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The investigation of cytotoxic and apoptotic activity of Cl-amidine on the human U-87 MG glioma cell line

Pınar Naile Öğüten, PhD[a,*], Selin Engür Öztürk, PhD[b], Miriş Dikmen, PhD[c]

Abstract

Background: Peptidyl (protein) arginine deiminases (PADs) provide the transformation of peptidyl arginine to peptidyl citrulline in the presence of calcium with posttranslational modification. The dysregulated PAD activity plays an important role on too many diseases including also the cancer. In this study, it has been aimed to determine the potential cytotoxic and apoptotic activity of chlorine-amidine (Cl-amidine) which is a PAD inhibitor and whose effectiveness has been shown in vitro and in vivo studies recently on human glioblastoma cell line Uppsala 87 malignant glioma (U-87 MG) forming an in vitro model for the glioblastoma multiforme (GBM) which is the most aggressive and has the highest mortality among the brain tumors.

Methods: In the study, the antiproliferative and apoptotic effects of Cl-amidine on GBM cancer model were investigated. The antiproliferative effects of Cl-amidine on U-87 MG cells were determined by 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate method at the 24th and 48th hours. The apoptotic effects were analyzed by Annexin V and Propidium iodide staining, caspase-3 activation, and mitochondrial membrane polarization (5,5', 6,6'-tetrachloro-1,1', 3,3' tetraethyl benzimidazolyl carbocyanine iodide) methods in the flow cytometry.

Results: It has been determined that Cl-amidine exhibits notable antiproliferative properties on U-87 MG cell line in a time and concentration-dependent manner, as determined through the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate assay. Assessment of apoptotic effects via Annexin V and Propidium iodide staining and 5,5', 6,6'-tetrachloro-1,1', 3,3' tetraethyl benzimidazolyl carbocyanine iodide methods has revealed significant efficacy, particularly following a 24-hour exposure period. It has been observed that Cl-amidine induces apoptosis in cells by enhancing mitochondrial depolarization, independently of caspase-3 activation. Furthermore, regarding its impact on healthy cells, it has been demonstrated that Cl-amidine shows lower cytotoxic effects when compared to carmustine, an important therapeutic agent for glioblastoma.

Conclusion: The findings of this study have shown that Cl-amidine exhibits significant potential as an anticancer agent in the treatment of GBM. This conclusion is based on its noteworthy antiproliferative and apoptotic effects observed in U-87 MG cells, as well as its reduced cytotoxicity toward healthy cells in comparison to existing treatments. We propose that the antineoplastic properties of Cl-amidine should be further investigated through a broader spectrum of cancer cell types. Moreover, we believe that investigating the synergistic interactions of Cl-amidine with single or combination therapies holds promise for the discovery of novel anticancer agents.

Abbreviations: BJ = human foreskin fibroblasts, Cl-amidine = chlorine-amidine, GBM = glioblastoma multiforme, IC_{50} = half maximal inhibitory concentration, JC-1 = 5,5', 6,6'-tetrachloro-1,1', 3,3' tetraethyl benzimidazolyl carbocyanine iodide, PAD = peptidyl arginine deiminase, PBS = phosphate buffer saline, PS = phosphatidylserine, U-87 MG = Uppsala 87 malignant glioma, WST-1 = 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

Keywords: Cl-amidine, glioblastoma multiforme, PAD inhibitor, U-87 MG

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The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

Ethics committee certificate is not required due to the use of secondary cell lines purchased from the company for a fee in the study.

[a] Faculty of Medicine, Department of Histology, Samsun University, Samsun, Turkey, [b] Department of Pharmacy Services, Tavas Vocational School of Health Services, Pamukkale University, Denizli, Turkey, [c] Department of Pharmacology, Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey.

** Correspondence: Pınar Naile Öğüten, Faculty of Medicine, Department of Histology and Embryology, Samsun University, Samsun, Turkey (e-mail: pinar.oguten@samu.edu.tr).*

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1. Introduction

The incidence of cancer, one of the most important health problems of our age, is increasing day by day. Among these, cancers of the central nervous system rank third in the list of fatal diseases in adults and second after leukemia in children. Gliomas are the most common tumors in the central nervous system and account for more than 70% of brain tumors.[1] The most malignant form is glioblastoma multiforme (GBM).[2] In the treatment, the spread of the tumor is prevented by surgical intervention and followed by radiotherapy and chemotherapy.[3] Patients lose their lives within 1 to 2 years because the drugs used in chemotherapy have many side effects and the development of resistance negatively affects the treatment.[4]

Peptidyl (protein) arginine deiminases (PADs) are enzymes that enable the conversion of peptidyl arginine to peptidyl citrulline in the presence of calcium by posttranslational modification.[5] This conversion, which causes the formation of citrullinated proteins with altered structure and function, leads to many pathological diseases such as inflammation, autoimmune diseases, and cancer through dysregulated PAD activity.[6] There are 5 defined types of PAD in mammals, including humans: PAD1, PAD2, PAD3, PAD4, and PAD6.[7] Of these PAD types, PAD4 in particular is highly expressed in various cancer cell lines and tumors.[6] PAD4 activity is also increased in human brain astrocytomas.[8] Chlorine-amidine (Cl-amidine), a PAD inhibitory substance, suppresses the activities of all other types of PAD, including PAD4.[9] The effect of this inhibitor has been examined in studies using both animal experimental models and various cancer cell lines.[6,8,10–13]

Cl-amidine is a potential anticarcinogenic molecule that can be used in in vitro cancer studies because it is an in vitro tumor suppressor, shows little toxicity at in vitro and in vivo doses, and does not have an immunosuppressive effect in disease models associated with PAD dysregulation.[14–18]

In this study, it has been aimed to determine the potential cytotoxic and apoptotic activity of Cl-amidine which is a PAD inhibitor and whose effectiveness has been shown in vitro and in vivo studies recently on human glioblastoma cell line Uppsala 87 malignant glioma (U-87 MG) forming an in vitro model for the GBM which is the most aggressive and has the highest mortality among the brain tumors.[1,2,19]

2. Materials and methods

2.1. Cell culture experiments

U-87 MG human glioblastoma cells (ATCC code: HTB-14) and human foreskin fibroblasts (BJ cells) (ATCC code: CRL2522) were grown in a special culture medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, Eagle Minimum Essential Medium at 37°C in 5% CO_2 incubator. Before applying the methods, cells were counted with a cell counting device (Cedex) and studies were carried out using appropriate numbers of cells for the experiments.

2.2. Determination of antiproliferative and cytotoxic effects by 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate method

4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) is a tetrazolium salt used to measure cell proliferation, cell viability, and cytotoxicity in mammalian cells. The experimental protocol is based on the conversion of the WST-1 tetrazolium salt to the formazan salt by cellular mitochondrial dehydrogenases. As the number of living cells increases, the activity of mitochondrial dehydrogenases and the resulting amount of formazan salt increases. It is faster and more sensitive than methods such as MTT and XTT because it does not include washing and solubilization steps other than incubation.[20–22]

After U-87 MG and BJ cells proliferated sufficiently, cell counts were made and they were seeded in 96-well plates with 3×10^3 cells in each well and incubated for 24 hours. Different concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.97 µM) were prepared freshly from the stock solution of Cl-amidine, carmustine, and cyclophosphamide by making the necessary dilutions in the cell culture medium and were applied to U-87 MG and BJ cells. The plates were then incubated for 24 and 48 hours. At the end of the incubation periods, WST-1 kit procedure instructions were applied to the cells in each 96-well, and the absorbance values of the plates were read in Cytation 3 Cell Imaging Multi-Mode Reader at a wavelength of 540 nm, 7 repetitions for each concentration. Dimethyl sulfoxide was used as the control group. The experiments were replicated for 3 times independently. The average absorbance values of the control wells were accepted as 100% and % viability values of other concentrations were calculated.[23]

2.3. Determination of apoptotic effect by Annexin V and Propidium iodide staining method in flow cytometry

Annexin V Apoptosis Detection Kit (Catalog no: 556547, BD) protocol was applied for this method. Cells were incubated by applying Cl-amidine (half maximal inhibitory concentration [IC_{50}]: 150 µM) and carmustine (IC_{50}: 45 µM) for 48 hours. U-87 MG cells were collected by detaching and centrifuged at 1200 RPM for 5 minutes and the supernatant was removed. The cell pellet was washed 2 times with 2 mL of cold phosphate buffer saline (PBS). After the last wash, the remaining pellet was resuspended with 100 µL PBS and transferred to the tube, and 5 µL Annexin V and 5 µL PI were added. After 20 minutes of incubation at room temperature and in the dark, 250 µL of "Annexin V binding buffer" was added to the tubes and apoptotic readings of the groups were performed on the flow cytometry device (Accuri C6, BD Bioscience, New Jersey, ABD) within 30 minutes.[24]

2.4. Determination of mitochondrial membrane integrity of cells in flow cytometry

Changes in the permeability of the mitochondrial membrane and loss of membrane potential are among the important changes that involve mitochondrial dysfunction in apoptosis.[13] 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethyl benzimidazolyl carbocyanine iodide (JC-1), a cationic voltage-dependent dye, is used to determine mitochondrial membrane potential. JC-1 indicates the polarization state of the mitochondrial membrane potential and mediates the measurement of mitochondrial membrane potential in cells.[25] Loss of mitochondrial membrane potential is an important indicator of apoptosis.[26]

Flow cytometry mitochondrial membrane potential determination kit (Catalog no: 551302, BD) was used to determine the mitochondrial membrane integrity of the cells. U-87 MG cells were incubated for 48 hours by applying Cl-amidine (IC_{50}: 150 µM) and carmustine (IC_{50}: 45 µM). Then, it was centrifuged at 1200 rpm for 5 minutes and the supernatant was removed. The working solution obtained by mixing the components in the kit appropriately was added to each tube and incubated in the incubator at 37°C for 15 minutes. At the end of the incubation period, the cells were washed with 1000 µL of assay solution included in the kit. After washing, the cells were suspended with 250 µL assay solution and analyzed by reading on a flow cytometry device (Accuri C6, BD).[27]

2.5. Determination of apoptotic effect by caspase-3 activation in flow cytometry

Caspase-3 can be activated by caspases involved in the apoptosis mechanism and through both extrinsic (apoptotic) and intrinsic (mitochondrial) apoptotic pathways. Therefore, determining caspase-3 enzyme activity in a cell is one of the most important indicators of the presence of apoptosis in that cell.[27]

Phycoerythin Active caspase-3 Apoptosis Kit (Catalog no: 550914, BD) was used to determine caspase-3 activation. U-87 MG cells were seeded in 6-well plates at 1×10^5 cells/well. The kit protocol was applied after the cells were incubated with IC_{50} concentrations of Cl-amidine and carmustine (Cl-amidine: 150 µM and carmustine: 45 µM) for 48 hours. Briefly, the cells were washed with PBS, 500 µL cytofix/cytoperm solution was added to the cell pellets to ensure fixation and permeability of the cells, and the cells were kept in ice for 20 minutes. Then, 10 µL antibody (Phycoerythin Rabbit anti caspase-3) was added and incubated at room temperature for 30 minutes. After the final washes, the samples were transferred to the flow tube and analyzed on the flow cytometry device.

2.6. Statistical analysis

Graph Pad Prism 6 program was used for statistical evaluations and the obtained data were analyzed by 1-way analysis of variance and post hoc Tukey test. Significance values: $P > .05$ was considered as no difference, $^*P < .05$ as a difference, $^{**}P < .01$, and $^{***}P < .001$ as a statistically significant.

3. Results

3.1. Evaluation of antiproliferative and cytotoxicity effects with the WST-1 method

After U-87 MG and BJ cells were cultured in the medium, the cells were seeded in 96-well plates and the effect of Cl-amidine, carmustine, and cyclophosphamide concentrations (0.97, 1.95, 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 µM) on cell viability in 24- and 48-hour of incubation was investigated via the WST-1 method. The results are shown in Figure 1. Additionally, IC_{50} values calculated in the excel program according to percentage proliferation values are shown in Table 1.

It was determined that Cl-amidine, carmustine, and cyclophosphamide decreased cell viability in both 24- and 48-hour incubation periods due to the increase in concentration in U-87 MG cells. Especially, the antiproliferative effects of Cl-amidine and carmustine were found to be statistically more significant compared to cyclophosphamide in U-87 MG control group. Statistical significance value was $^{**}P < .01$ at 0.97 µM Cl-amidine concentration and $^{***}P < .001$ at 1.95, 3.9, 7.81, 15.62, 31.25, 62.5, 125, 250, and 500 µM Cl-amidine concentrations at the

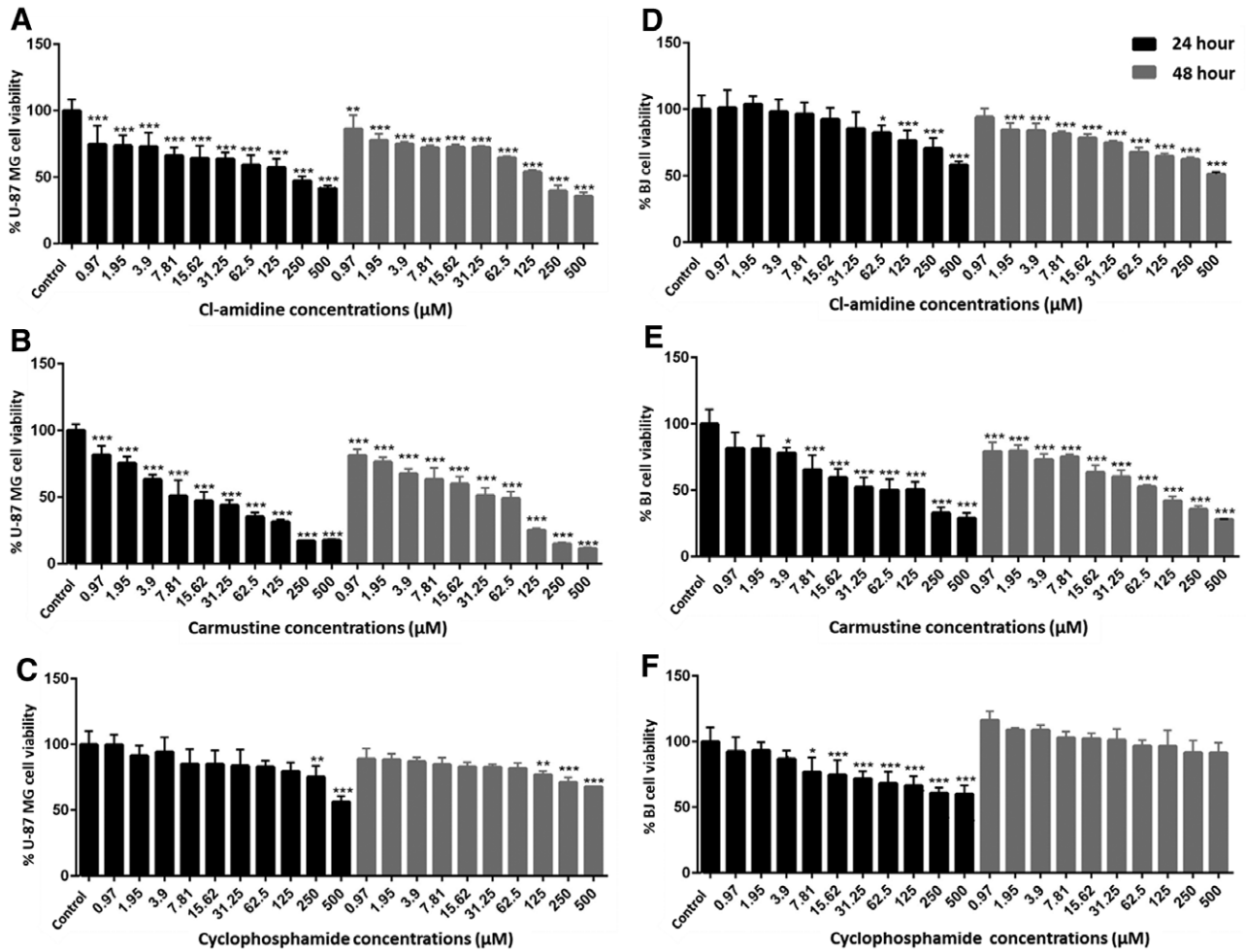

Figure 1. Viability (%) values and statistical evaluation of Cl-amidine, carmustine, and cyclophosphamide concentrations in U-87 MG and BJ cells at the 24th and 48th hours calculated according to the WST-1 method (control: 0.1% DMSO, mean ± standard deviation, n = 7, $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$). BJ = human healthy fibroblast cells, Cl-amidine = chlorine-amidine, DMSO = dimethyl sulfoxide, U-87 MG = human glioblastoma cells, WST-1 = 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

48th hour (Fig. 1A). Statistical significance value in carmustine concentrations was determined as ***$P < .001$ in all concentrations compared to the control group at the 24th and 48th hours (Fig. 1B). Statistical significance value for cyclophosphamide concentrations was determined as **$P < .01$ at 125 µM concentration and ***$P < .001$ at 250 and 500 µM concentrations at the 48th hour (Fig. 1C).

When the cytotoxic effects determined by the WST-1 method on BJ human healthy fibroblast cells were evaluated, although it was found that Cl-amidine and carmustine reduced cell viability in both 24- and 48-hour incubations, Cl-amidine was less anti-inflammatory in BJ cells, especially at high concentrations, compared to carmustine, which is used in the current treatment of glioma. Cyclophosphamide, which we used as a positive control, did not have a cytotoxic effect on BJ cells, especially at the 48th hour. Statistical significance value in Cl-amidine concentrations was observed as ***$P < .001$ in all Cl-amidine concentrations compared to the control group at the 48th hour (Fig. 1D). Similarly, the statistical significance value for carmustine concentrations was detected as ***$P < .001$ for all concentrations at the 48th hour (Fig. 1E). No statistical significance was found in at any concentration of cyclophosphamide compared to the control group at the 48th hour (Fig. 1F).

The IC_{50} values of Cl-amidine, carmustine, and cyclophosphamide in U-87 MG glioma cells, calculated according to WST-1 proliferation values, were determined as 256.09, 40.60, and 581.02 µM at the 24th hour and 150.40, 45.68, and 580.40 µM at the 48th hour, respectively. In BJ cells, IC_{50} values were detected as 604.27, 144.19, and 650.03 µM at the 24th hour and 549.20, 81.36, and 3972.062 µM at the 48th hour, respectively. According to these results, although the IC_{50} values of Cl-amidine were determined to be higher than the carmustine agent used in treatment, it is observed that they are lower than cyclophosphamide, which is used as a chemotherapy drug. Especially when the IC_{50} results in BJ human healthy fibroblast cells are compared, the fact that Cl-amidine has higher IC_{50} values than carmustine supports the conclusion that it has less cytotoxic effect on healthy cells (Table 1).

3.2. Evaluation of apoptotic effect by Annexin V and Propidium iodide staining method in flow cytometry

The 48th hour IC_{50} values of Cl-amidine and carmustine determined according to the results of the WST-1 method (Cl-amidine IC_{50}: 150.40 µM and carmustine IC_{50}: 45.68 µM) were applied to U-87 MG cells. The plates were then incubated for 48 hours. At the end of the incubation periods, U-87 MG cells from each group were detached and the kit protocol was applied. Then, the samples were analyzed by measuring on a flow cytometry device (Accuri C6, BD). The results are shown in Figure 2 and Table 2.

When the apoptosis results were evaluated, it was determined that both the early and late apoptotic effects of Cl-amidine and carmustine on U-87 MG cells at 48th hour increased compared to the control group at IC_{50} concentrations. Especially late apoptotic effects increased 2.5 times in the Cl-amidine group and approximately 3 times in the carmustine group compared to U-87 MG control group. No significant difference could be detected in necrotic cell death compared to the control.

3.3. Assessment of mitochondrial membrane integrity of cells in flow cytometry

The 48th hour IC_{50} values of Cl-amidine and carmustine (Cl-amidine IC_{50}: 150.40 µM and carmustine IC_{50}: 45.68 µM) were applied to U-87 MG cells. The plates were then incubated for 48 hours. At the end of the incubation periods, U-87 MG cells from each group were detached and JC-1 kit protocol was applied. Then, the samples were analyzed by measuring on a flow cytometry device (Accuri C6, BD). The results are shown in Figure 3 and Table 3.

Table 1

IC_{50} values according to WST-1 results at the end of 24 and 48 h of incubation in U-87 MG and BJ cells.

	U-87 MG			BJ		
	Cl-amidine	Carmustine	Cyclophosphamide	Cl-amidine	Carmustine	Cyclophosphamide
24 h (µM)	256.09	40.60	581.02	604.27	144.19	650.03
48 h (µM)	150.40	45.68	580.40	549.20	81.36	3972.062

BJ = human healthy fibroblast cells, Cl-amidine = chlorine-amidine, IC_{50} = half maximal inhibitory concentration, U-87 MG = human glioblastoma cells, WST-1 = 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

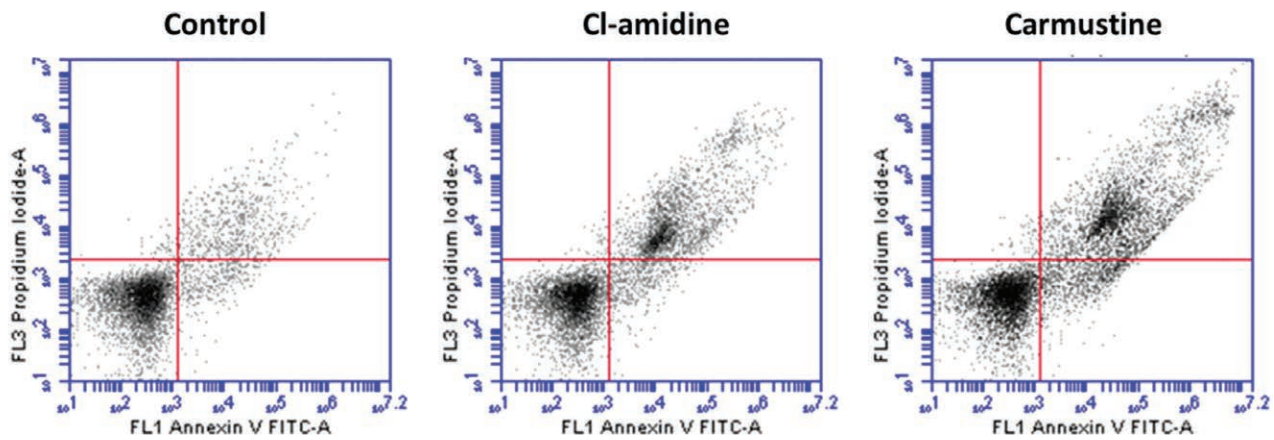

Figure 2. Apoptotic effect of Cl-amidine and carmustine IC_{50} concentrations on U-87 MG cells at the 48th hour. Cl-amidine = chlorine-amidine, IC_{50} = half maximal inhibitory concentration, U-87 MG = human glioblastoma cells.

When JC-1-positive cell results were evaluated, mitochondrial depolarization levels at the IC_{50} concentrations of Cl-amidine and carmustine on U-87 MG cell at the 48th hour were determined as 54.7% and 58.6%, respectively, and increased approximately 4.5 and 4.7 times, respectively, compared to the control. These findings support our late apoptotic cell results determined in Annexin-PI method.

3.4. Evaluation of apoptotic effect by caspase-3 levels in flow cytometry

The 48th hour IC_{50} values of Cl-amidine and carmustine (Cl-amidine IC_{50}: 150.40 µM and carmustine IC_{50}: 45.68 µM) were applied to U-87 MG cells. The plates were then incubated for 48 hours. At the end of the incubation periods, U-87 MG cells from each group were detached and the caspase-3 kit protocol was applied. Then, the samples were analyzed by measuring on a flow cytometry device (Accuri C6, BD). The results are shown in Figure 4 and Table 4.

Caspases are important mediators of both apoptotic and necrotic cell death, and many caspase types such as caspase-1, caspase-3, caspase-7, caspase-8, and caspase-9 can be effective in the apoptotic pathway. According to our results, the IC_{50} concentrations of Cl-amidine and carmustine on U-87 MG cells did not show any difference in caspase-3 levels at the 48th hour compared to the control. This result shows us that the apoptotic effects of Cl-amidine and carmustine may be mediated by other caspase types.

4. Discussion

PADs are enzymes that convert arginine to citrulline.[5] It has been observed that this enzyme activation is very high in many types of cancer and various autoimmune diseases.[6] Among PAD types, especially PAD4 isozyme has been associated with cancer. Chang et al[6] reported that PAD4 was absent in benign tumors and nontumor tissues, except for some acutely inflamed tissues, but showed high expression in cancerous tissues. It was also observed that PAD4 levels in the blood were higher in patients with malignant tumors than in patients with chronic inflammation and benign tumors.[6,28] PAD4, which has been identified as an oncogene in various studies,[29] has also been found at high levels in brain astrocytomas.[8]

Cl-amidine is a PAD inhibitor substance that inhibits PAD types at different rates (PAD1 > PAD4 > PAD3 > PAD2).[30–34] In animal experimental models of diseases with high PAD values, such as rheumatoid arthritis,[10] multiple sclerosis,[11] and ulcerative colitis,[13] it has been shown that PAD inhibitor agents suppress PAD activity and reduce the severity of developing clinical pictures. In in vitro studies, it has been presented that Cl-amidine has antitumorigenic effect in cancer cells such as HCT 116 (human, colon carcinoma),[17] MCF-7 (human, breast adenocarcinoma),[35] U20S (human, bone osteosarcoma),[36] HL60 (human, acute promyelocyte leukemia), HT-29 (human colorectal adenocarcinoma),[37] and PC3 (prostate cancer).[38] Antiproliferative activities have also been reported on various cancer cell lines.[6,8,10–13]

In our study, in which we investigated its effects on U-87 MG glioma cells in vitro, it was determined that Cl-amidine had significant antiproliferative and apoptotic activity compared to the control group. This activity reached its highest value at the 48th hour (% viability value at the highest concentration, 41.16 at 24 hours; 35.64 at 48 hours). It was, on average, half as effective as carmustine, a chemotherapeutic agent used in current treatment (% survival value at the highest concentration, 17.70 at 24 hours; 11.59 at 48 hours). The % viability values of cyclophosphamide, which is used in many types of cancer but is not

Table 2

Flow cytometry Annexin V-PI apoptosis results of IC_{50} concentrations of Cl-amidine and carmustine on U-87 MG cell line at the 48th hour (control: 0.1% DMSO, n = 3, mean ± standard deviation).

Groups	Viable cells % (lower left quadrant)	Necrotic cells % (upper left quadrant)	Early apoptotic cells % (upper right quadrant)	Later apoptotic cells % (lower right quadrant)
Control	84.2 ± 2.61	1.4 ± 1.13	4.1 ± 1.22	10.3 ± 2.77
Cl-amidine	68.8 ± 9.41	1.0 ± 0.55	4.4 ± 1.19	25.8 ± 10.41
Carmustine	57.6 ± 6.96	1.7 ± 1.43	9.2 ± 1.15	31.5 ± 4.72

Cl-amidine = chlorine-amidine, DMSO = dimethyl sulfoxide, IC_{50} = half maximal inhibitory concentration, U-87 MG = human glioblastoma cells, V-PI = Annexin V and Propidium iodide staining.

Table 3

Mitochondrial depolarization (JC-1) levels in flow cytometry of IC_{50} concentrations of Cl-amidine and carmustine in U-87 MG cells (control: 0.1% DMSO, n = 3, mean ± standard deviation).

Groups	Viable cells % (upper quadrant)	JC-1-positive cells % (lower quadrant)
Control	87.7 ± 2.0	12.3 ± 2.0
Cl-amidine	45.3 ± 11.51	54.7 ± 11.60
Carmustine	41.4 ± 3.80	58.6 ± 3.80

Cl-amidine = chlorine-amidine, DMSO = dimethyl sulfoxide, IC_{50} = half maximal inhibitory concentration, JC-1 = 5,5', 6,6'-tetrachloro-1,1', 3,3' tetraethyl benzimidazolyl carbocyanine iodide, U-87 MG = human glioblastoma cells.

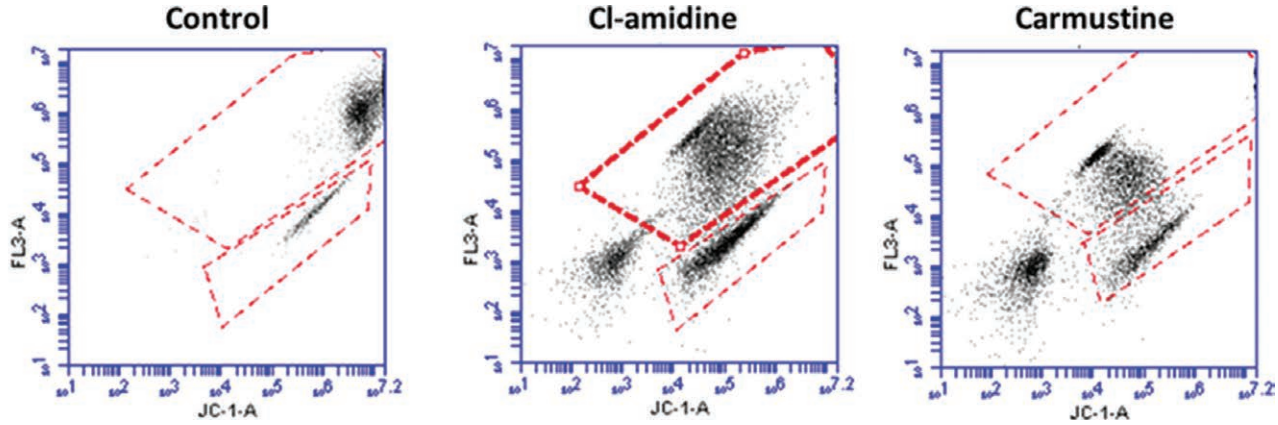

Figure 3. Determination of the effects of Cl-amidine (IC_{50}: 150.40 µM) and carmustine (IC_{50}: 45.68 µM) on mitochondrial membrane integrity in U-87 MG cells by flow cytometry. Cl-amidine = chlorine-amidine, IC_{50} = half maximal inhibitory concentration, U-87 MG = human glioblastoma cells.

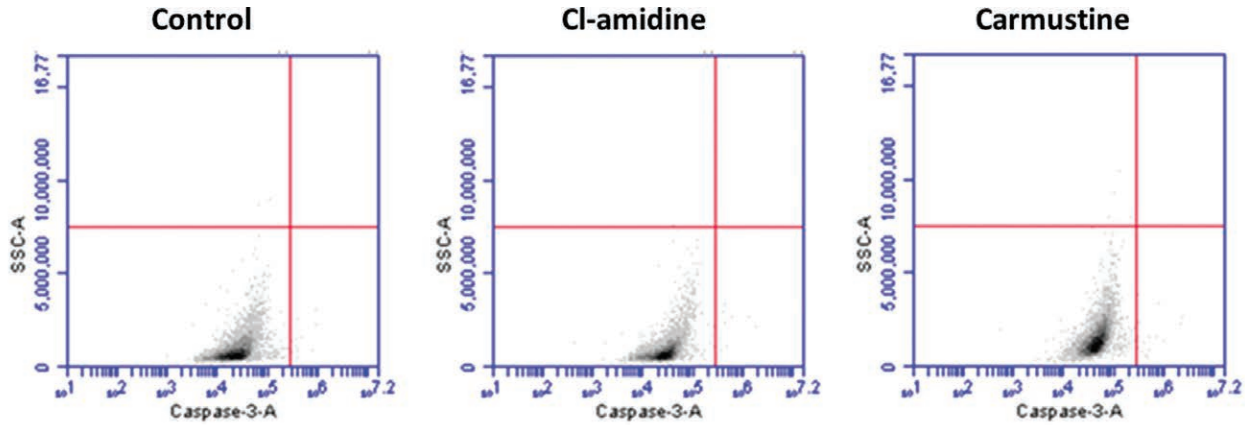

Figure 4. Determination of caspase-3 levels of Cl-amidine (IC_{50}: 150.40 μM) and carmustine (IC_{50}: 45.68 μM) in U-87 MG cells by flow cytometry. Cl-amidine = chlorine-amidine, IC_{50} = half maximal inhibitory concentration, U-87 MG = human glioblastoma cells.

Table 4

Caspase-3 levels of Cl-amidine and carmustine in U-87 MG cells (control: 0.1% DMSO, n = 3, mean ± standard deviation).

Groups	Viable cells % (lower left quadrant)	Caspase-3-positive cells % (lower right quadrant)
Control	99.4 ± 0.26	0.6 ± 0.3
Cl-amidine	97.2 ± 0.85	2.8 ± 0.9
Carmustine	96.2 ± 0.90	3.8 ± 1.0

Cl-amidine = chlorine-amidine, DMSO = dimethyl sulfoxide, U-87 MG = human glioblastoma cells.

preferred in glioma, were 56.30 in 24 hours and 67.80 at 48 hours. However, the available Cl-amidine and carmustine values were found to be very close at very low doses (0.97 and 1.95 μM). In current practices, while low percent viability values are desired for the cancer cell line, a low cytotoxic effect on healthy cells is desired. Therefore, when the IC_{50} results in BJ human healthy fibroblast cells are compared, the fact that Cl-amidine has higher IC_{50} values than carmustine shows that it has less cytotoxic effect on healthy cells, and this finding is among the important results of our study.

Phosphatidylserine (PS) is found on the cytoplasmic surface of the cell membrane of normal cells. During apoptosis, PS molecules are transported to the outer surface of the cell membrane. This change occurs in the early stages of apoptotic cells. Annexin V is used as a protein that can bind to PS on the outer surface. When labeled with fluorescent substances such as fluorescein isothiocyanate, apoptotic cells become apparent.[39–43] Propidium iodide, on the other hand, is used to detect late apoptotic and necrotic cells with damaged membrane structure, as it binds to DNA or double-stranded RNA.[44] According to our apoptotic effect results, it was determined that the late apoptotic effects of both Cl-amidine and carmustine increased compared to the control group. Some studies have shown that mitochondrial permeability is important evidence for the processes leading to apoptosis.[45] In our results, it was determined that Cl-amidine and carmustine increased mitochondrial depolarization levels in U-87 MG cells compared to the control, and our late apoptotic cell results were supported by these data. Caspases are proteases that play a role in both the death receptor pathway and mitochondrial apoptosis.[46] Caspase-3 is one of the caspases that are involved in the completion phase of the apoptosis mechanism and are responsible for the termination of apoptosis. Caspase-3 can be activated both through the extrinsic apoptotic pathway and the intrinsic (mitochondrial) apoptotic pathway.[47] According to our results, caspase-3 levels of Cl-amidine and carmustine on U-87 MG cell did not differ compared to the control. This result shows us that the apoptotic effects of Cl-amidine and carmustine may be mediated by other caspase types.

This study has limitations that should be considered when interpreting the results. First, the exclusive use of the U-87 MG cell line in the in vitro model may not fully capture the heterogeneity of glioblastoma tumors found in patients. Additionally, the relatively short exposure durations (24 and 48 hours) may not fully capture the sustained effects or potential development of resistance to Cl-amidine over an extended period. Furthermore, it is advisable for future research to explore the impact of Cl-amidine on particular targets implicated in the advancement of glioblastoma, using a more detailed molecular approach. Finally, the therapeutic significance of the study is limited due to its concentration on cellular and molecular aspects. Additional investigation, encompassing preclinical and clinical trials, is necessary to determine the practical effectiveness of Cl-amidine in treating glioblastoma.

This research shows that Cl-amidine may have a significant anticarcinogenic potential in GBM chemotherapy, as it has a significant antiproliferative and apoptotic effect on U-87 MG cells, as well as having a less cytotoxic effect on healthy cells than the current treatment agent, carmustine. We think that the antiproliferative effects of Cl-amidine should be also investigated in different cancer cell types. We also believe that Cl-amidine will contribute to the discovery of new anticancer drugs by studying it with single or combined agents.

Author contributions

Conceptualization: Pınar Naile Öğüten.
Formal analysis: Pınar Naile Öğüten, Selin Engür Öztürk.
Methodology: Pınar Naile Öğüten, Miriş Dikmen.
Project administration: Pınar Naile Öğüten.
Resources: Pınar Naile Öğüten.
Writing – original draft: Pınar Naile Öğüten.
Writing – review & editing: Pınar Naile Öğüten, Miriş Dikmen.
Data curation: Selin Engür Öztürk.
Investigation: Selin Engür Öztürk.
Software: Selin Engür Öztürk.
Validation: Miriş Dikmen.
Visualization: Miriş Dikmen.

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