M, Hanelçi RH, Ersoy Ü, Oskay Halaçlı S, Atilla P. Arachidonoylcyclopropylamide (ACPA) reduces viability and induces endoplasmic reticulum stress in glioma cells. Turk Hij Den Biyol Derg, 2025; 82(3): 419 - 426

flow cytometry on IC50 ACPA-exposed and untreated H4 cells. In addition, immunofluorescence labeling of anti-GRP78, anti-LC3B and anti-beta tubulin antibodies were analyzed to evaluate the effects on organelle-stress relationship (n=3).

Results: The IC50 dose ($2.1X10^{-7}$ M) of ACPA on H4 cells was determined by xCELLigence cell impedance assay and this dose reduced the proliferation capacity of H4 neuroglioma cells in a dose-time dependent manner ($p < 0.05$). Flow cytometry analysis showed that cells treated with IC50 ACPA had a higher late apoptotic rate (n=3). Immunofluorescence analysis showed a significant increase in GRP78 and LC3B labeling. Furthermore, a significant decrease in beta tubulin immunolabeling was observed compared to the untreated group ($p < 0.05$).

Conclusion: ACPA induced apoptosis, suppressed proliferation and was able to induce endoplasmic reticulum stress and autophagy in neuroglioma cells. These findings highlight the potential of ACPA as a therapeutic agent for neuroglioma refractory to clinical treatment and warrant further functional validation.

Key Words: Endocannabinoid, neuroglioma, xCELLigence, apoptosis, autophagy

edilmemiş H4 hücreleri üzerinde akım sitometrisi ile Annexin V/PI işaretlemesi gerçekleştirildi. Ayrıca organel-stres ilişkisi üzerine etkilerinin değerlendirilebilmesi için anti-GRP78, anti-LC3B ve anti-beta tübülin antikorlarının immünofloresan işaretlemesi analiz edilmiştir (n=3).

Bulgular: ACPA'nın xCELLigence hücre empedans testi ile H4 hücreleri üzerindeki IC50 dozu ($2,1X10^{-7}$ M) belirlenmiştir ve bu doz H4 nöroglioma hücrelerinin proliferasyon kapasitesini doz-zamana bağımlı bir şekilde azaltmıştır ($p < 0,05$). Akım sitometrisi analizi IC50 ACPA ile tedavi edilen hücrelerin daha yüksek geç apoptotik orana sahip olduğunu göstermiştir (n=3). İmmünofloresan analizi GRP78 ve LC3B işaretlenmesinde anlamlı bir artış olduğunu göstermiştir. Ayrıca, tedavi edilmeyen gruba kıyasla beta tübülin immün işaretlenmesinde anlamlı bir azalma gözlenmiştir ($p < 0,05$).

Sonuç: ACPA nöroglioma hücrelerinde apoptozu indüklemiştir, proliferasyonu baskılamış ve endoplazma retikulumu stresini ve otofajiyi tetikleyebilmişti. Bu bulgular, klinik tedaviye dirençli olan nöroglioma için ACPA'nın terapötik bir ajan olarak potansiyelini vurgulamakta ve daha fazla fonksiyonel doğrulama gerektirmektedir.

Anahtar Kelimeler: Endokannabinoid, nöroglioma, xcelligence, apoptoz, otofaji

INTRODUCTION

Cannabinoids are compounds derived from the plant Cannabis sativa, consisting mainly of phytocannabinoids such as Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD). These bioactive compounds affect the human body directly or indirectly and usually exert their effects through their ligands that interact with cannabinoid receptors (1).

Endocannabinoids are lipid-derived endogenous cannabinoid compounds produced *de novo* in living organisms. They are distributed through various systems, including nervous, immune, gastrointestinal, respiratory, circulatory, and reproductive systems (1). Their main ligands, Anandamide (AEA) and 2-Arachidonyl glycerol (2-AG), interact with cannabinoid 1 (CB1) and cannabinoid 2 receptors (CB2), mediating numerous physiological processes (2). Furthermore, endocannabinoids exhibit anti-cancer properties, such as reducing cell proliferation, inhibiting migration and metastasis, thus showing

potential in personalized cancer therapies (3-5).

Glioma is the most common tumor of the central nervous system and causing approximately 80% of malignant brain tumors. The World Health Organization (WHO) classifies gliomas between Stage I (benign) and Stage IV (malignant), with glioblastoma multiforme (GBM) being the most aggressive form. GBM, the most common primary brain tumor in adults, has an incidence rate of 3.19 per 100,000, with a two-year survival rate of only 30%. Despite present treatment modalities include chemotherapy, immunotherapy, radiation therapy, and surgical excision, the prognosis is generally poor (6, 7).

In vitro studies on glioma cells have demonstrated that cell-targeting applications can induce cell death types such as apoptosis and autophagy (8). These cells express CB1 and CB2 receptors, and CB2 receptor expression is increased in high-stage glioma samples, while CB1 receptor expression shows differences in the direction of increase and decrease (6).

Studies to date have revealed that cannabinoid ligands have various biological effects on glioma cells. For example, CBD has been found to increase reactive oxygen species (ROS) through CB1, CB2, and TRPV2 receptors, activating caspase-8, caspase-9, and caspase-3. Furthermore, CBD triggers ER stress by calcium ion imbalance, induces mitophagy, autophagy, and ferroptosis in glioblastoma cells via the ERK pathway (9-14). Another cannabinoid ligand, THC inhibits the proliferation and invasion of glioma cells by activating CB1 and CB2 receptors (15).

A synergistic anti-glioma effect of CBD and THC has been demonstrated in a xenograft mouse model, showing anti-proliferative, pro-apoptotic and anti-angiogenic properties in subcutaneous tumors derived from U-87MG cells (16). Also, Rupprecht and colleagues revealed that CBD + THC inhibits glioma cell energy metabolism by impairing mitochondrial electron transport chain (17).

Additionally, synthetic CB1 receptor agonist, arachidonylcyclopropylamide (ACPA), have been reported to have antiproliferative, pro-apoptotic, and autophagic effects in various cancers, including pancreatic, breast, and endometrial adenocarcinomas (3, 18-20).

Considering all these promising findings, endocannabinoids have therapeutic potential for glioma treatment. Therefore, in our study, we explored the possible antiproliferative, pro-apoptotic, and autophagic activity of ACPA, a specific cannabinoid 1 receptor agonist, on H4 cells and its effects on endoplasmic reticulum (ER) stress.

MATERIAL and METHOD

This study was designed as a controlled *in vitro* study. We investigated the effects of ACPA on H4 cells, specifically its antiproliferative, pro-apoptotic, antimigratory, and organelle-stress related effects.

Cell culture

The neuroglioma cell line H4 (#HTB-148, ATCC, USA) was purchased. The cells were expanded in culture media containing Dulbecco's Modified Eagle's Medium (DMEM) (#30-2002, ATCC, USA), supplemented with 10% Fetal Bovine Serum (FBS) (#30-2020, ATCC, USA), 2% L-Glutamine (#03-020-1C, Sartorius, USA) and 1% Penicillin-streptomycin (#30-2300, ATCC, USA) at 37°C, under 5% CO_2 conditions.

xCELLigence cell-impedance based real-time proliferation analysis

Once H4 cells reached 70-80% confluence, the culture medium was removed, and the cells were rinsed with phosphate-buffered saline (PBS). Subsequently, the cells were incubated with Trypsin/EDTA solution (3T4049, Sigma Aldrich, Germany) for 5 minutes and centrifuged at 177 g force. A cell suspension with a density of 5,000 cells per well was seeded into 96-well "E-plates". The cell index was continuously monitored by recording impedance changes caused by cell attachment and growth on the gold electrode-coated bases of the E-plates.

After 24 hours, ACPA was applied at concentrations ranging from 10^{-6} to 10^{-12} M and cells treated only

with culture medium was considered as the control group (n=6). Cellular indices were recorded every 15 minutes over a 96-hour period using the xCELLigence system (Agilent Technologies, Inc., Santa Clara, CA). The IC50 value for ACPA was calculated using Agilent software.

Flow cytometry

The IC50 dose of ACPA was administered to H4 cells for a period of 48 hours. Following this, the cells were washed and labeled with the FITC Annexin V/PI Apoptosis Detection Kit (640914, Biolegend, USA). Analysis was then conducted using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ) to detect apoptotic cells. Finally, the data was analyzed using FlowJo software (Treestar, Inc., San Carlos, CA). The experimental setup was replicated three times for both the ACPA-exposed and non-exposed control groups (n=3).

Immunofluorescence labeling

H4 cells were seeded in chambered slides. The cells were exposed to IC50 ACPA and only culture media as control group. On the 48th hour, media was removed and the cells were rinsed with PBS and fixed using paraformaldehyde for 10 min at RT. Afterwards, triton-X was applied for permeabilization for 10 min. Blocking with skimmed milk 5% was followed by primary antibody incubation for 3 hours at RT. Anti-GRP78 (#ab212054, Abcam, UK), anti-LC3B (#E-AB-65372, Elabscience, USA) and anti-acetylated ß-tubulin (#T8328, Sigma-Aldrich, USA) primary antibodies were applied on the cells. Secondary antibodies Alexa-488 and Alexa-596 (#ab150080, Abcam, UK; #ab150113, Abcam, UK) were applied and DAPI (#422801, Biolegend, USA) was used to impregnate the nuclei. The presence of specific immunolabeling was detected using a light microscope attached with a digital camera (Leica DM6B, Germany). The evaluation of immunolabeling was conducted using LASX software. At least three micrographs from non-overlapping regions of both positive and negative controls, and each sample's corrected total cell fluorescence (CTCF) was counted (CTCF= Integrated Density – (Area of selected cell x Mean fluorescence of background readings) (21).

Statistical analysis

The RTCA software was used to determine the IC50 of ACPA. For immunofluorescence analysis, the corrected total cell fluorescence (CTCF) formula was applied to calculate measurements, while GraphPad Prism 8 was employed for statistical analysis. Data normality was assessed using the Shapiro-Wilk test. For statistical comparisons, the Mann-Whitney U test was used for pairwise analyses, the Kruskal-Wallis test for multiple group comparisons, and the post hoc Dunn test for further evaluations. A total type-1 error rate of 5% was set as the threshold for statistical significance.

The study was approved by the Hacettepe University Health Sciences Research Ethical Committee (Date: 17.05.2024 and Number: SBA 24/481).

RESULTS

H4 cells were cultured under standard conditions, initially exhibiting a polygonal shape. Over time, these cells developed long fusiform extensions (Figure 1A, B).

Using the xCELLigence system, we observed that different doses of ACPA caused a dose- and time-dependent effect on cell growth. Lower doses of ACPA (10^{-12} M and 10^{-9} M) resulted in a slower proliferation capacity compared to the control group. The 10-6 M dose showed a response like the control, while the highest dose, 10^{-3} M, led to complete cell death (Figure 1C).

To determine the dose dependent specific efficacy, we calculated IC50 using ACEA software between 28^{th} and 75^{th} hours, as 2.1×10^{-7} M (Figure 1D).

The IC50 dose of ACPA increased the apoptotic ratio of H4 cells from 5.74% to 16%, with a notable rise in the late apoptotic cell population (Figure 1E).

Immunofluorescence analysis revealed that ACPA-treated H4 cells exhibited a significant increase in

the labeling of GRP78 and LC3B, markers associated with stress and autophagy pathways ($p < 0.05$). Conversely, there was a significant decrease in acetylated ß-tubulin immunolabeling compared to untreated controls ($p < 0.05$) (Figure 2A-I).

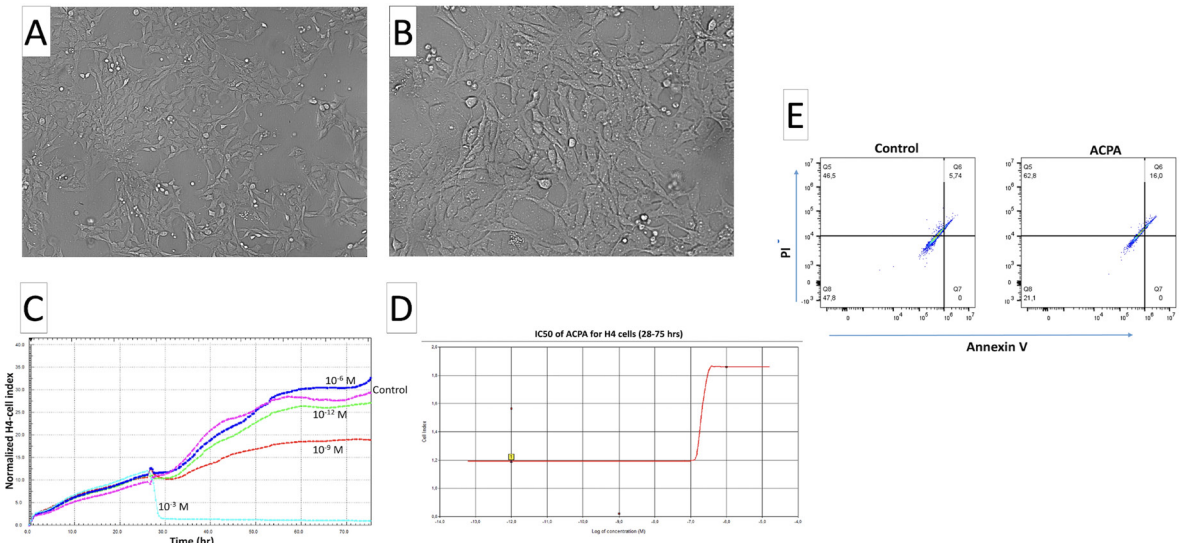

Figure 1. (A) H4 cells under phase-contrast microscope examination (10×) (B) H4 cells with extended long fusiform projections (20×) Images were captured using a Nikon Phase contrast microscope (Nikon Corporation, Tokyo, Japan) (C) 10^{-3}-10^{-12} M doses of ACPA reduced H4 cells' proliferation; (D) the IC50 dose of ACPA on H4 cells was determined between 28th and 78th hours as 2.1x10^{-7} M; (C) Annexin V/PI labeling showed that IC50 ACPA-exposed cells displayed higher apoptotic ratios.

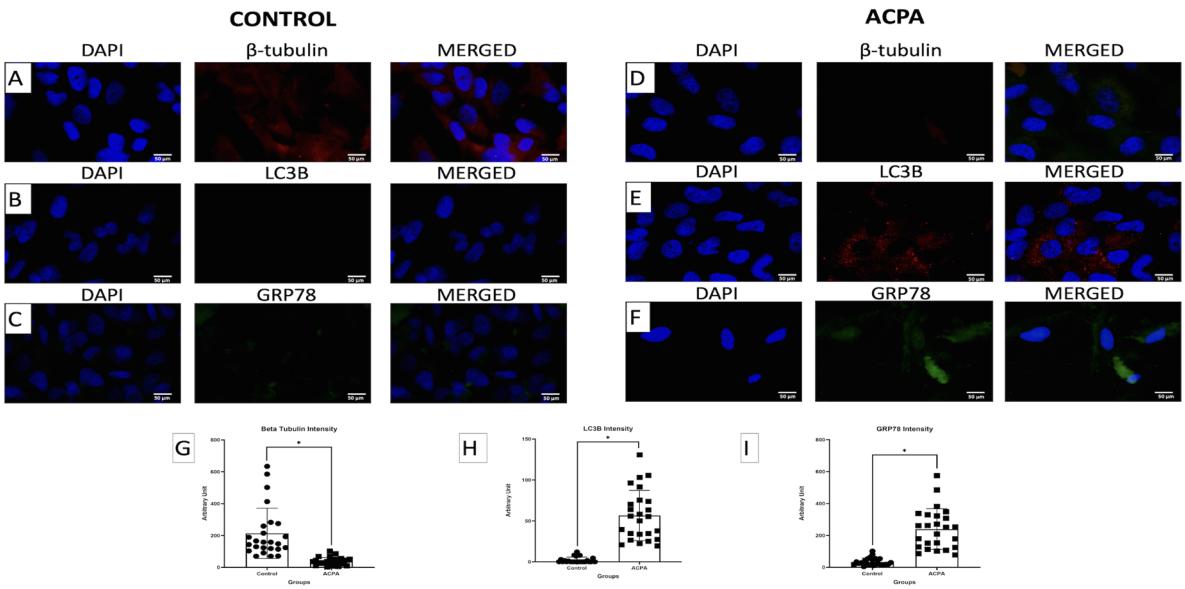

Figure 2. (A-I) ACPA increased LC3B and GRP78 immunolabeling while reducing ß-tubulin compared to the control (100×). Images were captured using a Leica DM6B microscope (Leica Microsystems, Wetzlar, Germany) and analyzed with LAS X software. Nuclei were counterstained with DAPI (blue); ß-tubulin and LC3B appear in red, and GRP78 in green.

DISCUSSION

Our study demonstrates that ACPA exerts potent antiproliferative and pro-apoptotic effects on H4 neuroglioma cells in a dose- and time-dependent manner. Using real-time impedance-based monitoring, we identified that IC50 of ACPA (2.1×10^{-7} M) not only inhibits the proliferation of H4 neuroglioma cells but also promotes apoptosis in a dose- and time-dependent manner. The xCELLigence analysis confirmed the suppressive effect of ACPA on cell viability, while flow cytometry revealed a notable increase in the late apoptotic cell population.

Similar findings have been reported with cannabinoid agonists in various cancer models (22, 23). For instance, cannabidiol (CBD) was shown to induce ER stress and apoptosis in ovarian cancer cell line through CB1 receptor activation (22). Additionally, CBD reduced proliferation and migration while inducing apoptosis and ER stress in colorectal cancer cells (23), aligning with our results showing that ACPA exerts similar effects on H4 glioma cells.

Increased GRP78 expression, an indicator of ER stress, a well-documented response to cellular damage and has been linked to apoptosis in various cancer models (24). Our findings aligned with Fu et al.'s study who reported increased GRP78 levels via western blotting following cannabinoid treatment in ovarian cancer model (22).

The increased labeling of LC3B suggests activation of autophagy, a process often associated with cell survival under stress but also with cell death when stress is prolonged (25). Previously, research has demonstrated that CB1 receptor agonists, including CBD and ACEA, induce autophagy via LC3 signaling in SH-SY5Y neuroblastoma cells (26). These findings align with previous studies linking ER stress and autophagy to cell death pathways in glioma cells (27).

In addition to ER stress and autophagy, we observed a reduction in ß-tubulin immunolabeling, suggesting cytoskeletal disruption. Cytoskeletal integrity is crucial for cancer cell migration and invasion, and its disruption may impair glioma progression. Previous studies identified ß-tubulin as a prognostic factor in glioma, while high levels of acetylated alpha tubulin was associated of post translational modification and mostly abundant in cytoplasmic extensions (28). ß-tubulin plays a key role in cytoskeletal dynamics and is critical for glioma cell function (29). In a study it is reported that Tubulin beta 2B class IIb (TUBB2B) inhibition decreased migration and proliferation of glioma cells (30). This suggests ACPA destabilizes microtubules in glioma cell line but also induce apoptosis and ER stress response.

Overall, our results indicate that ACPA, destabilizes microtubules, and activates apoptotic and autophagic pathways in H4 cells (Figure 3). This dual effect highlights ACPA's potential to suppress neuroglioma progression by impairing proliferation and migration while promoting cell death.

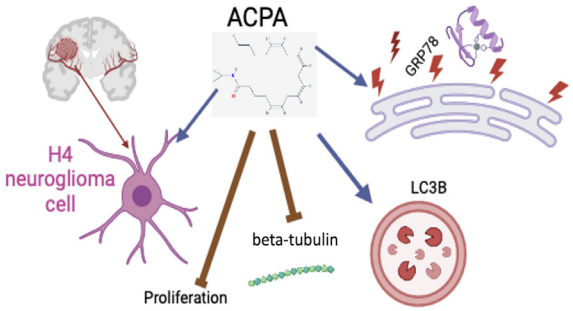

Figure 3. ACPA reduces H4 neuroglioma cell proliferation and induces apoptosis via ER stress, autophagy, and cytoskeletal disruption. The image was created in BioRender.

In conclusion, our study demonstrates that ACPA significantly reduces the proliferation capacity of H4 neuroglioma cells and induces apoptosis through mechanisms involving ER stress, autophagy, and cytoskeletal disruption. These findings suggest that ACPA has potential as a therapeutic agent for neuroglioma and could serve as a valuable tool for studying cell death and stress-related pathways. However, further functional assays and in vivo studies are necessary to confirm these results and explore the translational relevance of ACPA in neuroglioma treatment.

ACKNOWLEDGEMENTS

* The study was presented in poster format at the annual meeting of the International Cell Death Society, held in Ghent, Belgium, from 24th to 26th May 2024. The meeting was entitled "Cell Death: Mechanisms, Immunity and Clinical Applications". The study was partially supported by Scientific Research Project Council of Hacettepe University (#THD-2023-2042)

ETHICS COMMITTEE APPROVAL

* The study was approved by the Hacettepe University Health Sciences Research Ethical Committee (Date: 17.05.2024 and Number: SBA 24/481).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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