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Intracellular pH-mediated induction of apoptosis in HeLa cells by a sulfonamide carbonic anhydrase inhibitor

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ABSTRACT

Carbonic anhydrase IX (CAIX) is a hypoxia-associated transmembrane protein that is critical in the survival of cells. Because CAIX has a key role in pH regulation, its therapeutic effects have been heavily studied by different research laboratories. This study aims to investigate how a synthetic CAIX inhibitor triggers apoptosis in a cancer cell line, HeLa. In this regard, we investigated the effects of the compound **I**, synthesized as a CAIX inhibitor, on the survival of cancer cells. The compound **I** inhibited the proliferation of the CAIX+ HeLa cells, kept the cells in G0/G1 phase (74.7%) and altered the cells morphologies (AO/EtBr staining) and the nuclear structure (γ-H2AX staining). CAIX inhibition triggered apoptosis in HeLa cells with a rate of 47.4%. According to the expression of mediator genes (CASP-3, -8, -9, BAX, BCL-2, BECLIN, LC3), the both death pathways were activated in HeLa cells with the inhibition of CAIX with the compound **I**. The compound **I** was also determined to affect the genes and proteins that have a critical role in the regulation of apoptotic pathways (pro casp-3, cleaved casp-3, -8, -9, cleaved PARP and CAIX). Furthermore, CAIX inhibition caused changes in pH balance, disruption in organelle integrity of mitochondria, and increase intracellular reactive oxygen level of HeLa cells. Taken together, our findings suggest that CAIX inhibition has a potential in cancer treatment, and the compound **I**, a CAIX inhibitor, could be a promising therapeutic strategy in the treatment of aggressive tumours.

1. Introduction

Cancer treatment strategies have been subjected to intense scrutiny to remodel the modalities by combining the chemotherapies with specific enzyme inhibition drugs. Among the most important reasons for this shift, the side effects, inadequacy and ineffectiveness of the applications for the treatment of cancer are in the lead [1]. The major limitation of these standard treatments is the 'specificity' and the toxicity-related deaths in normal cells [2,3]. Therefore, side effects of the chemotherapy or radiotherapy are aimed to be minimized in enzyme-specific strategies [4,5]. Carbonic anhydrase IX (CAIX), which plays a role in the adaptive response of cells to cancer, has emerged as one of the most important targets of the most important 'enzyme inhibition' strategy in recent studies [6]. CAIXs, encoded by eight distinct gene families, function as zinc enzymes hydrating CO_2 to HCO_3^-, and this enzyme plays essential role in vital intracellular processes including metabolism and pH tampon mechanisms [7,8]. Moreover, it plays an important role in the formation of intracellular acid-base balance by promoting the survival of cancer cells in the hypoxic environment that occurs during tumour formation. It also contributes to the invasive and metastatic character of tumour cells by inducing the epithelial-mesenchymal transition (EMT) [9,10].

CAIX is localized to the cell membrane and functions especially in hypoxic tumour regions by the control of hypoxia inducible factor-1α (HIF-1α). Studies reported that CAIX is overexpressed in many solid tumours and has a poor prognosis [5,11–16]. Sulfonamide-derived compounds used in new CAIX-targeted treatment methods are a group of drugs known to suppress the proliferation of particularly aggressive

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cancer cells [17]. Following the emergence of theoretical proposals in the literature, experimental studies investigated the cytotoxic effects of compound **I** on cancer cells with high CAIX expression (HeLa [18–20], HT-29 [21], MDA-MB-231 [22] cell lines). However, none of these studies have revealed the molecular mechanism of the compound I in a cytotoxic environment. Together, in the present study, we aim to investigate the molecular mechanisms underlying the potential anticancer activity of the compound **I** by advanced functional experiments in the cell with a high cytotoxicity.

2. Materials and methods

2.1. Materials

We purchased the fetal bovine serum (FBS), cell-culture medium (RPMI 1640), penicillin, and streptomycin, from Gibco BRL (Life Technologies, Paisley, Scotland); DMEM-F12, acridine orange, ethidium bromide, dimethyl sulfoxide (DMSO), trypsin-EDTA solution, 2,7-dichlorofluorescein diacetate (DCFH-DA), 5-fluorouracil (5-FU), cisplatin and SLC-0111, from Sigma-Aldrich Chemical Company (Germany) and WST-1 (Roche, Basel, Switzerland); and the culture plates from Corning (Corning, New York, USA).

2.2. Cells and cell culture protocols

We used cell lines purchased from ATCC (American Type Culture Collection) and ECACC (European Collection of Authenticated Cell Cultures) stored in liquid nitrogen. We used HeLa (cervix cancer cells), MDA-MB-231 (breast cancer cells), HT-29 (colon cancer cells), PNT-1A (normal prostate cells), and HEK-293 (embryonic kidney epithelial cells) cell lines, and we incubated the cells according to the experiment protocol which was clearly described in our previous studies [4,5,23].

2.3. Drug preparation

The synthesis, characterization, spectral and analytical data of the sulfonamide derivatives used in this study were reported in our previous study. Compound **I** was re-synthesized and characterized as previously described [24,25]. Briefly, Compound I was synthesized by the reaction of 4-aminobenzenesulfonamide with 5-chloro-2-hydroxybenzaldehyde at a molar ratio of 1:1. The precursors dissolved in methanol were stirred at room temperature for 30 min and then refluxed for 3 h. Of formic acid, 2–3 drops were added as a catalyst. The mixture was then cooled to room temperature, filtered, and recrystallized from ethanol. Inhibition data for this Compound **I**: 4-[(5-Chloro-2-hydroxybenzylidene) amino] benzenesulfonamide (colour: Bright orange; mp: 199–201 °C, $C_{13}H_{11}ClN_2O_3S$ (310.76 g/mol)) against relevant CA isoforms are shown in Fig. 1.

2.4. Cytotoxicity analysis

The cytotoxic analysis was tested with WST-1 kit (Roche, Basel, Switzerland). After incubation in 96 well plate (10^4 cell for a well), compound **I**, **cisplatin** (cisplatin, interferes with DNA replication [26]) and **5-FU** (5-FU acts as a thymidylate synthase (TS) inhibitor [27]) was administered to the cell lines at the doses of 0, 2.5, 5, 10, 25, 50, 100 and 200 μM, for 24, 48 and 72 h. Following 4 h incubation with WST-1 reactive (10 μL), the measurements were carried with reader (Spectramax M5, Biotech, VT, USA). Consequently, the IC_{50} values of compound **I**, cisplatin, and 5-FU were calculated. Selectivity index (SI) can be defined as the ratio of the toxic concentration of a sample against its effective compound concentration.

2.5. Analysis of antiproliferative effects

The antiproliferative impacts of compound **I** were detected by BrdU kit (Bio vision, CA, USA) according to the protocol of manufacturer. The cells were planted in 96 well plates, and compound **I** and **SCL-0111,** is an ureido-substituted benzene sulfonamide small molecule inhibitor of CAIX [28], were administrated at the doses of 0, 2.5, 5, 10, 25, 50, 100 and 200 μM for 72 h. Experiment protocol was clearly defined in our previous studies [4,5,23].

2.6. AO/EtBr staining assay

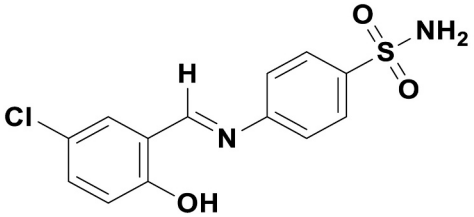
The morphological changes of HeLa cells were investigated with AO/EtBr dye under fluorescent microscopy (Olympus CKX 51, DP73, USA). Staining protocol was clearly defined in our previous studies [4,5,23]. Briefly, HeLa cells were seeded in 12 well plates and treated with different concentrations of compound I (10–25–50 μM) and cisplatin (10–25 μM). After 72 h, the medium was removed from the experimental slides. The morphology image was taken under a light microscope and the presence of apoptotic cells was observed under fluorescent light by AO/EtBr.

2.7. Annexin V-PI assay

The apoptotic effects of different concentration of compound **I** (10–25–50–100 μM) were detected by FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, NJ, USA). Briefly, HeLa cells were seeded in 6 well plates and treated with different concentrations of compound I (10–25–50–100 μM). After incubation, the cells were removed with trypsin, and after centrifugation, the pellet was washed with PBS and the dye-binding solution included in the kit. After adding 5ul Annexin V and PI dyes, they were incubated for 15 min in the dark and analysed by reading 10,000 cells in a BD Facs Via flow cytometer device (BD Biosciences, NJ, USA).

2.8. Detection of pHi levels

After administration of compound **I** at doses of 0–200 μM, the intracellular pH was measured with fluorometric intracellular pH assay kit (Sigma-Aldrich, MAK-150, Germany). Experiment protocol was clearly defined in our previous studies [4,5,23]. Briefly, the cells were seeded on opaque black plates, which provide effective fluorometric measurement by preventing light reflection, and incubated for 24 h according to the kit protocol. After the incubation, the cell medium was removed and 100 μl of BCFL-AM solution prepared in PBS was added and incubated at 37 °C, 5% CO_2 for 30 min in a dark environment. The measurement was performed at Ex/m: 490/535 nm in fluorimetry



	Ki(nM)		
Compound	**hCAI**	**hCAII**	**hCAIX**
I	>50.000	404.0	**47.9**

Fig. 1. The chemical structure and K_i (nM) value of novel compound **I**.

(Spectramax M5, BioTek, Vermont, USA).

2.9. Detection of pHe levels

Extracellular pH (pHe) was measured with a kit using the routine blood gas device (ABL90 FLEX PLUS, Radiometer, Copenhagen, Denmark) [5].

2.10. Reactive oxygen species detection

After incubation of the compound **I** at doses of 0–200 μM, intracellular free radicals were measured using ROS kit (Abcam-186027, Cambridge, UK). Detailed protocol of the kit was explained in our previous studies [4,5,23]. According to the kit protocol, HeLa cells were seeded on black plates and treated with different concentrations of compound I prepared in PBS. It was incubated at 37 °C in an atmosphere of 5% CO2 for 1 h (protected from light). After incubation, compound I was removed from the wells, ROS-Red fluorescence working solution was added, incubated under the same conditions, and analysed in a fluorometry device (Spectramax M5, BioTek, Vermont, USA) at Ex/m: 520/605 nm.

2.11. Mitochondrial membrane potential assay

After incubation of the compound **I** at doses of 0–200 μM, mitochondrial membrane potential (MMP) changes were spectrophotometrically detected in a fluorometry device at Ex/m: 490/530 nm (Spectramax M5, Biotech, VT, USA).

2.12. Cell cycle assay

The effects of compound **I** at concentrations of 10–25–50 μM on cell cycle was evaluated using BD Cycletest TM Plus DNA Reagent kit (BD Biosciences, NJ, USA) in accordance with the kit protocol which was clearly explained in our previous article [23]. Briefly, HeLa cells were seeded into 6 well plates and treated with different concentrations of compound I according to the kit protocol. After incubation, cells were removed with trypsin, and after centrifugation, the pellet was washed with PBS and the dye-binding solution included in the kit. Then, 250 μl solution A (Trypsin) was added and incubated for 10 min at RT; 200 μl solution B (Trypsin inhibitor) was incubated for 10 min at RT; and 200 μl solution C (PI dye) was incubated for 10 min at +4 °C. After these incubations, 10,000 cells were read in a BD Facs Via flow cytometer device (BD Biosciences, NJ, USA).

2.13. Immunofluorescence staining

DNA damage in HeLa cells were detected using with primary mouse monoclonal anti-γ-H2AX antibody (Cell Signalling Technology, MA, USA) and secondary goat anti-mouse Alexa-488-conjugated IgG (Invitrogen, CA, USA), and visualized using Olympus Inverted fluorescence microscope (Olympus CKX 51, DP73, USA). Antibody and microscope protocols were clearly explained in our previous studies [4,5,23].

2.14. mRNA extraction and RT-PCR

Apoptotic and autophagic genes expression levels were detected following 24 h incubation with the compound I at a dose of 25 μM. miRNeasy mini kit (Qiagen Hilden, Germany), Ipsogen RT Set (Qiagen Hilden, Germany), and QuantiTect SYBR Green PCR kit (Qiagen Hilden, Germany) were used to isolate mRNA, synthesize cDNA and analyse the expression level of the genes, CASP-3,8,9, BCL-2, LC3, BECLIN, NRF-2 and BAX. Primers used are listed in Supplementary Table S1.

2.15. Western blotting

Protein levels were detected using with the following primer and secondary antibodies according to clearly explained protocol in our previous studies [4,5,23]. HeLa cells were seeded in 6 well plates and treated with different concentrations of the compound I. After incubation, it was washed with cold PBS. Adjusting the protein concentration to 50 μg, the protein sample was conducted on 10% SDS-PAGE gel at 50 V for 30 min and at 80 V for 3 h, the proteins were blotted to PVDF membrane at 70 V for 5 min (Bio-Rad Turbo Transfer System). After blocking, the primary monoclonal antibodies were administered to the membrane overnight, and the antibodies were washed with 1xTBS-T at the end of incubation. The membrane was incubated with secondary antibodies-HRP for 60 min. For band imaging, ECL substrate (EMD Millipore Corp., MA, USA) was used in the imaging system (LI-COR Odyssey Fc, NE, USA).

2.16. Statistical analysis

The difference between the variable distribution to the normal distribution was tested by Shapiro-Wilk and Kolmogorov Smirnov tests. Student's *t*-test, Mann Whitney *U* test and One-way ANOVA tests were used to compare the mean of independent variables by using SPSS 25.0 packet program. Also, GraphPad 8 program was used to create graphs ($^*p < 0.05$; $^{**}p < 0.01$).

3. Results

3.1. Antiproliferative activity of compound I in cancer and normal cells

To test the effect of compound **I** (Fig. 1), cisplatin and 5-FU on cervix cancer cell proliferation and survival, WST-1 cell viability assay was performed. The compound **I** significantly inhibited the viabilities of HeLa (cervix), HT-29 (colon) and MDA-MB-231 (breast) cancers and HEK-293 and PNT1-A normal cells in dose (0, 2.5, 5, 10, 25, 50,100 and 200 μM) and time (24–48 and 72 h) dependent manners (Table 1). The lowest IC_{50} and the highest SI values were detected in HeLa cells. In normal cells, IC_{50} value was found to be 91.0 μM for HEK-293 and 231.5 μM for PNT-1A. Normal cells have a high IC_{50} value compared to cancer cells is the most important proof of that the compound I has a selective effect for cancer cells. HeLa cells were treated with chemical compounds (Compound I and CAIX inhibitor SLC-0111 [28,29]) and the BrdU cell proliferation assay was performed. While the IC_{50} value of the compound **I** was found to be 21.58 μM in HeLa cells, it was found to be 61.63 μM when the cells were treated with SLC-0111 under the same conditions (Fig. 2). Further experiments were carried out with the 10, 25 and 50 μM doses of the compound **I**. The effect of the compound **I** on HeLa cells was analysed by cell cycle assay after propidium iodide (PI) staining. With DNA content analysis of 10.000 cells, it was found that cell division was significantly suppressed with the increase in compound **I** doses. The amount of the cells in the G0/G1 phase was found to be 58.3% in WT, 59.5% in 10 μM, 69.3% in 25 μM, and 71.7% in 50 μM. In addition, the amount of cells in the Sub-G1 phase was found to be 1.2% in WT, 2.6% in 10 μM, 2.7% in 25 μM, and 3.1% in 50 μM (Fig. 3). Taken together, the data strongly indicates that the compound **I** has significant anti-proliferative activity against HeLa cells and might be a novel anticancer agent for therapeutic strategies.

3.2. Anticancer activity of compound I in cervix cancer cells

The anticancer potential of compound **I** on HeLa cells were tested by Annexin V/PI and AO/EtBr staining and gene and protein expression analyses. Firstly, HeLa cells were treated with compound **I** at the doses of 10–25 and 50 μM and then stained with AO/EtBr. As the first clue of apoptosis, changed cell morphologies were observed. Early-stage apoptotic cells with condensed or fragmented chromatin are stained

Table 1
Cytotoxicity of compound **I**, cisplatin and 5-fluorouracil on cancer and normal cell lines (IC_{50} and selectivity index (SI)).

IC_{50} (μM)															
Chemicals	HeLa			HT-29			MDA-MB-231			HEK-293			PNT-1A		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Compound **I**	66.1 ± 1.44	38.9 ± 1.04	**21.58** ± 2.32	334.0 ± 5.24	115.3 ± 3.21	91.0 ± 2.44	216.4 ± 13.44	56.3 ± 3.54	38.9 ± 4.23	102.1 ± 3.24	79.8 ± 4.32	59.7 ± 2.1	419.3 ± 9.14	43.6 ± 11.4	231.5 ± 10.02
Cisplatin	28.9 ± 1.04	27.6 ± 0.14	24.1 ± 0.28	23.7 ± 3.21	20.0 ± 2.01	16.3 ± 1.18	21.2 ± 3.74	10.2 ± 1.44	7.0 ± 0.14	29.6 ± 5.65	14.0 ± 3.14	2.0 ± 1.02	13.4 ± 7.08	11.4 ± 6.65	8.2 ± 4.53
5-FU	37.8 ± 2.24	31.2 ± 3.01	28.7 ± 1.74	47.3 ± 4.21	23.4 ± 1.78	19.3 ± 1.51	42.6 ± 4.2	37.9 ± 4.54	21.0 ± 2.14	44.6 ± 6.12	19.8 ± 5.84	14.5 ± 4.14	23.1 ± 5.51	21.2 ± 4.2	17.5 ± 7.21

SI (μM)			
	24 h	48 h	72 h
HEK-293/HeLa	>1.5	2	>2.7
HEK-293/HT-29	0.3	0.6	0.6
HEK-293/MDA-MB-231	0.007	1.4	1.5
PNT1-A/HeLa	>6	1.1	>10
PNT1-A/HT-29	1.2	0.3	>2
PNT1-A/MDA-MB-231	0.03	0.7	>5

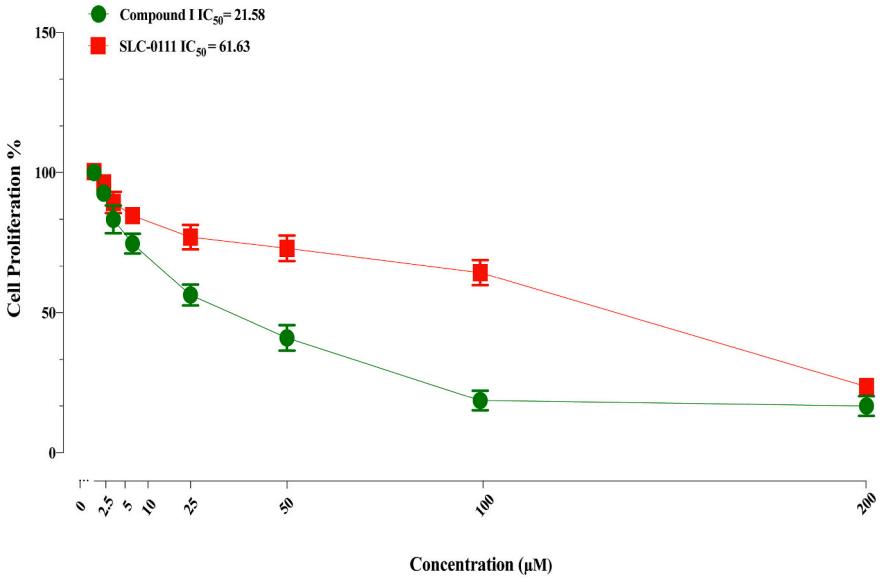


Fig. 2. Anti-proliferative activity of the compound **I**. HeLa cells was treated with compound **I** and SLC-0111 and IC_{50} values were calculated by BrdU cell proliferation assay (IC_{50}: 21.58- IC_{50}: 61.63). The experiments were performed in triplicate. IC_{50} values were calculated using the GraphPad Prism 8 program.

with AO, seems as bright green-yellow. Late-stage apoptotic cells with impaired cytoplasmic membrane integrity are stained by EtBr, seems as orange-red. Necrotic cells are stained dark orange-red, indicating that the cells are in the process of disintegration (Fig. 4).

The increase in expression of the pro-apoptotic gene (BAX) and the decrease in expression of the anti-apoptotic (BCL-2) gene showed that compound **I** triggered the intrinsic apoptosis pathway (CAS-3, -8, -9) in HeLa cells (two-fold or more increases were considered significant). Also, expressions of the mediators, BECLIN and LC3, of the secondary death pathways were increased. The gene expressions show that apoptosis and autophagy pathways were triggered (Fig. 5). Nuclear factor erythroid 2-related factor 2 (NRF-2) gene expression, which controls the expression of antioxidant response elements, was significantly increased in the CAIX-inhibited group compared to the control group. Annexin V/PI staining confirms these results that the compound **I** induced dose-dependent cellular apoptosis. The number of apoptotic cells was high especially at concentration of 25 μM. The apoptotic cells accumulated at the early apoptotic phase (47.4%) was relatively high compared to late apoptotic phase (10.9%), suggesting that the compound **I** may trigger early apoptosis in HeLa cells (Fig. 6). After application of the compound **I**, protein expression levels of the apoptotic mediator genes CASP-3, 8, 9 and cleaved Poly (ADP-Ribose) Polymerase 1 gene (PARP) were studied by western blot method in HeLa cells. In addition, the CAIX protein level was examined to partially determine the CAIX inhibitory property of compound **I**. When 10, 25 and 50 μM doses of compound **I** was compared with WT; the pro-caspase 3 protein level was found to be decreased; however, the cleaved caspase 3, 8 and 9 protein levels were found to be increased. In addition, CAIX protein level was decreased at 25 μM dose. Cleaved PARP protein level was found to be increased at 10 and 25 μM doses comparing to WT (Fig. 7). When results are collectively evaluated, the compound **I** was found to triggered apoptosis in cells by suppressing CAIX gene expression in HeLa cells.

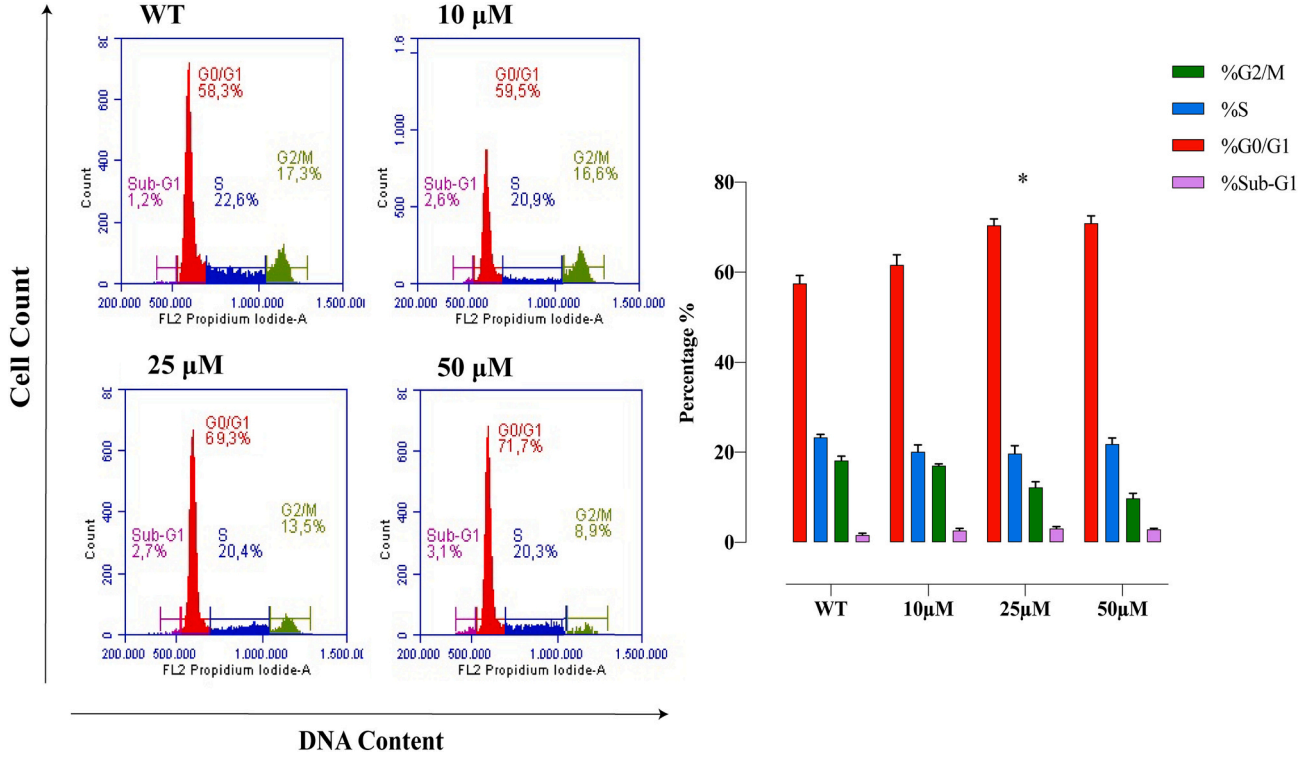


Fig. 3. Cell cycle analysis of HeLa cells. DNA contents were analysed as a result of the cell cycle assay. The experiments were performed in triplicate. *$p < 0.05$ was considered as significant.

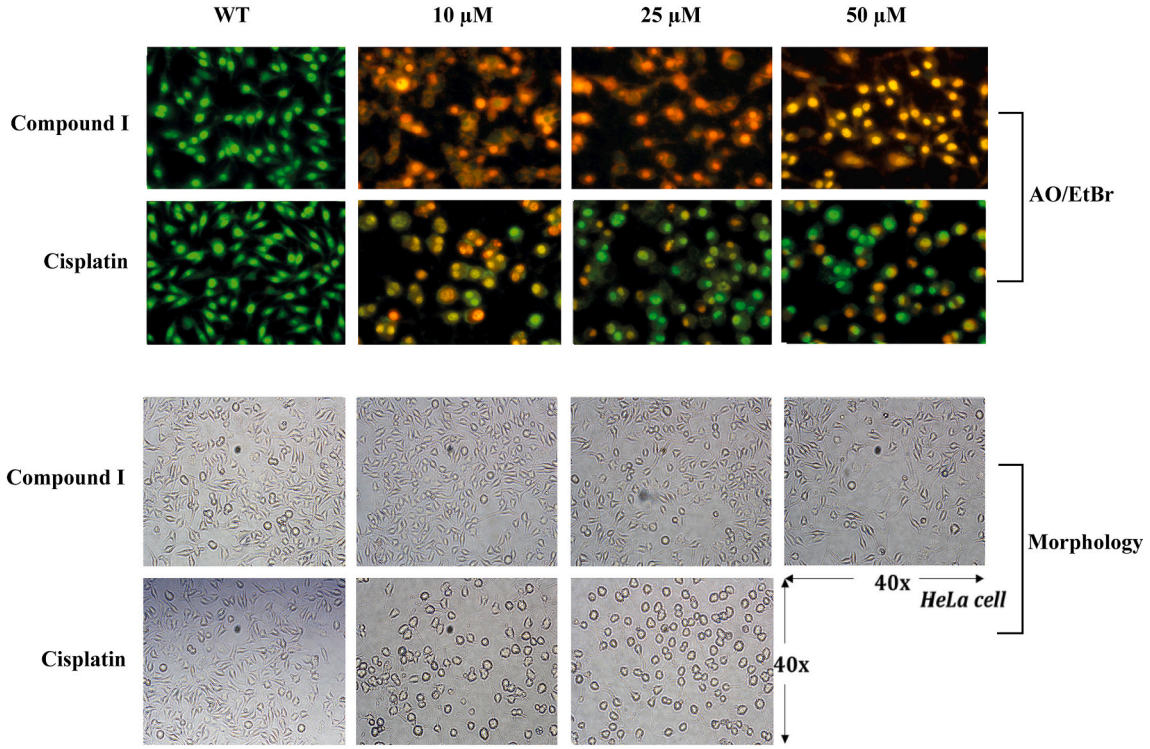


Fig. 4. Dual (AO/EtBr) staining. Morphological changes visualized under fluorescence microscopy of HeLa cells treated with different doses of compound **I**. (The live cells appear green and the apoptotic cells appear bright green-yellow and orange-red.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These results showed that the compound **I** can be nominated as a new-target specific anti-cancer drug especially for cancer types with metastatic character.

3.3. pH-ROS-MMP in cervix cancer cells

As a result of CAIX inhibition in cancer cells, intracellular acidosis

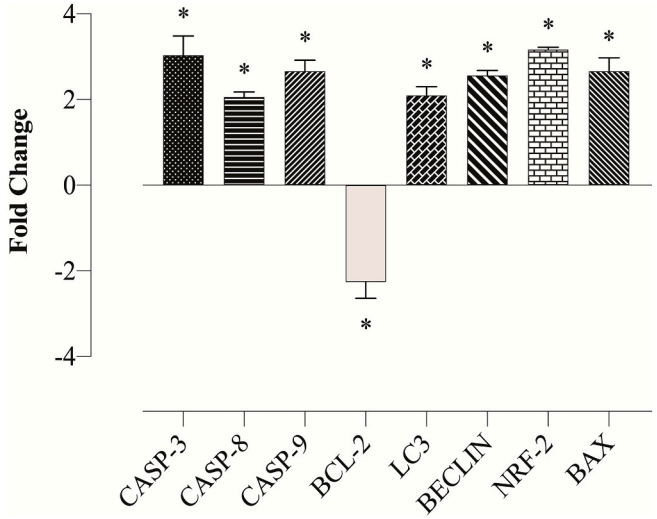


Fig. 5. Impacts of the compound **I** on the expression of apoptotic genes. The experiments were performed in triplicate. *p < 0.05 was considered as significant.

mechanism is disrupted and survival of the cells is prevented. In this context, it was found that the pHi level decreased significantly and the pHe level increased starting from the active dose in HeLa cells treated with compound **I** (Fig. 8A–B). The regulation of pHi and pHe is very important for maintaining homeostatic balance in both normal and cancer cells. Changes in pH of HeLa cells have been shown to induce cellular apoptosis by disrupting MMP (Fig. 9), leading to increased level of the ROS (Fig. 10) and DNA damage (γ-H2AX) (Fig. 11). These findings collectively suggest that the compound **I** triggered apoptosis by CAIX inhibition in HeLa cells.

4. Discussion

The focus of pharmacological research in recent years has been to identify new targets responsible for cancer disease progression and to characterize drugs specific to these targets. In this context, the CAIX gene, which is known to have a role in cancer development (cell transformation, growth and progression, invasion and metastasis), has become a remarkable target [30,31]. CAIX is a transmembrane protein that is responsible for pH regulation and has been shown to be overexpressed in cervix [23], renal [32], prostate [33], pancreas [34], breast [35], and head-neck squamous [36] cancer types [37]. Designing new drugs inhibiting CAIX and investigating their functional effects have been reported to be important for anticancer therapeutic strategies [38,39].

In this study, we demonstrated the anti-proliferative effects of compound **I** synthesized as a CAIX-inhibitor on HeLa cells using functional and advanced molecular techniques. As the most striking result, we showed that apoptosis was triggered in cells via the pH-ROS-MMP pathway after the treatment of cells with compound **I** in normoxic conditions. CAIX expression has been reported to be especially high in solid tumours and cancer cells with aggressive character [40,41]. HeLa, MDA-MB-231 and HT-29 cells, with high CAIX expression, were incubated with different concentrations of compound **I** for 24, 48, and 72 h. According to cell viability assay, we detected the lowest IC_{50} (21.58 μM) and the highest SI (>2.7) value in the HeLa cells compared with chemotherapy drugs (cisplatin IC_{50} (24.1 μM); 5-Fu IC_{50} (28.7 μM)), summarized in Table 1. Moreover, according to the BrdU staining, we found that the proliferation of HeLa cells was significantly reduced when compared with SLC-0111 (IC_{50} 61.63 μM) (Fig. 2). Studies indicated that CAIX inhibition reduces cell proliferation, slows down the division of cells and increases the arrest of cells in the G1 phase [38]. We showed that the compound **I** treatment arrested cell cycle in G0/G1 phase (69.3%) in HeLa cells. Also, the cell rate accumulated in Sub-G1 phase was increased to 2.7%, indicating triggered apoptosis (Fig. 3). Inhibition

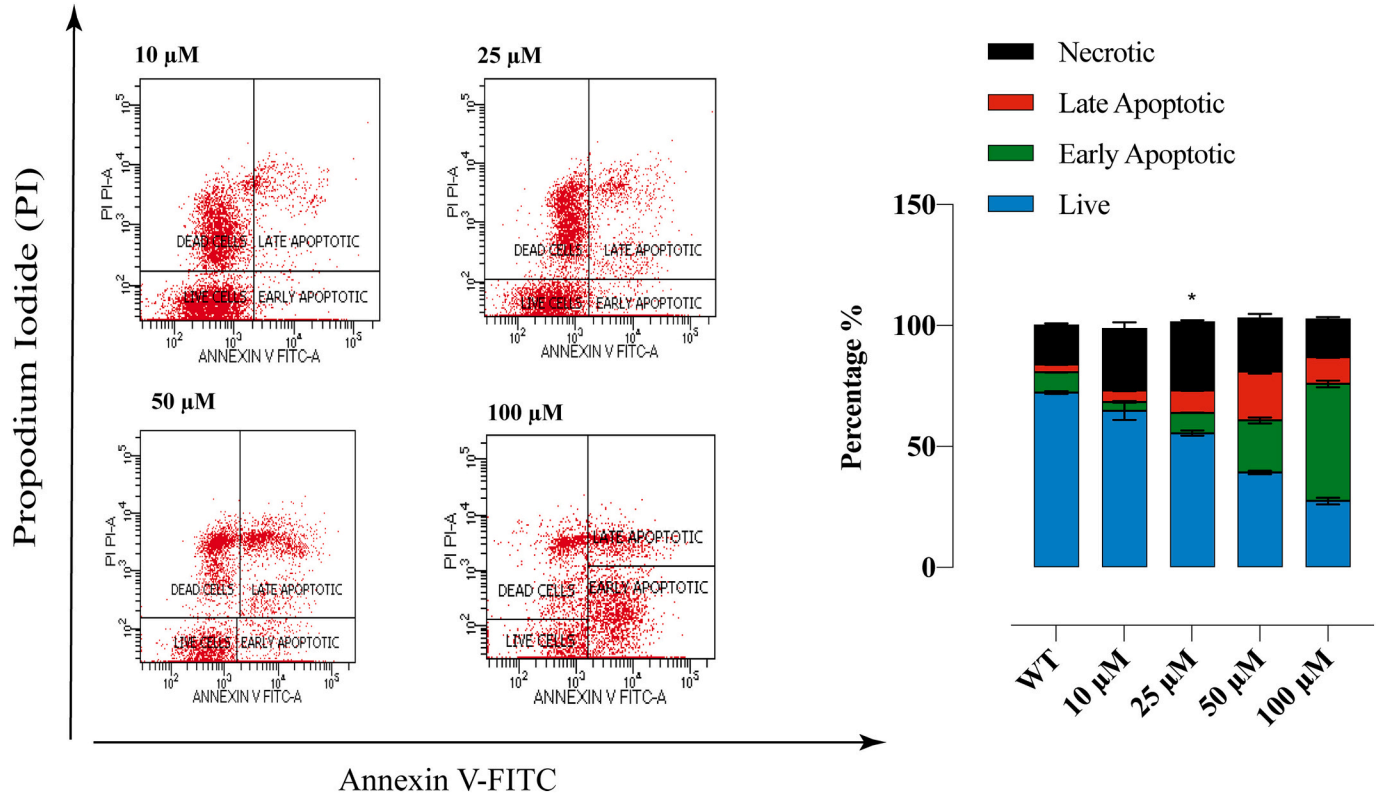


Fig. 6. Flow cytometry analysis of HeLa cells after Annexin-V/PI staining. The experiments were performed in triplicate. *p < 0.05 was considered as significant.

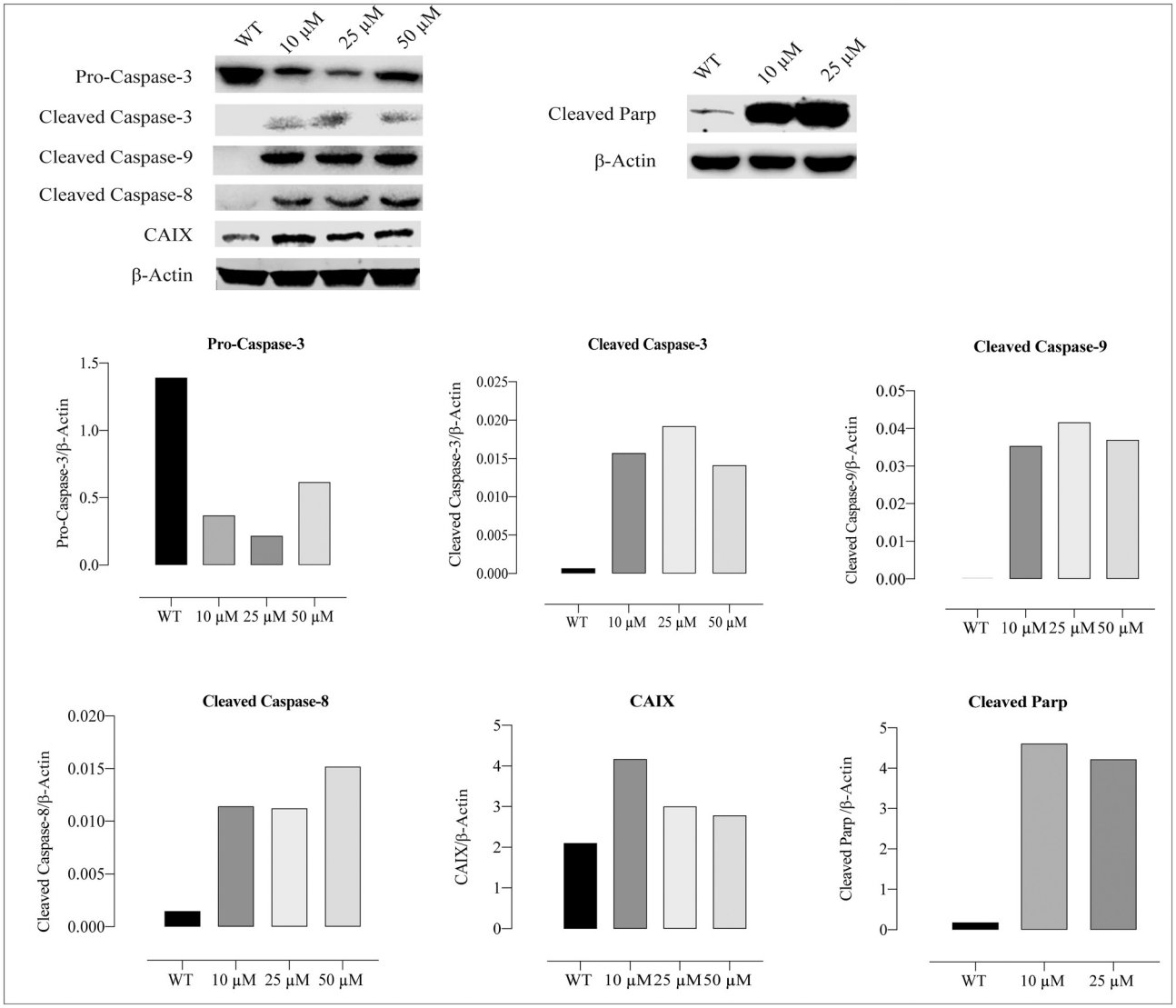


Fig. 7. Impacts of the compound **I** on apoptosis signalling proteins levels in HeLa cells. Protein levels were normalized to the respective β-actin. The densitometry quantification of blot was determined by software (Li-Cor Fc). The experiments were performed in triplicate.

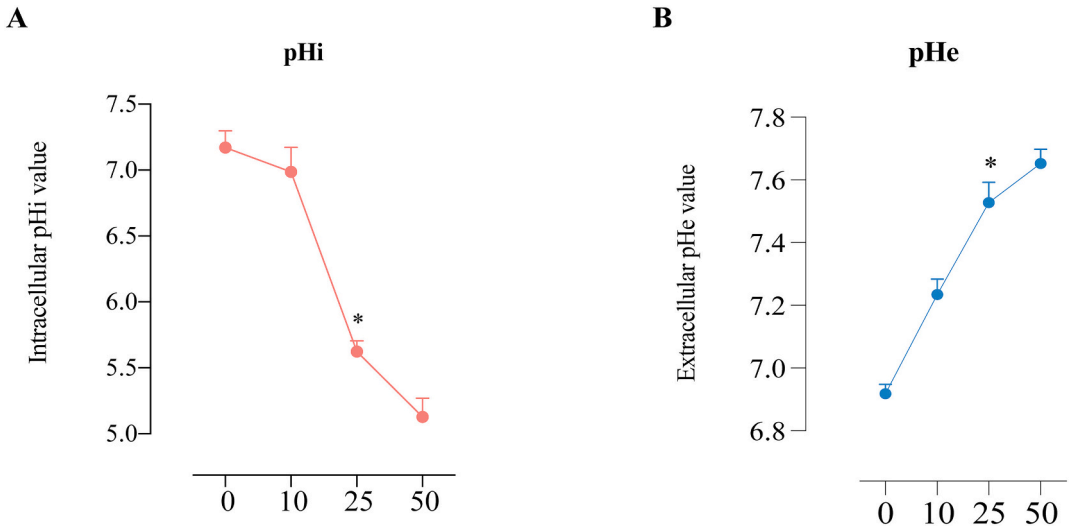


Fig. 8. Measurement of pH levels. A. The impacts of compound **I** on pHi in HeLa cells. B. The impacts of compound **I** on pHe in HeLa cells. The experiments were performed in triplicate. *$p < 0.05$ was considered as significant.

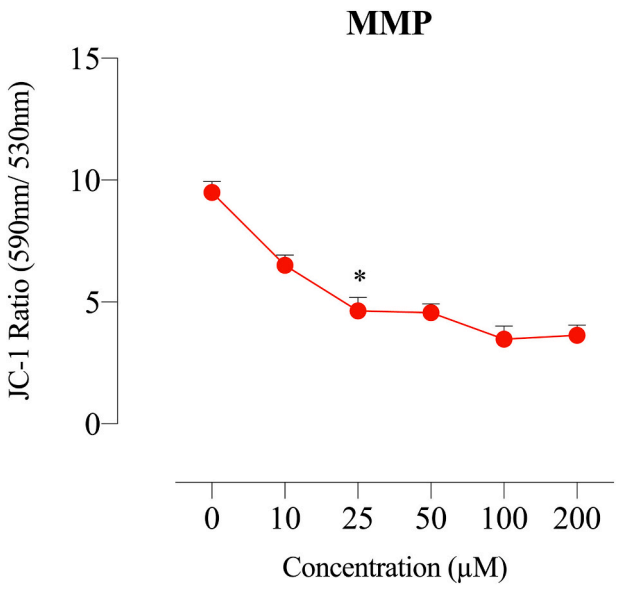


Fig. 9. Mitochondrial membrane potential (MMP) of the HeLa cells detected by spectrofluorimetric measurement. The experiments were performed in triplicate. *p < 0.05 was considered as significant.

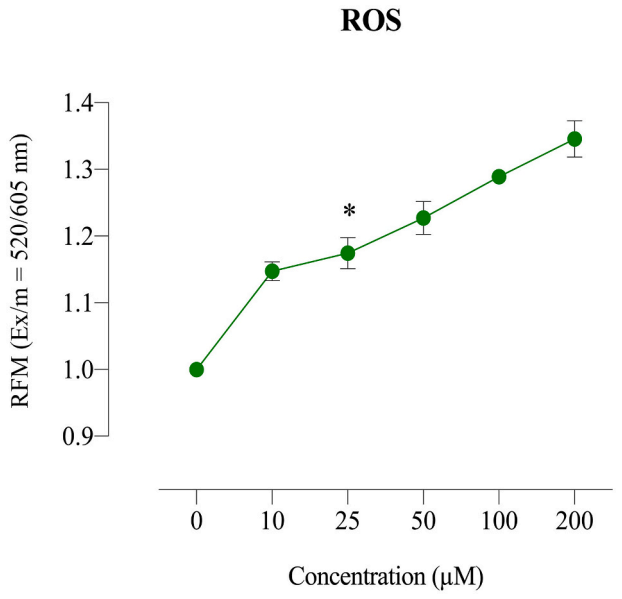


Fig. 10. Reactive oxygen species (ROS) levels of the HeLa cells detected by spectrofluorimetric measurement. The experiments were performed in triplicate. *p < 0.05 was considered as significant (RFM: red fluorescence measurement).

of CAIX in cancer cells changes cell morphology, leading to disruption of nucleus integrity and formation of apoptotic characters in cells [4,5,23]. In HeLa cells treated with compound **I**, we observed a dose-dependent change of cell morphology and reduced cell number. In addition, we observed under fluorescence microscopy, that the nuclei of cells were mostly stained bright green-yellow with AO/EtBr (Fig. 4) [42,43]. The most important evidence is the activation of caspase, which are responsible for the degradation process in cells [38,44]. The autophagy pathway plays a role in carcinogenesis. Autophagy has been shown to work with a 'dual strategy' by suppressing tumour growth in cells or promoting their survival [45]. BECLIN-1 and LC-3 genes are autophagy pathway regulators. Performed in breast, ovarian, colorectal and prostate cancer cells, a study reported that the increase in the expression of these genes suppressed autophagy-supported tumour growth and decreased aggressive character in the cells [46,47]. In HeLa cells, we found that the mediators of the cell death pathways (CASP-3, 8, 9, BAX, BECLIN-1 and LC-3) have been activated, the anti-apoptotic BCL-2 gene expression has been reduced. NRF-2 is a regulator of cellular resistance to reactive oxidants [48]. It controls the expression of a number of antioxidant response elements to regulate the physiological and pathophysiological consequences of increased oxidant level in the cell. In this study, we think that NRF-2 gene expression has been increased to suppress enhanced level of the ROS in the cells (Fig. 10). Based on these data, we suggest that with inhibition of the CAIX, tumour formation was suppressed by triggering both death pathways in HeLa cells. We tested the effects of CAIX inhibition on the apoptotic pathway with Annexin V/PI staining and observed 47.4% of cells accumulating in the early apoptotic phase at the 25 μM dose (Fig. 6) (*p < 0.05). In addition, when we measured the protein levels of apoptotic genes, we found that procaspase-3 levels decreased, while cleaved caspase-3,8 and 9 levels increased. Furthermore, the protein level of cleaved-PARP, an indicator of DNA damage, increased (Fig. 7). According to these results, inhibition of CAIX in HeLa cells caused caspase activation. Caspase activation in cells initiates cellular degradation [44] that causes pH (pHi-pHe) disturbance [49], increased intracellular ROS level due to MMP degradation [50], and DNA damage [51].

The increase of CAIX expression disrupts pH balance (7.4) and causes substantial increase in H^+ concentration to a pH of 6.5 in tumour cells [49,52]. This low pH of tumour cells has been associated with tumourigenic transformation, chromosomal rearrangements, extracellular matrix fragmentation, migration and invasion, induction of cell growth factors expression, and protease activation [49,52,53]. CAIX inhibiting strategies prevent dramatic increases of extracellular pH; however, this strategy also enhances the rate of soluble CO_2 amount in extracellular space, which may trigger anaerobic oxidation pathways. Anaerobic oxidation prompts apoptotic machinery by accumulating reactive oxygen species [4,5,23]. In this study, we examined the changes in pH, MMP and ROS levels of HeLa cells treated with compound **I**, as well as their effects on DNA damage. We found that in HeLa cells, the pHi level decreased and the pHe level increased (Fig. 8A–B). The pH balance in the cells was abolished due to CAIX inhibition. In addition, we found that it degrades MMP that increases intracellular ROS level (Figs. 9, 10), and causes DNA damage (Fig. 11) in HeLa cells (*p < 0.05). These results indicated that the apoptotic mechanism triggered in HeLa cells was triggered via the pH-MMP-ROS pathway. Together, we demonstrated that compound **I**, which we designed as a potent and specific inhibitor of CAIX, inhibits CAIX in HeLa cells with high CAIX expression, which triggers a series of mechanisms in the cell. According to our findings, the most important output of this intracellular cycle is that the inhibition of CAIX changes pH homeostasis, causes MMP degradation, increases ROS level, and activates the apoptotic pathway. This study supports the literature recommendations and provides a strong suggestion for the CAIX inhibition targeted therapies in aggressive cancer cells with high CAIX expression.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2021.12.190.

Abbreviations

CAIX	Carbonic anhydrase IX
pHi	Intracellular pH
pHe	Extracellular pH
ROS	Reactive oxygen species
MMP	Mitochondrial membrane potential
AO/EtBr	Acridine orange/ethidium bromide
PI	Propidium iodide
HIF-1	Hypoxia inducible factor-1
EMT	Epithelial-mesenchymal transition

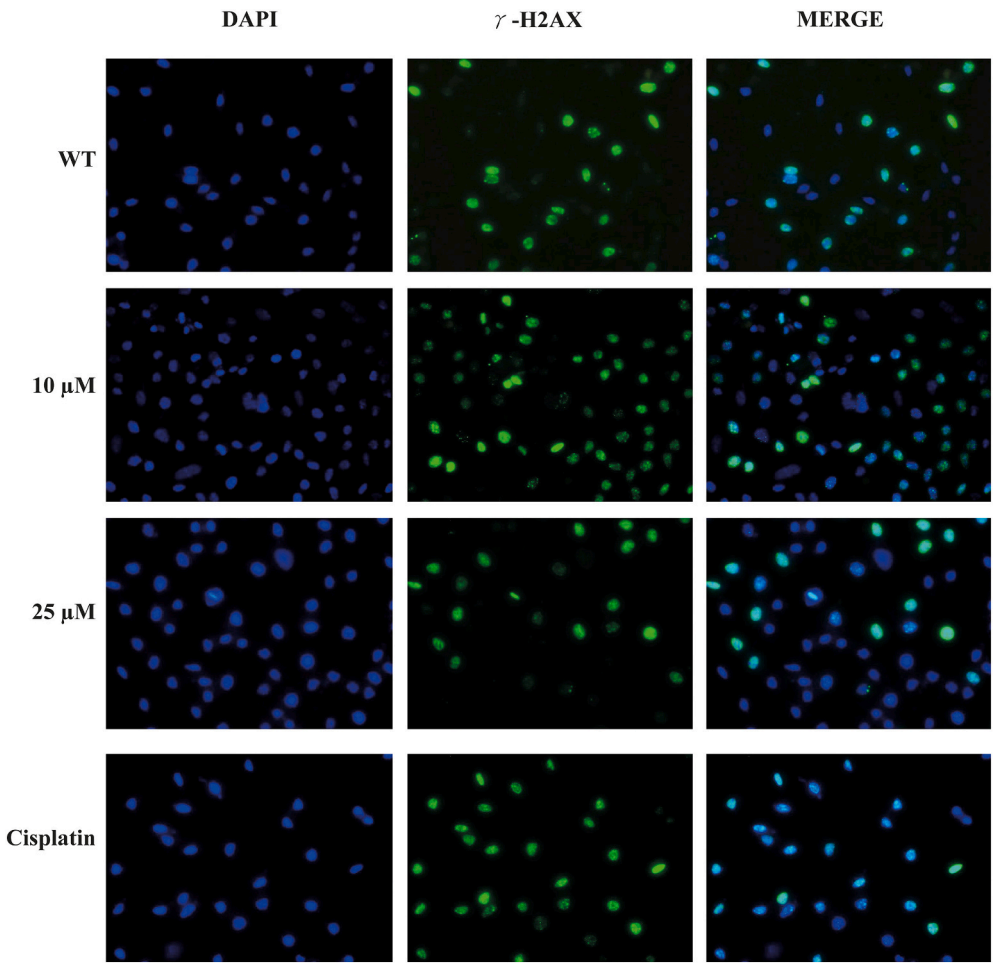


Fig. 11. Microscopy images of HeLa cells treated with compound **I**. γ-H2AX staining is green; nuclei are stained with DAPI blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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