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Anticarcinogenic activity of Cl-Amidine on non-small cell lung cancer

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Abstract

Peptidyl arginine deiminases (PADs) are enzymes that convert arginine to citrulline. They play a role in embryogenesis and cell signal transduction activities. However, it has been determined that excess or dysregulated PAD levels increase in many diseases and may be associated with diseases. Cl-amidine, used as a PAD inhibitor, has been shown to suppress PAD activity and reduce the severity of developing clinical pictures in animal experimental models such as rheumatoid arthritis and ulcerative colitis. Anti-proliferative activities have also been reported due to their cytotoxic effects on various cancer cell lines. In our study, the anti-carcinogenic activity of Cl-amidine was investigated in A549 human non-small cell lung cancer cells. The anti-proliferative effects of Cl-amide on A549 cells were determined by the WST-1 method. Apoptotic effects were analyzed on the flow cytometry device with Annexin V-PI, caspase-3 activation and mitochondrial membrane depolarization (JC-1) methods. It was determined by the WST-1 method that Cl-amidine had a significant anti-proliferative effect on A549, depending on time and concentration. The apoptotic effects analyzed by Annexin V-PI and JC-1 methods were found to be especially significant at the 48th hour. It has been determined that Cl-amidine, in particular, causes cells to undergo apoptosis by increasing mitochondrial depolarization. In addition, it was observed that Cl-amidine had a less cytotoxic effect on CCD-19Lu healthy lung cells than cisplatin, one of the existing treatment agents. This study shows that Cl-amidine has the potential to be an important chemotherapy agent in the lung cancer treatment, as it has a significant anti-proliferative and apoptotic effect on A549 cells, as well as being less cytotoxic when compared to existing cisplatin treatment on healthy lung cells. We believe that Cl-amidine will contribute to the discovery of new anti-cancer drugs by investigating it with single or combined agents.

Keywords: peptidyl arginine deiminase inhibitor, Cl-amidine, lung cancer, A549 cell line

1. Introduction

Lung cancer is the most dangerous type of cancer in terms of survival time and prognosis and is responsible for 34% of cancer deaths in men and 22% in women. Non-small cell lung cancer accounts for 85% of lung cancers. Lung adenocarcinomas, considered in the non-small cell lung cancer group, constitute 60% of all lung cancers. A549 human lung adenocarcinoma cell line was used to investigate this type of lung cancer at the *in vitro* level (1).

Peptidyl arginine deiminases (PADs) are enzymes that convert arginine to citrulline (2). It has been observed that the activation of this enzyme is very high in many types of cancer and various autoimmune diseases (3-5). As a PAD inhibitor substance, Cl-amidine has been shown to suppress PAD activity and reduce the severity of developing clinical pictures in animal experimental models such as rheumatoid arthritis and ulcerative colitis. Anti-proliferative activities have also been reported on various cancer cell lines (3,5,6-9).

This study aimed to determine the anti-proliferative and apoptotic effects of Cl-amidine on A549 human non-small cell lung cancer cell. The cytotoxic effects of Cl-amidine on A549 cells were determined by the WST-1 method, and the apoptotic effects of it were determined by the Annexin V-PI, Caspase-3 and JC-1 methods. Additionally, cisplatin, an antineoplastic agent, was used as a positive control in the study. Also, the effect of Cl-amidine on healthy human lung fibroblast cell line was evaluated.

2. Materials and Methods

2.1. Proliferation of cells in culture medium

A549 human non-small cell lung cancer cells (ATCC code: CCL-185™) were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and CCD-19Lu human healthy lung fibroblast cells (ATCC code: CCL-210™) were grown in Eagle's Minimum Essential Medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, in a culture medium at 37°C in 5% CO_2 incubator. Proliferating cells were passaged into flasks and used for experimental studies, and some of them were stocked for use in future experiments. Before applying the methods, the cells were removed with trypsin, stained with trypan blue solution, counted with a cell counting device (Cedex), and studies were carried out using appropriate numbers of cells for the experiments.

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2.2. Determination of cytotoxicity by WST-1 method

WST-1(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) is a tetrazolium salt and binds specifically to the succinate-dehydrogenase enzyme in the mitochondria of living cells and turns into water-insoluble formazan salts. This conversion depends on the glycolytic production of nicotinamide adenine dinucleotide phosphate in living cells. Therefore, the amount of formazan formed directly indicates metabolically active cells (living cells) in culture. The absorbance value measured spectrophotometrically in the WST-1 method shows the metabolic activities of the cells in culture, and this value is related to the number of living cells. As proliferation increases, the absorbance value also increases due to formazan salt formation (10,11).

After A549 and CCD-19Lu cells proliferated sufficiently, cell counts were made and they were seeded in 96-well plates with 3,000 cells in each well. All cells were incubated for 24 hours to allow them to adhere. Different concentrations (500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.97 µM) were freshly prepared by making the necessary dilutions in cell culture medium from the stock solution of Cl-amidine and cisplatin prepared with DMSO (Dimethylsulfoxide) and applied to A549 and CCD-19Lu cells. DMSO was also used as a control group. The plates were then incubated for 24 and 48 hours. At the end of the incubation period, 10 µL of WST-1 reagent was added to the cells in each 96-well according to the WST-1 kit procedure instructions, the cells were incubated in the incubator for 3 hours, and at the end of the incubation, the absorbances were read on the ELISA device at a wavelength of 420 nm. The experiments were replicated for three times independently. The absorbance values obtained according to colour formation were directly related to cell viability, and the anti-proliferative effect was evaluated by determining the % viability values and the IC_{50} value was calculated.

2.3. Determination of apoptotic effect by Annexin V-PI method in flow cytometry

This method is based on the principle that phosphatidylserine, which is found on the inside of healthy cell membranes, is released to the outside of the cell by the breakdown of the cell membrane in apoptotic cells (12). Since annexin V is a protein that can bind to phosphatidylserine that translocates to the outer surface of the cell, the apoptotic cell can be made visible by labeling it with a fluorescent substance (e.g. fluorescein isothiocyanate (FITC)) (13-16). Propidium iodide (PI), 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 (17). For this method, the Annexin V FITC Apoptosis Detection Kit (Catalog no: 556547, BD) protocol was applied. After waiting for A549 cells to proliferate sufficiently, cell counts were made and the cells were seeded in 6-well plates with $1x10^5$ cells in each well. The cells were incubated for 24 hours to allow them to adhere. Then, the cells were incubated with the 48-hour IC_{50} concentration of Cl-amidine for 48 hours. At the end of the incubation period, the cells in each 6-well were removed and the supernatant was removed by centrifugation at 1200 rpm for 5 minutes. The cell pellet was washed 2 times with 2 mL of cold PBS. After the last wash, the remaining pellet was resuspended with 100 µL PBS and transferred to the flow tube, and 5 µl Annexin V and 3 µl PI were added. The tubes were incubated for 20 minutes at room temperature and in the dark, and at the end of the incubation, 250 µl of "Annexin V binding buffer" was added and read on the flow cytometry device within 30 minutes.

2.4. Determination of mitochondrial membrane integrity of cells by JC-1 staining method in flow cytometry

Some studies have shown that mitochondrial permeability transition pore opening is important evidence for the processes leading to apoptosis (18). Two important factors have been revealed in apoptosis, including mitochondrial dysfunction. One of these factors is the change in the permeability of the mitochondrial membrane and the subsequent loss of membrane potential, and the second is the release of apoptotic proteins such as AIF (Apoptosis inducing factor) and cytochrome c from the inner membrane space of the mitochondria into the cytosol (19). JC-1 (5,5', 6,6'-tetrachlo-1,1', 3,3' tetraethylbenzimidazolylcarbocyanine iodide), a cationic voltage-dependent dye, is used to determine mitochondrial membrane potential. This is a dye that shows the polarization state of the mitochondrial membrane potential (20). Loss of mitochondrial membrane potential is an indicator of apoptosis. JC-1 measures mitochondrial membrane potential in cells. In healthy cells, JC-1 stains mitochondria red. In apoptotic cells, the mitochondrial membrane potential collapses. JC-1 cannot accumulate inside mitochondria. In these cells, JC-1 remains in its green fluorescent monomeric structure in the cytoplasm (21).

Flow cytometry mitochondrial membrane potential determination kit (Catalog no: 551302, BD) was used to determine the mitochondrial membrane integrity of the cells. A549 cells were seeded in 6-well plates at $1x10^5$ cells/well. Cells were incubated with the 48-hour IC_{50} concentration of Cl-amidine for 48 hours. At the end of the incubation period, the cells in each 6-well were removed and 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 on the flow cytometry device.

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

Caspases are proteases that play a role in both the death receptor pathway and mitochondrial apoptosis (22). Caspase-3 is one of the (executioner) 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. Caspase-3 is responsible for chromatin condensation, DNA fragmentation and degradation of cytoskeletal proteins. For this reason, determining caspase-3 enzyme activity in a cell is one of the most important evidence that that cell is undergoing apoptosis (23).

PE Active Caspase-3 Apoptosis Kit (Catalog Nn: 550914, BD) was used to determine caspase-3 activation. A549 cells were seeded in 6-well plates at $1x10^5$ cells/well. Cells were incubated with the IC_{50} (336 µM) concentration of Cl-amidine at 48 hours for 48 hours. At the end of the incubation period, the cells in each 6-well were dissociated and centrifuged at 1200 rpm for 5 minutes and the supernatant was removed. The cell pellet was washed 2 times with cold PBS. After the last wash, the supernatant on the tubes was poured and 500 µL of cytofix/cytoperm solution was added to the pellet to ensure fixation and permeability of the cells and the mixture obtained was kept in ice for 20 minutes. At the end of this period, it was centrifuged at 1200 rpm for 5 minutes and the pellet was washed 2 times with 500 µL "perm wash". After the last wash, the cell pellet was resuspended with 50 µL perm wash, 10 µL antibody (PE Rabbit Anti caspase-3) was added and incubated for 30 minutes at room temperature. At the end of the incubation period, 1000 µl perm wash was added to each tube and centrifuged. At the end of centrifugation, the pellet was transferred to the flow tube with 350 µL perm wash 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 one-way ANOVA and post-hoc Tukey test. Significance values; $p>0.05$ was considered as no difference, $p<0.05$* as there was a difference, $p<0.01$** as a significant difference, and $p<0.001$*** as a very significant difference.

3. Results

3.1. Evaluation of cytotoxicity with the WST-1 method

The % viability values of A549 and CCD-19Lu cells at 24 and 48 hours are shown in Fig. 1 as % values at Cl-amidine and cisplatin concentrations, respectively, compared to the control group. The IC_{50} values of Cl-amidine and cisplatin determined in A549 and CCD-19Lu cells are shown in Table 1.

Table 1. IC_{50} values according to WST-1 results after 24 and 48 hours of incubation in A549 and CCD-19Lu cells

	A549		CCD-19Lu	
	Cl-amidine	Cisplatin	Cl-amidine	Cisplatin
24 hour (µM)	385.58	361.50	388.74	182.44
48 hour (µM)	336.67	84.56	360.79	100.93

According to the results of the WST-1 method, it was statistically determined that Cl-amidine and cisplatin reduced cell viability in A549 lung cancer cells after both 24 and 48 hours of incubation periods, and the most antiproliferative effect was observed at 500 µM Cl-amidine concentration (**Fig. 1A**). The effects of cisplatin were found to be statistically similar to Cl-amidine in A549 cells, and the highest anti-proliferative effects were determined at 125, 250 and 500 µM cisplatin concentrations after 48 hours of incubation periods (**Fig. 1B**).

In the results of CCD19-Lu healthy lung cells, the anti-proliferative effects of Cl-amidine (**Fig. 1C**) were found to be less statistically significant than cisplatin (**Fig. 1D**), especially in the 48-hour incubation. This result shows us that Cl-amidine has a less cytotoxic effect on healthy lung cells than cisplatin, a drug used in current treatment.

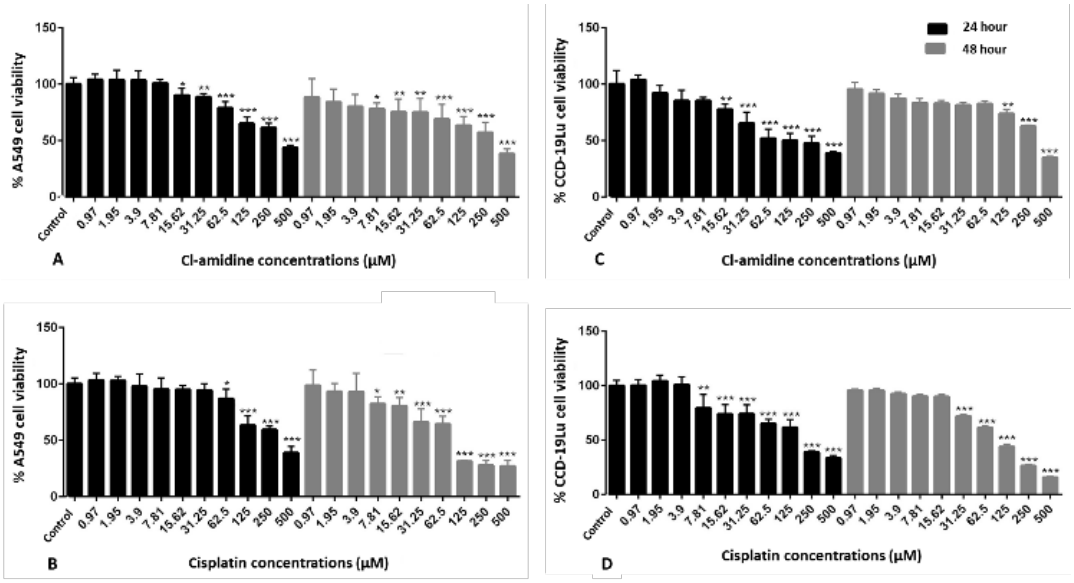

Fig. 1. Viability (%) values and statistical evaluation of Cl-amidine and cisplatin concentrations at the 24th and 48th hours calculated according to the WST-1 method in A549 and CCD-19Lu cell lines (mean ± standard deviation). (n=7); $p<0.05$*, $p<0.01$**, $p<0.001$***

According to WST-1 results, it was determined that Cl-amidine had anti-proliferative effects due to the increase in concentration in A549 cells at both 24 and 48 hours incubation periods. Moreover, it was observed based on the IC_{50} values in healthy lung cells that Cl-amidine had a less cytotoxic effect than cisplatin, one of the existing therapeutic agents used in the treatment of lung cancer.

3.2. Evaluation of apoptotic effect with Annexin V-PI method in flow cytometry

The IC50 concentration of Cl-amidine, determined by WST-1 results, was applied to A549 cells for 48 hours, and then the samples were measured and analysed on the flow cytometry device (Accuri C6, BD) by applying the kit protocol. The results are shown in Fig. 2 and Table 2.

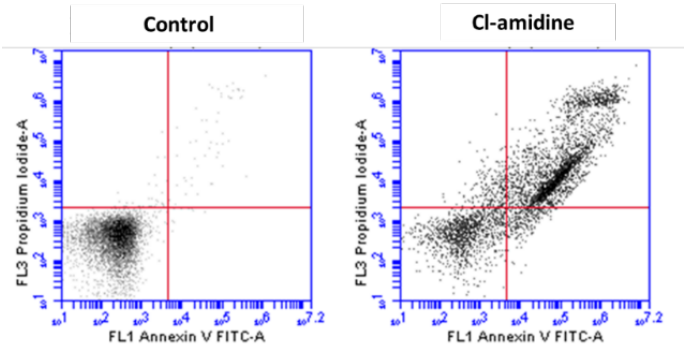

Fig. 2. Apoptotic effect of control and IC_{50} concentrations of Cl-amidine (Cl-amidine: 336 µM) on A549 cells at 48 hours

Table 2. Flow cytometry Annexin V-PI apoptosis results of control and Cl-amidine on A549 cell line at 48 hours (mean±; standard deviation, n=3)

GROUPS	Viable cells % (lower left quadrant)	Necrotic cells % (upper left quadrant)	Early apoptotic cells % (upper right quadrant)	Late apoptotic cells % (lower right quadrant)
Control	97.6±1.15	0.7±0.2	0.9±0.85	0.8±0.2
Cl-amidine	40.8±8.40	4.0±0.9	4.5±2.7	50.7±5.9

The binding rate of FITC and Annexin V to phosphatidylserine on the cell surface can be measured by flow cytometry. Since Annexin V binding can also be seen on the surfaces of necrotic cells, propidium iodide is added as a second dye. Cells stained simultaneously with Annexin V-FITC (green fluorescence) and the non-vital dye propidium iodide (red fluorescence) allows living cells ([Annexin-V - /PI -]), early apoptotic cells ([AnnexinV + /PI -]), late apoptotic cells ([Annexin-V + /PI +]) and necrotic cells ([Annexin-V- /PI +]) to be distinguished from each other (15,17). According to the results, the late apoptotic effect of Cl-amidine, especially at the 48th hour, on A549 cells at the IC_{50} concentrations increased significantly compared to the control group.

3.3. Evaluation of mitochondrial membrane integrity of cells by JC-1 staining method in flow cytometry

The IC50 concentration of Cl-amidine, determined by WST-1 results, was applied to A549 cells for 48 hours, and then the samples were measured and analysed on a flow cytometry device (Accuri C6, BD) to determine the mitochondrial depolarization of the cells by applying the kit protocol. The results are shown in Figure 3 and Table 3.

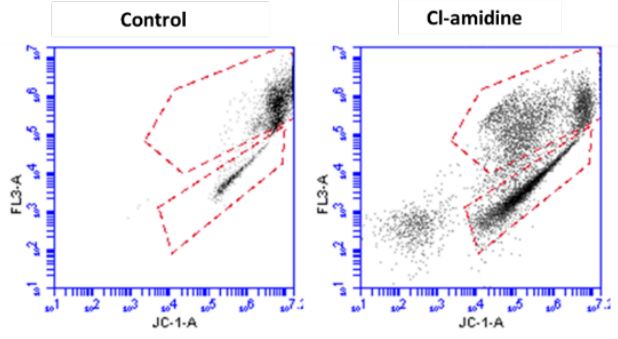

Fig 3. Effect of the IC_{50} concentrations of control and Cl-amidine (Cl-amidine: 336 µM) on the mitochondrial membrane integrity on A549 cells at the 48th hour

According to the results in Figure 3 and Table 3, the mitochondrial depolarization value at the 48th hour on A549 cell increased in the IC_{50} concentrations of Cl-amidine compared to the control.

Table 3. Mitochondrial depolarization (JC-1) results of control and Cl-amidine in flow cytometry on A549 cell line (mean ± standard deviation, n=3)

GROUPS	Viable cells % (upper quadrant)	JC-1 positive cells % (lower quadrant)
Control	85.9±4.9	14.1±5.0
Cl-amidine	54.7±3.84	45.3±3.90

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

The IC_{50} concentration of Cl-amidine, determined by WST-1 results, was applied to A549 cells for 48 hours, and then the samples were measured and analyzed on a flow cytometry device (Accuri C6, BD) to determine the caspase-3 levels of the cells by applying the kit protocol. The results are shown in Fig. 4 and Table 4.

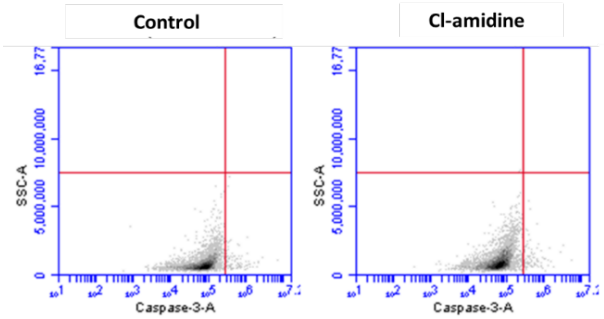

Fig. 4. Effect of control and IC50 concentrations of Cl-amidine (Cl-amidine: 336 µM) on A549 cells on caspase-3 activation at the 48th hour

Table 4. Caspase-3 activation results of control and Cl-amidine on A549 cell line at the 48th hour in flow cytometry

GROUPS	Viable cells % (upper left quadrant)	Caspase-3 positive cells % (lower right quadrant)
Control	97.7±1.38	2.3±1.4
Cl-amidine	95.7±0.56	4.3±0.6

According to the results in Fig. 4 and Table 4, an increase in the active caspase-3 values on the A549 cell at the 48th hour was determined to be 2.3 and 4.3%, respectively, in the IC_{50} concentrations of control and Cl-amidine.

4. Discussion

The incidence of cancer, one of the important health problems, is increasing day by day. According to the data of the International Cancer Research Center, lung cancer is the most frequently diagnosed and most common type of cancer that causes death worldwide. While lung cancer ranks first in terms of mortality in both genders, its incidence ranks second after breast cancer in women and prostate cancer in men (1).

PAD, the enzyme that converts arginine to citrulline (2), has been observed at high levels in diseases such as rheumatoid arthritis (5), multiple sclerosis (7), and ulcerative colitis (9). In animal experimental models of these diseases, it has been shown that the severity of disease symptoms is reduced with PAD inhibitor agents (6,7,9). It has also been observed that the activation of this enzyme is very high in many types of cancer and various autoimmune diseases (3-5). It is known that Cl-amidine inhibits all PAD (PAD1, PAD2, PAD3, PAD4 and PAD6) enzymes at different levels (24). Of these PAD types, PAD4 activity in particular has been shown to be overexpressed in many types of cancer, including esophagus, stomach, duodenum, colon, rectum, bladder, prostate, ovary, thyroid and lung adenocarcinomas and lung squamous carcinoma (5). Studies using cell lines for acute promyelocyte leukemia and colorectal adenocarcinoma (25), colon carcinoma (26), breast adenocarcinoma (27) and bone osteosarcoma (28) have shown that Cl-amidine has an anti-proliferative effect. In their study on colon cancer cells, Cui and colleagues showed that Cl-amidine activates p53, an important tumor suppressor gene, by suppressing peptidyl arginine deiminases, which in turn activates miRNA-16, which is involved in the regulation of gene expression. With miRNA-16 activation, cell division is stopped in the G1 phase by targeting cyc D, cyc E and cdk 6 (26).

In our study investigating the effectiveness of the PAD inhibitor substance Cl-amidine on A549 non-small cell lung cancer cells, it was determined that the IC_{50} values of Cl-amidine showed results close to the IC_{50} values of cisplatin, which is widely used in this lung cancer. It is also desirable that healthy cells exposed to the chemotherapeutic agent used in the cancer treatment process are not damaged as much as possible. One of the important results of our study is that Cl-amidine, a PAD inhibitor, we used showed less cytotoxic effects on CCD-19Lu healthy lung cells than the cisplatin included in the treatment. Moreover, it was determined in this study that Cl-amidine showed significant apoptotic effects on A549 cells by using different flow cytometry methods.

As a result, we think that Cl-amidine will contribute to the discovery of anti-cancer drugs with single or combined agents. We believe that the use of Cl-amidine, a PAD inhibitor, in lung cancer treatment is supported by advanced molecular studies both *in vitro* and *in vivo*.

Conflict of interest

The authors declared no conflict of interest.

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None to declare.

Authors' contributions

Concept: P.N.Ö., S.E.Ö., M.D., Design: P.N.Ö., Data Collection or Processing: P.N.Ö., S.E.Ö., M.D. Analysis or Interpretation: P.N.Ö., S.E.Ö., M.D., Literature Search: P.N.Ö., S.E.Ö., M.D., Writing: P.N.Ö, S.E.Ö.

Ethical Statement

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

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