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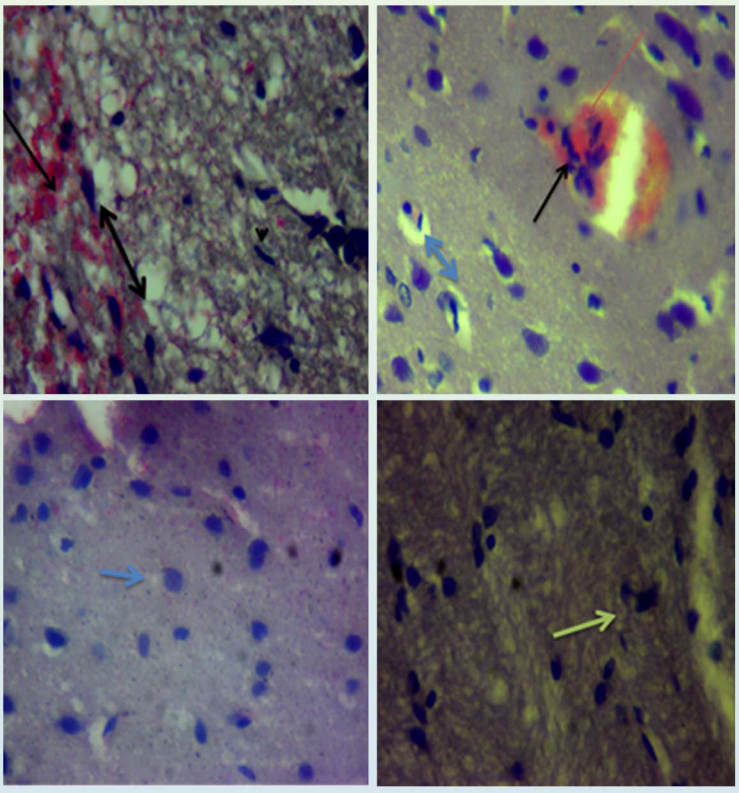

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AIM AND SCOPES
Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

READERSHIP

Biophysics	Biochemistry
Biology	Biomedical Engineering
Pharmacology	PhysiologyGenetics
Cardiology	Neurology
Oncology	Psychiatry
Neuroscience	Neuropharmacology

Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide, ageing, antioxidants, neuropathy, traumatic brain injury, pain, spinal cord injury, Alzheimer's Disease, Parkinson's Disease.

RESEARCH ARTICLE

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Clostridium botulinum neurotoxin A inhibits DBTRG glioblastoma cell proliferation and TRPV1 channel signaling pathways

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Abstract

Prevalence of glioblastomas is high within the adult brain tumors and the proliferation of the glioblastomas was induced by excessive Ca^{2+} influx. Ca^{2+} permeable TRPV1 channel is gated by capsaicin and reactive oxygen species (ROS), although its activity was decreased in neurons by AMG and antioxidants. **Clostridium botulinum** neurotoxin A (BotxA) acted antioxidant action in several cells and its treatment modulated TRPV1 in neurons. Hence, treatment of BotxA may modulate glioblastoma cell proliferation and death via inhibition of TRPV1 in the DBTRG glioblastoma in vitro cell line model.

The DBTRG cells were divided into three groups as control, BotxA (5 IU for 24 hours) and BotxA+TRPV1 channel blocker (AMG and 1 μM for 30 min).

Intracellular Ca^{2+} response to TRPV1 activation was increased in the cells from capsaicin, although it was reduced by the BotxA and AMG. BotxA treatment decreased cell proliferation, although its treatment increased cell death (propidium iodide/Hoechst rate). In addition, BotxA decreased mitochondrial membrane depolarization levels, cytosolic and mitochondrial ROS generation in the cells. Their levels were further decreased in the BotxA+AMG group by the AMG treatment.

The antiproliferative and neurotoxic effects of BotxA were shown to be exerted via modulation of oxidative stress and TRPV1 activation. BotxA could be used as an effective agent in the treatment of glioblastoma proliferation.

Keywords: Glioblastoma; Mitochondria; Oxidative stress; Proliferation; TRPV1 channel.

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List of Abbreviations;

***$[Ca^{2+}]_c$**, cytosolic free Ca^{2+} concentration; **AMG**, AMG 9810 (TRPV1 receptor antagonist); **BotxA**, **clostridium botulinum** neurotoxin A; **Ca^{2+}**, calcium ion; **CPS**, capsaicin; **DCFH-DA**, 2',7'-Dichlorofluorescin Diacetate (DCFH-DA); **DHR123**, Dihydrorhodamine 123 (DHR123); **JC-1**, 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; **ROS,** reactive oxygen species; **TRPV1**, transient receptor potential vanilloid 1; **VGCC**, voltage gated calcium channel*

Introduction

Most aggressive type of adult primary brain tumor is glioblastomas (Hanif et al. 2017). Despite significant advances in the understanding of the molecular and

cellular basis of tumor origin and progression, survival rate of glioblastomas is 14-15 months after diagnosis (Kanu et al. 2009). The incidence of glioblastomas is higher in men as compared to women. There is limited information on the etiology of glioblastomas. Most common factors in the etiology of glioblastomas are environmental factors such as radiation and environmental toxins and oxidative stress (Krylova et al. 2019; Xiao et al. 2019).

Several physiological and pathophysiological functions in normal and cancer cells are arranged by the calcium ion (Ca^{2+}) signaling. Tumor cell proliferation, including glioblastoma cell proliferation is controlled by the cytosolic basal neuronal Ca^{2+} changes (Amantini et al. 2007; Nazıroğlu 2007; Deveci et al. 2019). On the other hand, during apoptosis and death, brain and glioblastoma cells enhance intracellular Ca^{2+} influx (Kanu et al. 2009; Kumar et al 2014) and activate a pathway of factors such as reactive oxygen species (ROS) generation and inflammation that when produced in excess can lead to tumor cell death (Morrone et al. 2016; Ataizi et al. 2019). Enhance of ROS generation results in cancer cell proliferation via additional activation Ca^{2+} channels such as voltage gated calcium channel (VGCC) and NMDA receptors (da Silva et al. 2015). Hence, blockade of the Ca^{2+} channels decreases proliferation of tumor cells via inhibition of ROS. A member of Ca^{2+} channels is the transient receptor potential (TRP) superfamily with 28 members and 6 subgroups in mammalian (Clapham 2003; Sakaguchi and Mori 2020). A member of the TRP superfamily is TRP vanilloid 1 (TRPV1) calcium permeable cation channel and it is activated by low pH, noxious high temperature, and the endogenous vanilloid or exogenous capsaicin (CPS) (Caterina et al. 1997; Clapham 2003; Nazıroğlu 2012; Sakaguchi and Mori 2020). In addition to the activators, TRPV1 in tumor cells such as glioblastoma and neuroblastoma cells is activated by excessive ROS generation (Amantini et al. 2007; Stock et al. 2012; Nabissi et al. 2016). A correlation between TRPV1 channel expression and patients with glioblastoma multiforme was also reported (Alptekin et al. 2015). Involvement of TRPV1 activation on the tumor cell proliferation and ROS production in several cancer types was recently reported (Qian et al. 2016; Liu et al. 2016). In addition, antioxidant treatments via inhibition of the TRPV1 in the cancer cells decreased oxidative stress (Qian et al. 2016; Liu et al. 2016).

The Clostridium botulinum neurotoxin has seven serotypes (A-G) and it is produced by the bacterium Clostridium botulinum. A protease content of Clostridium botulinum neurotoxin A (BotxA) blocks the release of acetylcholine at the neuromuscular junction. Hence, the BotxA is extensively being used as a therapeutic agent against pain, epilepsy, ophthalmological and neuromuscular disorders (Oh and Chung 2015). In addition, accumulating evidence suggests that natural toxins, including BotxA via inhibition of VGCC and glutamate receptors acted therapeutic action in cancer therapy (Yonei et al. 1995; Zhang et al. 1996; Shu et al. 2008; da Silva et al. 2015). Recent data indicated anticancer actions of BotxA against cancer cell proliferation in human prostate cancer (Karsenty et al. 2009), breast cancer (Bandala et al. 2013) and human neuroblastoma cells (Rust et al. 2016). However, the anticancer action of BotxA via inhibition of TRPV1 has not been clarified yet.

There is no report the modulator action of BotxA against the cancer cell proliferation and death via modulation of TRPV1 channel in the DBTRG glioblastoma cell line. In the present study I have investigated whether BotxA exerts neuroprotection against in vitro proliferation, Ca^{2+} influx, oxidative stress. Moreover, I have correlated the possible anticancer actions of the BotxA with their ability to modulate TRPV1 channel.

Materials and methods

Cell culture

The DBTRG cell line was bought from Şap Institute (Ankara, Turkey). A 90% Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Istanbul, Turkey) and 10% fetal bovine serum (FBS, Gibco, Istanbul, Turkey) mixture was used for growing the cells. The cells were kept in a humidified atmosphere in 5% CO_2 at 37°C (NB-203QS, N-BIOTEK, Istanbul, Turkey). After counting the cell in an automatic cell counter (Casy Modell TT, Roche, Germany), they were seeded in T-25 flask (surface area 25 cm^2 and capacity 50 ml with filter cap) at a density of $1x10^6$ cells per flask.

Groups

The DBTRG cells were mainly divided into three groups as follows;

***Control group*:** The cells were kept in under the same cell

culture medium and conditions for 24 hours without a TRPV1 channel blocker (AMG) and BotxA treatments.

***BotxA group*:** The cells in the group were pre-incubated with BotxA (5 IU) for 24 hours as described in a previous study (Yowler et al. 2002).

***BotxA+AMG group*:** The cells in the group were pre-incubated with BotxA (5 IU) for 24 hours and then they were further incubated with 1 μM AMG 9810 for 30 min (Mandadi et al. 2011).

The CPS was purchased from Sigma-Aldrich Inc. (Istanbul, Turkey), although AMG 9810 [(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4 dioxin-6-yl) acrylamide] was purchased from Tocris Bioscience (Istanbul, Turkey). Stock solutions of CPS and AMG were prepared in DMSO (1%) before dilation in extracellular buffer with Ca^{2+} (1.2 mM). BotxA (Onabotulinum toxin A, BOTOX, Allergan Inc., Istanbul, Turkey) was diluted in 1 ml sterile serum physiologic solution.

Assay of cell viability

After removing medium by centrifugation (1500 g for 5 min), the DBTRG cells were diluted in the Casy tone solution as described in previous studies (Ataizi et al. 2019; Ertilav 2019). The cell viability was monitored through electrical current exclusion by a cell counter (Casy Modell TT, Roche, Germany). The cell viability level was expressed as %.

Bride filed and death (PI)/live (Hoechst) analyses in the laser scan confocal (LSC) microscope

As described in a previous study (Özkaya and Nazıroğlu 2020), death/live cell in the LSC microscope (LSM800, Zeiss, Ankara, Turkey) were determined by using Hoechst 33342 and propidium iodide (PI) staining, respectively. Briefly, the neuronal cells ($1x10^6$) were seeded into the glass bottom dishes (Mattek Corporation Inc., Ashland, MA, USA) and then they were incubated with PI (5 μg/ml) and Hoechst 33342 (1 μM) (Cell Signaling Technology) at 37 °C for 30 min. The viable and dead cells are distinguished by their fluorescent color under the LSC microscope. Images of bride field (black/white), blue and red cells were captured under the identical capture settings using the LSC microscope. ImageJ/Imaris software was used for analysis of cells stained with PI and Hoechst. The samples were analyzed by the LSC microscopy fitted with a 20× objective. Death/live cell rate was expressed as %.

Measurement of intracellular Ca^{2+} fluorescence intensity through TRPV1 potentiation in the DBTRG cells by using LSC microscope

I investigated BotxA-induced TRPV1 potentiation in the DBTRG cells by using an LSC microscope (LSM800) analyses as described in previous studies (Deveci et al. 2019; Ertilav 2019). The cells were seeded on 35 mm glass bottom dishes (Mattek Corporation**)** before the analyses**.** Intracellular changes in the Ca^{2+} fluorescence intensity in the DBTRG cells were monitored by using 1 μM fluorescent dye (Fluo-8, Calbiochem, Darmstadt, Germany). The Fluo-8 is a single wavelength excitation and emission dye that excited by a 488 nm argon laser from the confocal microscope (LSM 800). The cells were treated with AMG (1 μM) to inhibit Ca^{2+} entry before stimulation of TRPV1 via CPS (1 μM). The neurons were analyzed at 515 nm by the LSC microscopy fitted with a 40x oil objective by using ZEN program. The results of Fluo-8 were expressed as the mean fluorescence intensity as arbitrary units per cell.

Measurement of mitochondrial membrane potential (ΔΨm) formation

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimida-zolylcarbocyanine iodide (JC-1, Molecular Probes, Eugene, OR, USA) accumulates in mitochondria according to ΔΨm level (Keil et al. 2011). Details of the ΔΨm formation analyses were given in previous studies (Joshi and Bakowska 2011; Ertilav 2019). Briefly, the cells ($1x10^6$) were incubated with 5 μM JC-1 at 37 °C for 30 min. Fluorescence intensity of each cells was monitored in the LSC microscope equipped with 40× oil objectives. Images were acquired with ZEN program and analyzed using Image J/Imaris software. The data are presented as arbitrary unit.

Measurement of cytosolic ROS formation in the DBTRG cells

2′,7′-Dichlorofluorescin Diacetate (DCFH-DA) and dihydrorhodamine 123 (DHR123) are two oxidation-sensitive stains. They are two non-fluorescent compounds, but they are converted to fluorescent forms in the cytosol of cell, when it was taken up into cells (Keil et al. 2011). ROS formation was monitored using the LSC

microscope (LSM800) described previously (Ataizi et al. 2019). Briefly, the cells ($1x10^6$) were incubated with 10 μM DCFH-DA and DHR123 for 30 min at 37 °C in dark. The fluorescence increase, which is due to the hydrolysis of DCFH-DA to DCF, and hydrolysis of DHR123 to Rh123 by nonspecific cellular esterase and its subsequent oxidation by peroxides, was excited with an Argon laser at 488 nm. DHR123 and DCFH-DA were excited with a diode laser at 488 nm. Excitation and emission detection wavelengths of DHR123 were 507 nm and 529 nm, respectively. Excitation and emission detection wavelengths of DHR123 were 504 nm and 525 nm, respectively. Fluorescence intensity of each cells was monitored in the ZEN program of LSC microscope equipped with 20× oil objectives. The both data are presented as arbitrary unit.

Imaging ROS generation in the mitochondria by using LSC microscope analyses

Mitochondrial ROS (MitoROS) generation in the laser confocal microspore analyses (LSM 800) was assayed by using MitoTracker Red CM-H2Xros (Life Technologies) florescent dye. In the assay, we followed manufacturer's instructions. After applicated to indicated treatments, the cells were incubated in extracellular buffer with Ca^{2+} (1.2 mM) containing 100 nM MitoTracker Red CM-H2Xros for 30 min for 30 min at 37 °C in dark (Özkaya and Nazıroğlu 2020). The cells were washed and maintained in 1xPBS before imaging the cells. MitoTracker Red CM-H2Xros was excited (Excitation: 576 nm and Emission: 598 nm) with a diode laser at 561 nm. ZEN program was used for analyzing the fluorescence intensity results of each cell. The results of MitoROS were expressed as arbitrary unit.

Statistical analyses

All data were indicated as means ± standard deviation (SD). To assess the differences between treatment groups for each treatment, we used the one-way ANOVA. I used a post hoc test only when an ANOVA gave a statistically significant difference. I performed a Kruskal-Wallis in all data. The $p \leq 0.05$ value was accepted statistically significant.

Results

I tested effects of BotxA on the proliferation of DBTRG cells by using Casy Tone Cell counter. The images of bride field (black/white) and mean results of cell number are shown in the Figure 1a and b, respectively. The cell number was lower in the BotxA group than in the Ctr group ($p \leq 0.05$).

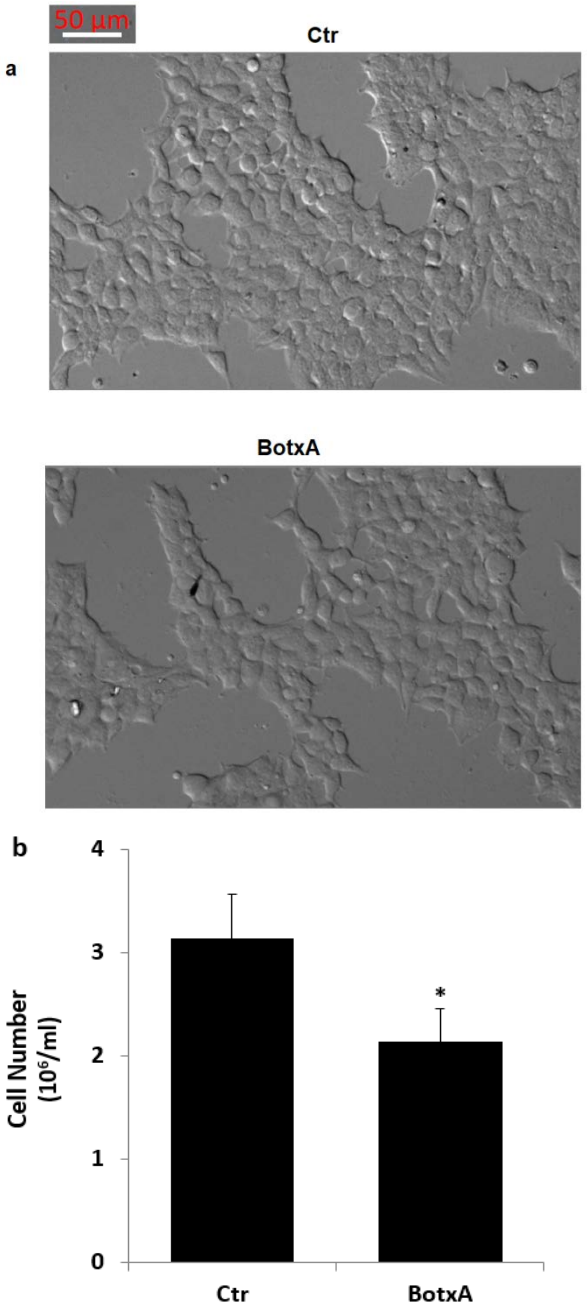


Figure 1. BotxA decreased DBTG cell number. *(Mean±SD and n=3).* ***a)*** *Representative confocal image showing bride field (BF) in the four groups of the DBTRG cells. The scale bar is 50 μm. The neurons were analyzed in a laser confocal microscope fitted with a 10x objective.* ***b)*** *Summary of the mean cell number under control and treatment conditions. The cell values of the four groups were measured in an automatic cell counter by using the Casytone counting solution. (*p ≤ 0.05 vs. Ctr).*

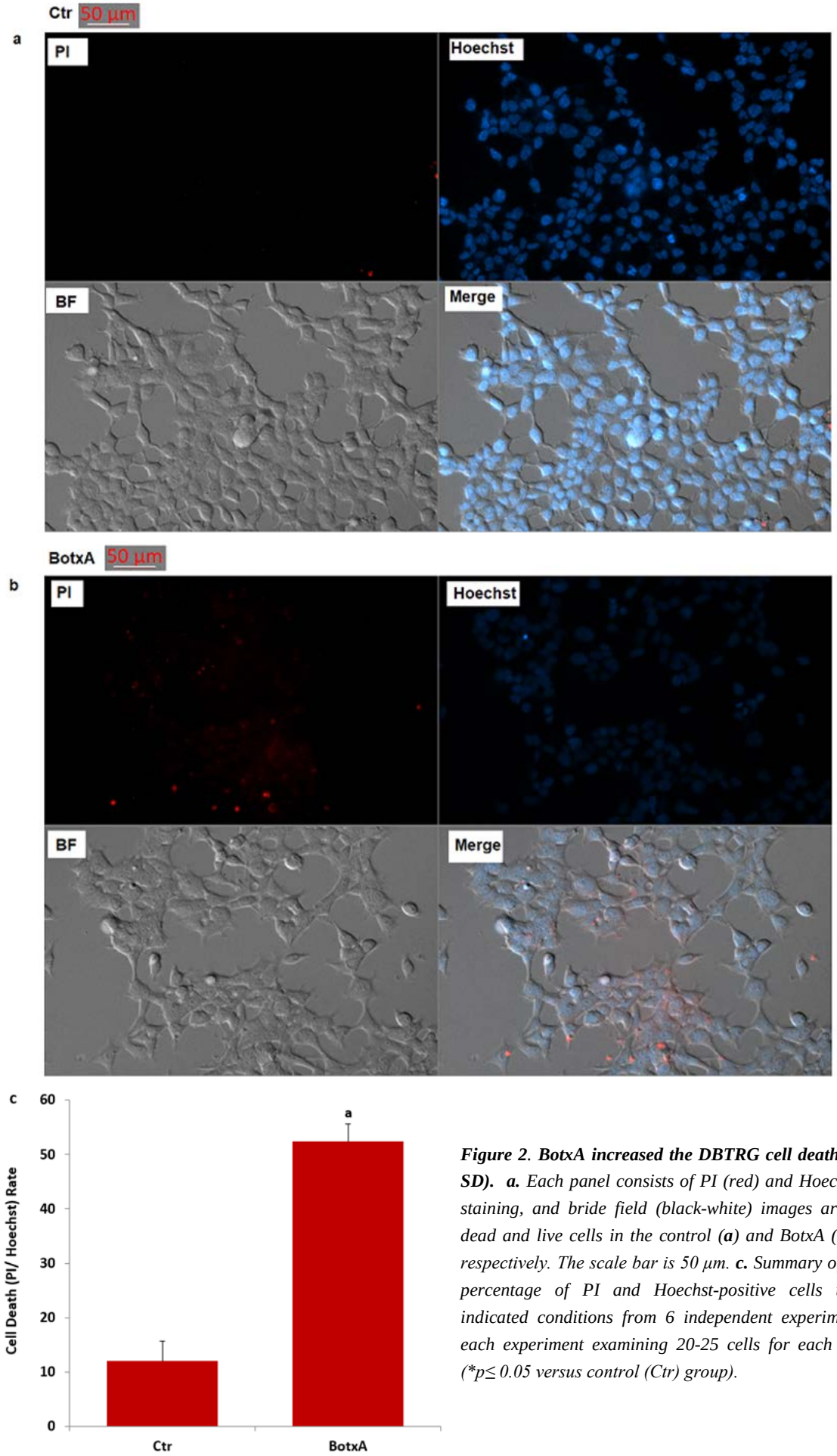


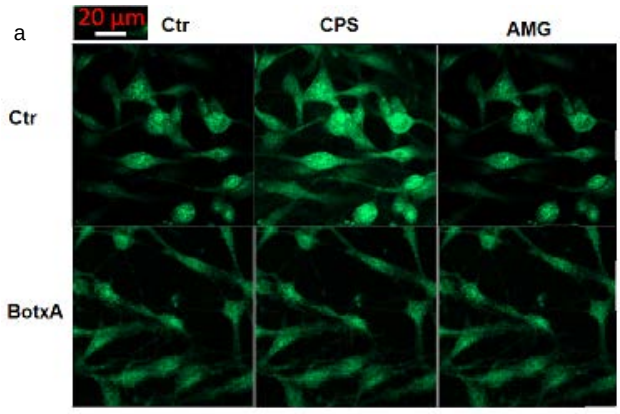
Figure 2. BotxA increased the DBTRG cell death. (mean ± SD). ***a.*** *Each panel consists of PI (red) and Hoechst (blue)-staining, and bride field (black-white) images are showing dead and live cells in the control* ***(a)*** *and BotxA* ***(b)*** *groups, respectively. The scale bar is 50 µm.* ***c.*** *Summary of the mean percentage of PI and Hoechst-positive cells under the indicated conditions from 6 independent experiments, with each experiment examining 20-25 cells for each condition. (*p≤ 0.05 versus control (Ctr) group).*

BotxA increased death cell rate in the DBTRG cells

After observing decrease of the glioblastoma cell line with the treatment, I suspected decrease of life cell rate in the DBTRG cells. Hence, BF and PI (red)/Hoechst (blue) cell images of the control and BotxA groups were shown in Figure 2a and b, respectively. The death cell rate was markedly higher in the BotxA group than in the Ctr group (Figure 2c) ($p \leq 0.05$).

CPS-induced increase of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) fluorescence intensity was decreased by BotxA and AMG treatments

The increases of cancer cell proliferation were induced by the overload Ca^{2+} influx via CPS and activation of several Ca^{2+} channels, including activation of TRPV1 (Liu et al. 2016; Nabissi et al. 2016; Qian et al. 2016). In the current study, I investigated involvement of BotxA via inhibition of TRPV1 channel response to CPS stimulation. The effect of BotxA on $[Ca^{2+}]_c$ was measured by detection of $[Ca^{2+}]_c$ using the TRPV1 channel activator (CPS) and blocker (AMG). In the Fluo-8 results of the LSC microscope image analyses (Figure 3a) and mean column results (Figure 3b), the fluorescence intensity of $[Ca^{2+}]_c$ was increased in the Ctr group by CPS stimulation (activation of TRPV1) ($p \leq 0.05$) and its concentration was higher in the Ctr+CPS group than in the Ctr group. However, the CPS-induced increase of $[Ca^{2+}]_c$ fluorescence intensity was decreased in the Ctr+AMG+CPS group by the AMG treatment and its concentration were significantly ($p \leq 0.05$) lower in the Ctr+AMG+CPS group groups as compared to Ctr+CPS group. In the BotxA groups, there was no action of CPS and AMG on the fluorescence intensity of $[Ca^{2+}]_c$ in the DBTRG cells.



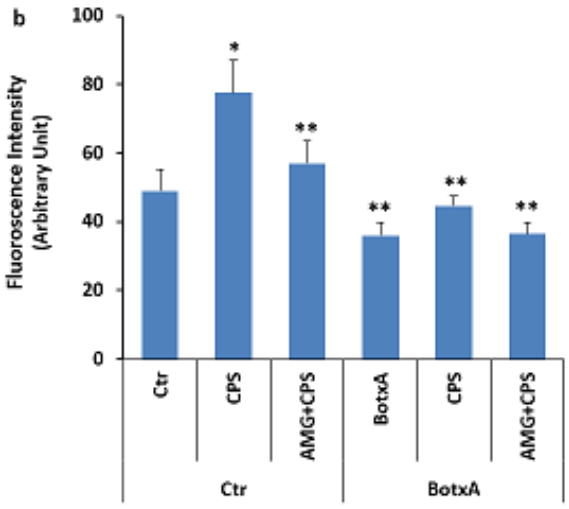


Figure 3. BotxA treatment reduced $[Ca^{2+}]_c$ fluorescence intensity via modulation of TRPV1 channel in the DBTRG cells. (Mean±SD and n=6). *In the both Ctr and BotxA groups, all cells were stained with 1 µM Fluo-3 calcium ion dye for 30 min. Then they were stimulated by CPS (1 µM), although they were inhibited by AMG (1 µM). a. The images were taken with the LSC microscope fitted with 10x objective from the both groups. (*$p \leq 0.05$ vs. Ctr group. **$p \leq 0.05$ vs. Ctr+CPS group).*

BotxA and AMG treatments modulated mitochondrial membrane depolarization (JC-1), cytosolic ROS (DHR123) generation changes in the DBTRG cells

The electron transport system of mitochondria induces loss of mitochondrial membrane depolarization in the mitochondria resulting in excessive ROS generation (Joshi and Bakowska 2011). For this reason, mitochondrial membrane depolarization is an important parameter of mitochondrial function and it was used as an indicator of ROS generation in DBTRG neurons. Mitochondrial membrane depolarization and cytosolic ROS generation was assayed by using JC-1 (Figure 4a and b) and DHR123 (Figure 4a and c), respectively. The JC-1 level and DHR123 generation in the cells were higher in the Ctr group than in the BotxA groups ($p \leq 0.05$). However, the JC-1 level and DHR123 generation were further decreased in the BotxA+AMG group as compared to BotxA group ($p \leq 0.05$).

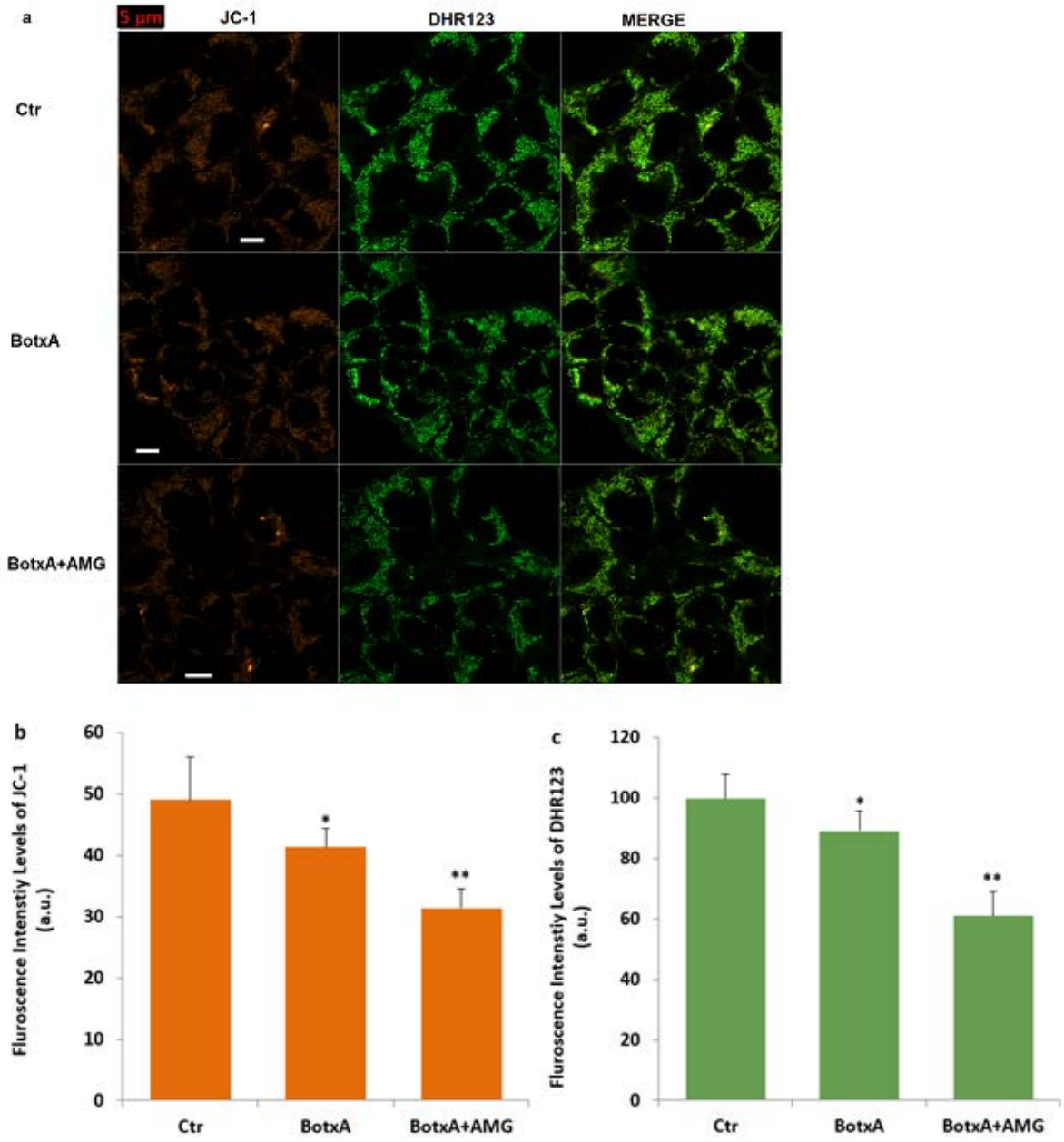


Figure 4. Effect of BotxA and AMG on the mitochondrial membrane depolarization (JC-1) and cytosolic (DHR123) ROS generation levels in the DBTRG cells. *Mean ± SD of fluorescence in 15 mm^2 of the cells as arbitrary unit (a.u.) is presented; n = 20 independent experiments. The DBTRG cells were incubated by BotxA (5 IU for 24 hours) and BotxA+TRPV1 channel blocker (AMG and 1 µM for 30 min). The samples were analyzed by the LSC microscope fitted with a 20 objective. The scale bar was 5 µm. Representative images (**a**) and column of fluorescence intensities of the JC-1 (**b**) and DHR123 (**c**) in the cells are shown in the Figure 4. (*p ≤ 0.05 vs. control (Ctr) group. **p ≤ 0.05 vs BotxA group).*

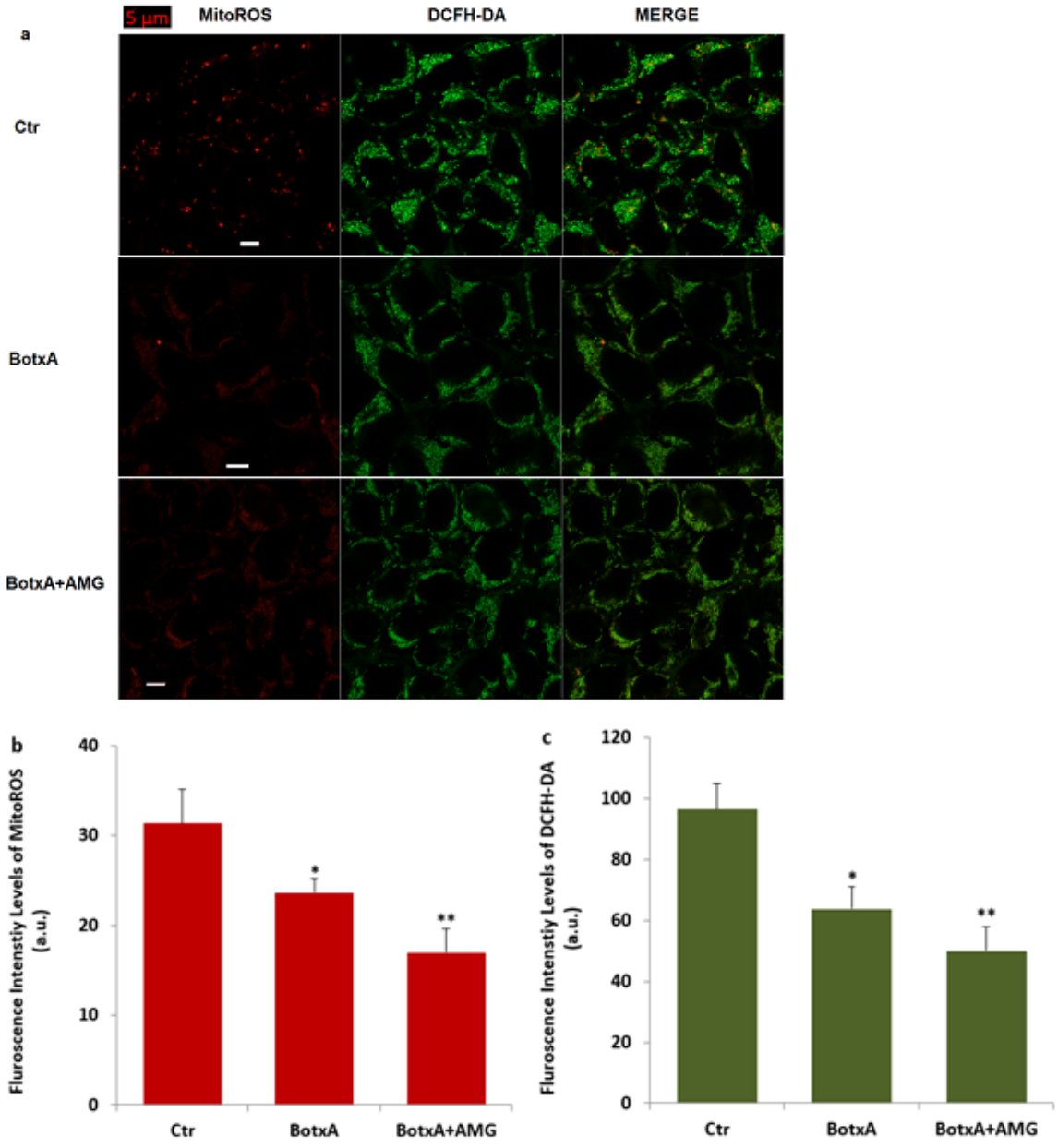


Figure 5. Effect of BotxA and AMG on the cytosolic (DCFH-DA) and mitochondrial (MitoROS) ROS generations in the DBTRG cells. *Mean ± SD of fluorescence in 15 mm^2 of the cells as arbitrary unit (a.u.) is presented; n = 20 independent experiments. The DBTRG cells were incubated by BotxA (100 mM for 24 hours) and BotxA+TRPV1 channel blocker (AMG and 1 µM for 10 min). The samples were analyzed by the LSC microscope fitted with a 20 objective. The scale bar was 5 µm. Representative images (**a**) and column of fluorescence intensities of the DCFH-DA (**b**) and MitoROS (**c**) in the cells are shown in the Figure 5. (*p ≤ 0.05 vs. control (Ctr) group. **p ≤ 0.05 vs BotxA group).*

BotxA and AMG diminished increase of fluorescence intensities cytosolic (DCFH-DA) and mitochondrial (MitoROS) ROS generation in the DBTRG cells

In addition to the analyses of mitochondrial membrane depolarization (JC-1) in the DBTRG cells, I want to further confirm the changes in the mitochondria of the DBTRG cells by using LSC microscope analyses. Compared with control, BotxA treatment diminished the fluorescence intensity of MitoROS (Fig. 5a and b) and DCFH-DA (Figs. 5a and c) ($p \leq 0.05$). Importantly, I found BotxA+AMG further reduced the fluorescence intensities of the MitoROS and DCFH-DA in the cells ($p \leq 0.05$). The present cytosolic and mitochondrial ROS data further suggested that cytosolic and mitochondrial ROS generation, which could be diminished by BotxA treatment through inhibition of TRPV1 channel.

Discussion

There are currently no effective protective agents against glioblastoma cell proliferation. Recently, BotxA as VGCC blocker and the glutamate receptor inhibitor toxin has attracted attention as a potential therapeutic anticancer agent based on its potential antioxidant or anti-proliferative actions (Tsutsuki et al. 2007; Yao et al. 2016). There are structural and functional interactions between BotxA and TRPV1 in the dorsal root ganglion (DRG) neurons (Li and Coffield 2016). In the current study, I observed that BotxA treatment ameliorated glioblastoma cell proliferation, death and ROS generation via modulating the intracellular Ca^{2+} hemostasis in the DBTRG cells. Based on our results, I proposed the beneficial effects of BotxA against glioblastoma cell proliferation via inhibition of TRPV1 channel (Figure 6).

Several pathophysiological functions such as apoptosis and cell proliferation in cancer cells are induced by Ca^{2+} homeostasis (Morrone et al. 2016). TRPV1 within TRP superfamily members was mostly expressed in neurons, such as DRG and hippocampus, because it has a main role in the Ca^{2+} homeostasis of the neurons (Clapham 2003). Function and presence of TRPV1 in the DBTRG cell line were recently reported (Nazıroğlu et al. 2019). Cancer cell proliferations in colorectal (Hou et al. 2019) and breast (So et al. 2020) cancers were induced by activation of TRPV1 channels, although the cancer cell proliferations were diminished by TRPV1 channel blocker. In the current study, I observed the modulator role of DBTRG glioblastoma cell proliferation and death via activation of TRPV1 channel. Similar to the current results, the protective role of TRPV1 inhibition in the glioblastoma proliferation was also reported (Amantini et al. 2007). C3 toxin is an exoenzyme secreted by the bacterium Clostridium botulinum and it was selectively block cancer cell proliferation via its antioxidant property (Sillar et al. 2019). The positive role of TRPV1 in the survival of patients with glioma was also reported (Alptekin et al. 2015; Nabissi et al. 2016).

In addition to the CPS, ROS also induce activation of the TRPV1 channel via promoting ROS, although TRPV1 inhibition by antioxidant agents induced protective actions against cytosolic and mitochondrial ROS generation (Sakaguchi and Mori 2020). Consistent with the reports, I observed decrease of Ca^{2+} influx and excessive ROS production through the modulation of TRPV1 activation in the DBTRG cells by the BotxA treatment. Similarly, the protective role of BotxA in the acute myeloid leukemia was recently reported (Sillar et al. 2019). Melanoma cancer cell proliferation and oxidative toxicity were reduced by the treatment of C3 Botulinum Toxin Substrate 2 (Hu et al. 2019). Pretreatment with BTX-A inhibited CAPS-induced increases in expression of ROS in a dose-dependent manner in the brainstem and lung tissue of rats (Chien et al. 2012).

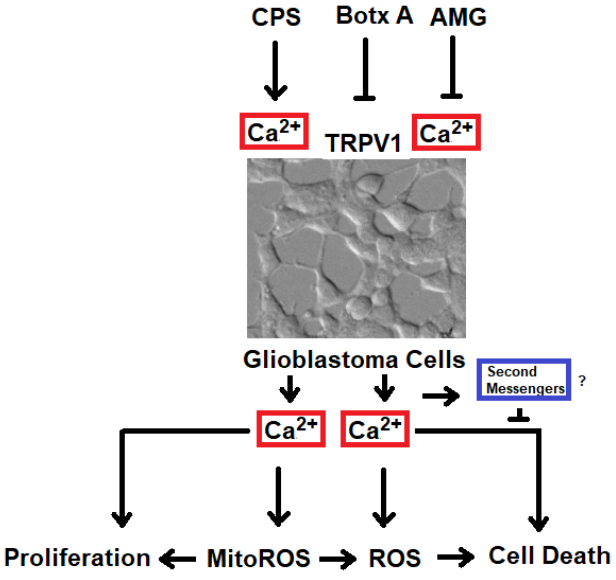


Figure 6. Possible protective pathways of BotxA on the tumor cell proliferation and TRPV1 activity in the DBTRG cells. *Excessive Ca^{2+} influx via TRPV1 activation induces glioblastoma cell proliferation, mitochondria (MitoROS) and cytosolic ROS generation. TRPV1 channel is activated in the*

cells by capsaicin (CPS), although it is inhibited by AMG and BotxA. Hence, glioblastoma cell proliferation, MitoROS and ROS were modulated via inhibition of TRPV1 by BotxA and AMG.

In summary, the present study provides the first evidence that BotxA treatment of the DBTRG cells can modulate the glioblastoma cancer cell proliferation, death, cytosolic and mitochondria ROS generation in the DBTRG glioblastoma cells by modulating TRPV1 activation (Figure 6). These effects resulted from an increase in the restoration of mitochondrial function via modulation of TRPV1-induced calcium signaling. The current findings suggest a new property of BotxA treatment via modulation of TRPV1 activation in the DBTRG cells.

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Conflict of Interest Statement

Dr. Yener Akyuva declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Statement

The author confirms that human and animal samples did not use in the current study. The study was performed in the commercial DBTRG cell line.

Competing Interest Statement

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References

Akpınar H, Nazıroğlu M, Övey İS, Çiğ B, Akpınar O. (2016). The neuroprotective action of dexmedetomidine on apoptosis, calcium entry and oxidative stress in cerebral ischemia-induced rats: Contribution of TRPM2 and TRPV1 channels. Sci Rep. 6:37196.

Alptekin M, Eroglu S, Tutar E, Sencan S, Geyik MA, Ulasli M, Demiryurek AT, Camci C. (2015). Gene expressions of TRP channels in glioblastoma multiforme and relation with survival. Tumour Biol. 36(12):9209-9213.

Amantini C, Mosca M, Nabissi M, Lucciarini R, Caprodossi S, Arcella A, Giangaspero F, Santoni G. (2007). Capsaicin-induced apoptosis of glioma cells is mediated by TRPV1 vanilloid receptor and requires p38 MAPK activation. J Neurochem. 102(3):977-990.

Ataizi ZS, Ertilav K, Nazıroğlu M. (2019). Mitochondrial oxidative stress-induced brain and hippocampus apoptosis decrease through modulation of caspase activity, Ca(2+) influx and inflammatory cytokine molecular pathways in the docetaxel-treated mice by melatonin and selenium treatments. Metab Brain Dis. 34(4):1077-1089.

Bandala C, Perez-Santos JL, Lara-Padilla E, Delgado Lopez G, Anaya-Ruiz M. (2013). Effect of botulinum toxin A on proliferation and apoptosis in the T47D breast cancer cell line. Asian Pac J Cancer Prev. 14(2):891-894.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature 389(6653):816-24.

Chien CT, Lee HM, Wu CC, Li PC. (2012). Inhibitory effect of botulinum toxin type A on the NANC system in rat respiratory models of neurogenic inflammation. Arch Biochem Biophys. 524(2):106-113.

Clapham DE. (2003). TRP channels as cellular sensors. Nature 426(6966):517-524.

da Silva LB, Poulsen JN, Arendt-Nielsen L, Gazerani P. (2015). Botulinum neurotoxin type A modulates vesicular release of glutamate from satellite glial cells. J Cell Mol Med. 19(8):1900-1909.

Deveci HA, Akyuva Y, Nur G, Nazıroğlu M. (2019). Alpha lipoic acid attenuates hypoxia-induced apoptosis, inflammation and mitochondrial oxidative stress via inhibition of TRPA1 channel in human glioblastoma cell line. Biomed Pharmacother. 111:292-304.

Ertilav K. (2019). Pregabalin protected cisplatin-induced oxidative neurotoxicity in neuronal cell line. J Cell Neurosci Oxid Stress. 11 (1): 815-824.

Hanif F, Muzaffar K, Perveen K, Malhi SM, Simjee ShU. (2017). Glioblastoma multiforme: A review of its epidemiology and

pathogenesis through clinical presentation and treatment. Asian Pac J Cancer Prev. 18(1):3-9.

Hou N, He X, Yang Y, Fu J, Zhang W, Guo Z, Hu Y, Liang L, Xie W, Xiong H, Wang K, Pang M. (2019). TRPV1 induced apoptosis of colorectal cancer cells by activating calcineurin-NFAT2-p53 signaling pathway. Biomed Res Int. 2019:6712536.

Hu W, Zhu L, Pei W, Pan S, Guo Z, Wu A, Pei H, Nie J, Li B, Furusawa Y, Konishi T, Hei TK, Zhou G. (2019). Overexpression of Ras-related C3 botulinum toxin substrate 2 radiosensitizes melanoma cells in vitro and in vivo. Oxid Med Cell Longev. 2019:5254798.

Joshi DC, Bakowska JC. (2011). Determination of mitochondrial membrane potential and reactive oxygen species in live rat cortical neurons. J Vis Exp 51: 2704.

Kanu OO, Mehta A, Di C, Lin N, Bortoff K, Bigner DD, Yan H, Adamson DC. (2009). Glioblastoma multiforme: a review of therapeutic targets. Expert Opin Ther Targets. 13(6):701-718.

Karsenty G, Rocha J, Chevalier S, Scarlata E, Andrieu C, Zouanat FZ, Rocchi P, Giusiano S, Elzayat EA, Corcos J. (2009). Botulinum toxin type A inhibits the growth of LNCaP human prostate cancer cells in vitro and in vivo. Prostate. 69(11):1143-1150.

Keil VC, Funke F, Zeug A, Schild D, Müller M. (2011). Ratiometric high-resolution imaging of JC-1 fluorescence reveals the subcellular heterogeneity of astrocytic mitochondria. Pflugers Arch. 462:693-708.

Khan MSS, Asif M, Basheer MKA, Kang CW, Al-Suede FS, Ein OC, Tang J, Majid ASA, Majid AMSA. (2017). Treatment of novel IL17A inhibitor in glioblastoma implementing 3rd generation co-culture cell line and patient-derived tumor model. Eur J Pharmacol. 803:24-38.

Krylova NG, Drobysh MS, Semenkova GN, Kulahava TA, Pinchuk SV, Shadyro OI. (2019). Cytotoxic and antiproliferative effects of thymoquinone on rat C6 glioma cells depend on oxidative stress. Mol Cell Biochem. 462(1-2):195-206.

Kumar VS, Gopalakrishnan A, Nazıroğlu M, Rajanikant GK. (2014). Calcium ion--the key player in cerebral ischemia. Curr Med Chem. 21(18):2065-2075.

Li X, Coffield JA. (2016). Structural and functional interactions between transient receptor potential vanilloid subfamily 1 and botulinum neurotoxin serotype A. PLoS One. 11(1):e0143024.

Liu T, Wang G, Tao H, Yang Z, Wang Y, Meng Z, Cao R, Xiao Y, Wang X, Zhou J. (2016). Capsaicin mediates caspases activation and induces apoptosis through P38 and JNK MAPK pathways in human renal carcinoma. BMC Cancer. 16(1):790.

Mandadi S, Armati PJ, Roufogalis BD. (2011). Real-Time translocation and function of PKCβII isoform in response to nociceptive signaling via the TRPV1 pain receptor. Pharmaceuticals (Basel). 4(11):1503-1517.

Morrone FB, Gehring MP, Nicoletti NF. (2016). Calcium channels and associated receptors in malignant brain tumor therapy. Mol Pharmacol. 90(3):403-409.

Nabissi M, Morelli MB, Arcella A, Cardinali C, Santoni M, Bernardini G, Santoni A, Santoni G, Amantini C. (2016). Post-transcriptional regulation of 5'-untranslated regions of human Transient Receptor Potential Vanilloid type-1 (TRPV-1) channels: role in the survival of glioma patients. Oncotarget 7(49):81541-81554.

Nazıroğlu M, Taner AN, Balbay E, Çiğ B. (2019). Inhibitions of anandamide transport and FAAH synthesis decrease apoptosis and oxidative stress through inhibition of TRPV1 channel in an in vitro seizure model. Mol Cell Biochem. 453(1-2):143-155.

Nazıroğlu M. (2007). New molecular mechanisms on the activation of TRPM2 channels by oxidative stress and ADP-ribose. Neurochem Res. 32(11):1990-2001.

Nazıroğlu M. (2012). Molecular role of catalase on oxidative stress-induced Ca(2+) signaling and TRP cation channel activation in nervous system. J Recept Signal Transduct Res. 32(3):134-141.

Oh HM, Chung ME. (2015). Botulinum toxin for neuropathic pain: A review of the literature. Toxins (Basel). 7(8):3127-3154.

Özkaya D, Nazıroğlu M. (2020). Curcumin diminishes cisplatin-induced apoptosis and mitochondrial oxidative stress through inhibition of TRPM2 channel signaling pathway in mouse optic nerve. J Recept Signal Transduct Res. 40(2):97-108.

Qian K, Wang G, Cao R, Liu T, Qian G, Guan X, Guo Z, Xiao Y, Wang X. (2016). Capsaicin suppresses cell proliferation, induces cell cycle arrest and ROS production in bladder cancer cells through FOXO3a-mediated pathways. Molecules. 21(10). pii: E1406