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ORIGINAL ARTICLE

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Expression of cyclin Y after treatment with icaritin and cisplatin in the lung cancer cell lines

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ABSTRACT

Introduction: Cyclin Y has been defined as a protein that is involved in both — cell cycle control and transcription processes. This study aimed to determine the association between cyclin Y expression and events triggered by cisplatin and icaritin treatment.

Material and methods: Apoptosis and the cell cycle distribution were evaluated by flow cytometry. Protein expression was measured by conducting an immunofluorescence staining. The study revealed that icaritin induces apoptosis and cell cycle arrest in the G2/M phase in A549 and H1299 cells.
Results and conclusions: The results of the study showed that the cells of the A549 and H1299 lines were sensitive to cisplatin and icaritin. Furthermore, it was shown that cisplatin increased the percentage of cells of both lines in the G2/M phase of the cell cycle. The expression of cyclin Y increased with increasing doses of cisplatin and icaritin. The cyclin Y upregulation was correlated with cell cycle arrest, an increase in the percentage of apoptotic cells, and the inhibition of the migration potential of cells of both lines.
Keywords: cyclin Y, lung cancer, icaritin, cisplatin

Introduction

One of the main features of cancer cells is uncontrolled proliferation, which is why cancers are often called “cell cycle diseases” due to the aberrations occurring in them [1]. The cell cycle is regulated by several proteins and checkpoints whose task is to monitor the course of individual phases of the cell cycle and determine the conditional transition to its subsequent stages. The main group of cell cycle regulators include cyclins and cyclin-dependent kinases (CDKs) [2].

Deregulation of the expression of cell division proteins is currently becoming the subject of intensive research, because disturbances in their expression and regulation may contribute to the process of carcinogenesis. Abnormal expression of cyclins has been demonstrated in many types of malignant tumours, where the excessive proliferation of cancer cells, the ability to metastasize, as well as impaired cell death processes and drug resistance, were associated with their overexpression. It is therefore justified to thoroughly understand the mechanisms of action of regulatory proteins of the cell life cycle in terms of their importance in the prevention, diagnosis and treatment of cancer [3].

Cyclin Y is a highly conserved protein from the cyclin family, with a proven role in regulating the cell cycle and the transcription process [4]. It is encoded by the CCNY gene located on the short arm of chromosome 10 at position p11.21 [36]. Cyclin Y's structure differs from the structure of conventional cyclins. Most cyclins contain two cyclin cassettes, whereas Y-cyclin has only one cyclin cassette [5]. Cyclin Y was originally identified as a protein that binds to CDK14/PFTK1 in yeast, increasing its activity and altering its

intracellular localization [6]. Later studies showed that cyclin Y also binds to CDK16/PCTK1, forming an active complex capable of controlling many biological processes [7].

Other reports suggest that cyclin Y together with CDK14, affects the transduction of the Wnt pathway through phosphorylation of LRP6. It has been shown that the cyclin Y-CDK14 complex prevents the degradation of β-catenin through phosphorylation of LRP6, which enables Wnt pathway signalling. Wnt receptor phosphorylation and signalling are maximal in the G2/M phase, as is cyclin Y expression, which may suggest a link between the cell cycle and the transcription process [8].

Cyclin Y plays an important role in many cellular processes such as: maintaining the properties of mammalian stem/progenitor cells, regulating the development of Drosophilia, as well as controlling the process of adipogenesis and lipid production. It has also been shown that it participates in the regulation of the proliferation of cancer cells, including lung and kidney cancers, and reducing the level of cyclin Y expression reduces the proliferation and growth of non-small cell lung cancer and laryngeal cancer cells [5, 7, 9].

This study aimed to determine the effect of increasing doses of cisplatin and icaritin on survival, morphology, cell cycle, cell migration potential, type of induced cell death and expression of cyclin Y in A549 and H1299 cell lines.

Materials and methods

Cell culture and treatment

Human non-small cell lung cancer cell lines A549 and H1299 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured as a monolayer at 37ºC in a humidified CO_2 incubator (5% CO_2). A549 cell line was cultured in DMEM medium containing (CORNING, Mediatech, USA) with 10% fetal bovine serum (FBS; Gibco, Life Technologies Corp., Carlsbad, CA, USA) and 50 mg/mL of penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA). However, the H1299 cell line was cultured in RPMI-1640 medium containing (Lonza, Verviers, Belgium) supplemented with 10% FBS (Gibco, Life Technologies Corp., Carlsbad, CA, USA) and 50 mg/mL of penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Logarithmically growing A549 and H1299 cells were harvested and seeded on 6-, 12-, 24-, or 96-well culture plates for further studies. After overnight proliferation, the adherent cells were incubated with cisplatinum (CP) (cis-Diamineplatinum (II) dichloride; Sigma Aldrich, St. Louis, MO, USA) at final concentrations of 10 or 30 mM for 24 or 48 hours and with icaritin (ICA) (Sigma-Aldrich, St.

Louis, MO, USA) at a final concentration of 30 or 60 mM for 24 or 48 hours. While cells grown in such under the same conditions without the addition of compounds were controlled.

Analysis of cell survival in the MTT assay

Cells grown in 96-well culture plates at a density of 1×10^4 cells/well and treated for 24 and 48 hours with selected compounds by the above-mentioned method were removed from the medium and rinsed with Phosphate Buffered Saline (PBS; CORNING, Mediatech, USA). The next step was to add 100 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) working solution per well and incubate at 37ºC for 2 hours. After the allotted time had elapsed, the MTT working solution was removed. The resulting formazan crystals were dissolved in 100 µL DMSO (dimethylsulfoxide; CHEMPUR, Poland) and incubated again at 37°C for 10 minutes. The last stage was the analysis and measurement of absorbance spectrophotometrically at a wavelength of 570 nm. From the data obtained, the percentage of growth inhibition was calculated in cells in the presence of the test compound, taking the absorption of the control cell solution as 100%.

Cell cycle analysis

Cell cycle analysis of A549 and H1299 lines treated with cisplatin and icaritin was performed using a flow cytometer (Merck Guava 6HT-2L, Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in 6-well culture plates at a density of 5×10^4 cells/well in the A549 cell line and at a density of 4×10^4 cells/well in the H1299 cell line, and stained according to the method described previously [10]. Samples were placed in 200 µL per well of a 96-well plate and analysed on a Guava EasyCyte 6HT-2L flow cytometer.

Colony formation assay analysis

Cells were cultured in 6-well culture plates using the method described above. After the culture reached approximately 70% confluence, the cells were treated with cisplatin and icaritin. Untreated cells served as control. After 48 hours, the cells were detached and reseeded at 1,000 cells/well. Colony growth lasted 10 days, during which the culture medium was changed every 2 days. Then the medium was drained and 4% PFA was added for 20 minutes. After incubation, the wells were rinsed with PBS (3 × 5 minutes) and incubated in methanol for 10 minutes. The cells fixed in this way were washed PBS again (3 × 5 minutes) and flooded with crystal violet (CORNING, Mediatech) for 20–30 minutes. After the allotted

time, the cells were washed until the crystal violet was completely washed out and washed out of the wells. BIO-RAD ChemiDoc was used to evaluate the results.

Cell migration analysis

Cells were cultured in 24-well culture plates. After the culture reached approximately 70% confluence, the cells were treated with cisplatin and icaritine at the appropriate concentrations. Untreated cells served as control. After 48 hours of trypsin incubation, cells were detached. New 24-well plates with medium with 10% FBS (750 µL/well) were loaded with inserts (pore size 8 µm), seeded with previously detached cells at a density of 25–50 × 10^4 cells/insert, and medium without FBS was added. Cells were incubated for 16 hours in an incubator. After incubation, the medium was drained and the cells were fixed with 3.7% PFA (2 min). The inserts and wells were then rinsed twice with PBS. The next step was permeabilization of the cell membrane with 100% colled methanol for 20 min in a hood. The cells were again rinsed twice with PBS and crystal violet (CORNING, Mediatech) was added to the inserts and incubated for 15 minutes. The inserts were rinsed in PBS and the inserts were gently cleaned inside with cotton buds. An Eclipse E800 (Nikon) with a light microscope was used to assess cell migration using a computer image analysis system, based on the NIS-elements 4.00 program and a CCD camera (DS.-5Mc-U, Nikon).

Analysis of the type of induced cell death

In order to analyse the death process of the tested cells, a flow cytometer was used. Cells were cultured in 6-well plates at a density of 10 x 10^4 cells/well in the A549 cell line and at a density of 8 × 10^4 cells/well in the H1299 cell line for 48 hours. The cells were harvested and stained with an Elab Science Apoptosis kit following the manufacturer's protocol. The resulting samples were analysed on the Guava Easycyte cytometer by adding 200 µL of sample per well.

Analysis of the expression of cyclin Y, RRM2 protein and β-catenin using a flow cytometer

The analysis of the expression of cyclin Y, Ribonucleotide Reductase Regulatory Subunit M2 (RRM2) and β-catenin in the A549 and H1299 lines was performed using a flow cytometer. Cells were grown in 6-well plates at a density of 10 x 10^4 cells/well in the A549 cell line and at a density of 8 × 10^4 cells/well in the H1299 cell line for 48 hours. The cells were detached and centrifuged, the medium was aspirated, rinsed with PBS and centrifuged again. PFA (500 µL/1 million cells) was then added for fixation and incubated for 20–30 min.

The next step was to wash the cells with PBS. The cells were centrifuged and the supernatant fluid was withdrawn. The last stage of fixation was the addition of 1 ml of 80% methanol and leaving at –20°C for 24 hours. After 24 hours, the cells were centrifuged (650 × g, 5 min) and the supernatant was collected. 1 ml of PBS was added to the pellet to wash the cells, pipetted and centrifuged again (500 × g, 7 min). After centrifugation, the supernatant was collected, 1 ml of bovine serum albumin (BSA) was added and incubated for 20 min. After incubation, the supernatant was centrifuged and collected. The next step was the addition of a primary rabbit monoclonal IgG antibody against cyclin Y (Anti-cyclin Y; Sigma-Aldrich), RRM2 (Santa Cruz Biotechnology) or β-catenin (Invitrogen) at a dilution of 1:200 in the presence of BSA and incubating again for 30 minutes. The cells were then washed with PBS, centrifuged and the supernatant collected. A secondary anti-rabbit IgG antibody conjugated with Alexa Fluor 647 (Invitrogen, Thermo Fisher) was added at a dilution of 1:200 for Cyclin Y, and an anti-mouse IgG antibody conjugated with Alexa Fluor 488 (Invitrogen, Thermo Fisher) was added for RRM2 and β-catenin and incubated in the dark for 30 minutes. Cells were washed with PBS, centrifuged, and suspended in 200 µL of PBS. A flow cytometer was used to evaluate the results adding 200 µL of sample per well to the plate.

Cyclin Y expression analysis using fluorescence microscopy

Cells were stained using the standard protocol described in a previous study [10]. Briefly, the cells were fixed with 4% paraformaldehyde blocked with 4% BSA and stained with appropriate primary and secondary antibodies. F-actin was stained using Alexa Fluor 488 phalloidin (cat. no. A12379; 1:40; Life Technologies; Thermo Fisher Scientific, Inc).

Statistical analysis

Data were expressed as pooled means ± standard error of the mean (S.E.M.) of at least three independent experiments. Results were normalized and expressed as a percentage of the control value. Statistically significant differences between results were determined by the univariate analysis of variance (ANOVA) or Student's *t-test* and appropriate post hoc analysis using GraphPad Prism 9.0 software (La Jolla, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

Icaritin and cisplatin affect the viability and proliferation of A549 and H1299 cell lines

Flow cytometry was used to analyse the cell cycle to estimate the percentage of cells in each phase. In A549 cells, after using compounds that induce cell death, statistically significant changes were observed in the percentage of cells in individual phases of the cell cycle compared to control cells. In the G0/G1 phase, the greatest decrease in the percentage of cells was obtained after treatment with cisplatin at a concentration of 10 μM ($p = 0.0007$). In the case of the percentage of cells treated with icaritin at a concentration of 60 μM, an increase was noticeable, which may indicate that cells are arrested in this phase of the cell cycle. However, the remaining concentrations used in the study do not affect statistically significant changes in the percentage of cells. In all doses of both compounds, the percentage of cells in the S phase compared to the control showed no statistically significant changes. However, in the G2/M phase, statistically significant changes occurred between the control and the CP10 dose, where $p = 0.0035$.

For the H1299 line, the percentage of control cells compared to cells treated with cisplatin and icaritin, as in the case of the A549 line, changed in individual phases of the cell cycle. Analysing the results of the G0/G1 phase, it can be seen that there is statistical significance between the control and CP10 and CP30, where $p < 0.0001$. It can also be noticed that as the dose of cisplatin increases, the number of cells in this phase of the cycle decreases. However, the percentage of cells treated with icaritin compared to the control group is at the same level. In the S phase, the use of cisplatin caused a decrease in the number of cells in this phase, and the use of icaritin caused a minimal increase in cells compared to the control. In the G2/M phase of the cell cycle, the percentage of cells increases after both doses of cisplatin (for both $p < 0.0001$). In both lines, the use of cisplatin caused a decrease in the percentage of cells in the G0/G1 phase and an increase in the G2/M phase. The inhibitory effect of the drugs was confirmed by colony formation assay, where the number of colonies was dramatically reduced after drug intervention (Figs. 1–4).

Assessment of cell migration

The present results show that both cisplatin and icaritin have the potential to decrease the migratory potential of A549 and H1299 cell lines significantly. Lower doses of cisplatin and icaritin reduce the number of cells passing through the membrane, whereas higher doses prevent the cells from migrating (Fig. 5).

Assessment of the type of induced cell death

Annexin V/PI staining reveals that cisplatin and icaritin trigger a moderate level of apoptosis in A549 and H1299 cell lines. The extent of apoptotic cells was slightly higher in the H1299 cell line but did not exceed 40% of early and late apoptotic cells (Fig. 6).

Assessment of cyclin Y, RRM2 and β-catenin protein expression using flow cytometry

Conducting the flow cytometry, the authors analysed the relative expression of cyclin Y in the A549 and H1299 cell lines. The results from both cell lines were comparable. The expression of cyclin Y increased with the dose of cisplatin and icaritin. The result suggests the potential involvement of cyclin Y in the response to drug treatment. Those observations were confirmed under the fluorescent microscope. In both cell lines, the lowest level of cyclin Y was observed in control cells.

It was also checked how icaritin and cisplatin affect RRM2 and β-catenin expression. RRM2 limits deoxyribonucleotide supply and is crucial for cell cycle and DNA repair. It was shown that after cisplatin treatment relative RRM2 expression increases much more than after icaritin treatment. The effect was similar in both cell lines. Next, was measured relative β-catenin expression, and a similar increase in protein levels after cisplatin and icaritin treatment in both cell lines was observed (Figs. 7, 8).

Discussion

Every year, there is an increase in cancer cases and deaths whole the world. The conventional treatment methods used, such as chemotherapy, radiotherapy and surgery, are becoming less and less effective in the fight against cancer, which is becoming more aggressive and resistant to most of the drugs introduced so far. Therefore, the search for new treatment methods and the improvement of already used therapies becomes the main task [11]. However, it is not only the continuous development of methods of treating more or less advanced cancers that is the subject of research. Researchers are also interested in the constant search for prognostic markers. Earlier detection of cancer gives a greater chance of curing the patient, which reduces the number of deaths. Biomarkers are an essential tool in oncology that are used to assess a patient's current outcome and determine the likelihood of cancer recurrence after treatment. However, few prognostic markers have developed in recent years from discovery to validation as tools used in clinical practice [12, 13]. That is why the

constant search for new preventive tools, i.e. prognostic markers and less invasive and more effective ways of treating cancer, is so important.

This study assessed the expression of a protein from the cyclin family — cyclin Y — and changes, cell cycle, migration potential, as well as the type of cell death induced in non-small cell lung cancer lines A549 and H1299 under the influence of factors that induce cell death. To achieve the assumed goal of the study, cisplatin at concentrations of 10 and 30 μM and icaritin at concentrations of 30 and 60 μM were used. The first stage of the research was to determine the effect of cisplatin and icaritin on the cell cycle of lung cancer lines. Analysing data from cytometric measurements of cell distribution in individual phases of the cell cycle, it can be seen that cisplatin in both lines affects the arrest of cells in the G2/M phase. In the A549 line, a statistically significant increase occurred after a dose of 10 μM cisplatin. However, in the H1299 line, statistical significance was demonstrated for both doses. Analogous results were obtained by Sarin et al., who treated the A549 line and the platinum-resistant A549rCDDP2000 line with cisplatin. The authors showed that in the A549 line, the cycle was stopped in the G2/M phase, while in the A549rCDDP2000 line, no such changes were observed [14]. In turn, after the use of icaritin in the studies presented, the cell cycle stopped in the G0/G1 phase, and the percentage of cells in the S phase increased compared to the control group. According to Liu et al., icaritin treatment affects the arrest of glioblastoma cells in the G0/G1 phase of the cell cycle depending on the dose and incubation time with the compound [15].

Based on the analyses of the colony formation test, cell migration and migration rate, the migration potential of A549 and H1299 cells treated with cisplatin and icaritin was assessed. Both invasion and migration are inherent features of tumour metastasis, which causes death in cancer patients. Through various mechanisms, including collective cell migration (collective migration) and mesenchymal cell migration, cancer cells can migrate and spread, which reduces the effectiveness of anti-metastatic therapy [16]. Collective migration occurs in the case of epithelial cells due to the large number of intercellular connections. This type of mechanism is most often used by cancers that show a high degree of differentiation, including prostate cancer, breast cancer, and NSCLC [17]. However, epithelial cells can acquire a phenotype similar to mesenchymal cells through the EMT (epithelial-mesenchymal transition) [18]. Wang et al., in their studies on the effect of cisplatin on the migration potential of breast cancer cells, showed that cisplatin, even at low concentrations (2 mg/kg), inhibited the proliferation, colony formation and migration of cancer cells. The authors suggest that cisplatin inhibits the formation of metastases by blocking the early stages

of EMT [19]. In the case of icaritin, research by Xu et al. proves that this compound significantly inhibits the invasion of glioma cells by stopping EMT [20]. Although the presented work proves that cisplatin and icaritin reduce the migration potential of lung cancer cells, which may be related to EMT, the detailed mechanism of the influence of the cytostatics used on this process still remains unclear.

The presented studies also assessed the impact of cytotoxic compounds on the type of cell death of the A549 and H1299 lines. It has been shown that after 48 hours of incubation with icaritin, the percentage of viable cells decreases, and as a result, the percentage of early- and late-apoptotic cells increases. In their research, Liu et al. draw attention to the fact that 48 hours of incubation with icaritin promoted the apoptotic death of glioma cells by increasing the expression of caspase 3 and the pro-apoptotic protein Bax, as well as decreasing the Bcl-2 protein [15]. There are also reports in the literature regarding the effect of icaritin on the induction of apoptosis in NSCLC. Żuryń and Krajewski showed that icaritin induced apoptosis in the A549 lung cancer line [10]. In turn, after the use of cisplatin in the studies conducted in this study, the induction of the apoptosis process was not as significant as in the case of icaritin.

The subject of this study was the assessment of cyclin Y expression in cancer cells after the induction of cell death. The studies showed an increase in the level of cyclin Y in NSCLC lines after the use of cytotoxic compounds, which depended on the dose and type of compound, incubation time and cell line characteristics. An increase in cyclin Y expression was observed just 3 hours after treating cells of both lines with cytotoxic compounds, and the highest expression was demonstrated after 48 hours of incubation with cisplatin. As previously suggested, both compounds used in the study induced cell death and cell cycle arrest by affecting the AMPK pathway. The AMPK pathway plays an important role in maintaining proper cell energy homeostasis by coordinating many cellular processes, such as cycle progression, autophagy, mitochondrial dynamics, glycolysis, and lipogenesis [21]. In vitro studies have confirmed the therapeutic potential of AMPK activation in many types of cancer. Interestingly, the action of AMPK in cancer progression may be related to blocking or even reversing EMT [22]. Based on the collected information, in relation to the present research, it is suggested that the use of both cisplatin and icaritin affects the activation of the AMPK pathway, which may increase the expression of cyclin Y. As a result, cyclin Y through the AMPK pathway may contribute to the inhibition of proliferation, cell migration, invasion and induction of apoptosis in NSCLC. The assessment of RRM2 protein and β-catenin showed an increase in the level of both proteins after the use of cisplatin and icaritin, which

correlated with an increase in the expression of cyclin Y. RRM2 is a protein that catalyzes the formation of deoxyribonucleotides from ribonucleotides [23]. Overexpression of this protein has been demonstrated in many types of cancer, including melanoma, head and neck cancer, breast cancer and lung cancer. RRM2 overexpression is associated with drug resistance and worse prognosis in cancer patients. Wang et al., examining the expression of RRM2 in NSCLC patients receiving platinum-based chemotherapy, showed that lower protein expression reduced tumour progression and drug resistance of cancer cells. Patients with lower RRM2 expression also showed longer survival [24]. Krajewski et al., examining melanoma lines (A375 and RPMI-7951) treated with cisplatin, showed an increase in the expression of the tested protein after the use of the cytotoxic compound. A review of the above literature confirmed the results obtained in this study, which showed an increase in RRM2 protein expression in cisplatin-treated NSCLC lines. The increase in expression depends on the activity of the p53 protein and affects the arrest of the cell cycle and the reduction of drug resistance of cancer cells. Assessment of β-catenin showed an increase in the expression of the tested protein after the use of cell death-inducing factors. The results of the conducted research and the analysis of the available literature suggest that the initial assumptions that overexpression of the Wnt/β-catenin signaling pathway promotes the initiation, promotion and progression of cancer, and also leads to a worse clinical outcome, may be an oversimplification. Instead, it appears that the Wnt pathway, either dependent or independent of β-catenin, can induce or inhibit carcinogenesis in a context-dependent manner.

Conclusion

The results showed that the expression of cyclin Y increases in a dose-dependent and time-dependent manner after using cell death-inducing agents. The cyclin Y upregulation was correlated with cell cycle arrest, an increase in the percentage of apoptotic cells, and the inhibition of the migration potential of cells of both lines. Based on the presented results, it is considered reasonable that a thorough understanding of the mechanisms of action of cell cycle proteins is important in the prevention, diagnosis, and treatment of cancer.

Article information

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Ethics statement: *Not applicable.*

Author contributions: *AO carried out experiments and wrote the paper, AK interpreted the data and prepared figures, KP carried out experiments and cured the data, AG, AW, and WN wrote the paper, and did literature research, AŻ designed and supervised the paper.*

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References

1. Matthews HK, Bertoli C, de Bruin RAM. Cell cycle control in cancer. Nat Rev Mol Cell Biol. 2022; 23(1): 74–88, doi: 10.1038/s41580-021-00404-3, indexed in Pubmed: 34508254.
2. Heuvel Sv. Cell-cycle regulation. WormBook. 2005, doi: 10.1895/wormbook.1.28.1.
3. Lim S, Kaldis P. Cdks, cyclins and CKIs: roles beyond cell cycle regulation. Development. 2013; 140(15): 3079–3093, doi: 10.1242/dev.091744, indexed in Pubmed: 23861057.
4. Liu D, Finley RL. Cyclin Y is a novel conserved cyclin essential for development in Drosophila. Genetics. 2010; 184(4): 1025–1035, doi: 10.1534/genetics.110.114017, indexed in Pubmed: 20100936.
5. Cho E, Kim DH, Hur YN, et al. Cyclin Y inhibits plasticity-induced AMPA receptor exocytosis and LTP. Sci Rep. 2015; 5: 12624, doi: 10.1038/srep12624, indexed in Pubmed: 26220330.
6. Jiang M, Gao Y, Yang T, et al. Cyclin Y, a novel membrane-associated cyclin, interacts with PFTK1. FEBS Lett. 2009; 583(13): 2171–2178, doi: 10.1016/j.febslet.2009.06.010, indexed in Pubmed: 19524571.
7. Mikolcevic P, Sigl R, Rauch V, et al. Cyclin-dependent kinase 16/PCTAIRE kinase 1 is activated by cyclin Y and is essential for spermatogenesis. Mol Cell Biol. 2012; 32(4): 868–879, doi: 10.1128/MCB.06261-11, indexed in Pubmed: 22184064.
8. Wang X, Jia Y, Fei C, et al. Activation/Proliferation-associated Protein 2 (Caprin-2) Positively Regulates CDK14/Cyclin Y-mediated Lipoprotein Receptor-related Protein

5 and 6 (LRP5/6) Constitutive Phosphorylation. J Biol Chem. 2016; 291(51): 26427–26434, doi: 10.1074/jbc.M116.744607, indexed in Pubmed: 27821587.

9. Shi K, Ru Q, Zhang C, et al. Cyclin Y Modulates the Proliferation, Invasion, and Metastasis of Hepatocellular Carcinoma Cells. Med Sci Monit. 2018; 24: 1642–1653, doi: 10.12659/msm.906075, indexed in Pubmed: 29557391.
10. Żuryń A, Krajewski A. Icaritin induces apoptosis and downregulates RhoA/ROCK pathway in the A549 adenocarcinoma cell line. Medical Research Journal. 2022; 7(3): 242–248, doi: 10.5603/mrj.2022.0045.
11. Debela DT, Muzazu SGy, Heraro KD, et al. New approaches and procedures for cancer treatment: Current perspectives. SAGE Open Med. 2021; 9: 20503121211034366, doi: 10.1177/20503121211034366, indexed in Pubmed: 34408877.
12. Costantini S, Budillon A. New Prognostic and Predictive Markers in Cancer Progression. Int J Mol Sci. 2020; 21(22), doi: 10.3390/ijms21228667, indexed in Pubmed: 33212936.
13. van Gool AJ, Bietrix F, Caldenhoven E, et al. Bridging the translational innovation gap through good biomarker practice. Nat Rev Drug Discov. 2017; 16(9): 587–588, doi: 10.1038/nrd.2017.72, indexed in Pubmed: 28450744.
14. Sarin N, Engel F, Kalayda GV, et al. Cisplatin resistance in non-small cell lung cancer cells is associated with an abrogation of cisplatin-induced G2/M cell cycle arrest. PLoS One. 2017; 12(7): e0181081, doi: 10.1371/journal.pone.0181081, indexed in Pubmed: 28746345.
15. Liu Y, Shi L, Liu Y, et al. Activation of PPARγ mediates icaritin-induced cell cycle arrest and apoptosis in glioblastoma multiforme. Biomed Pharmacother. 2018; 100: 358–366, doi: 10.1016/j.biopha.2018.02.006, indexed in Pubmed: 29453045.
16. Wu JS, Jiang J, Chen BJ, et al. Plasticity of cancer cell invasion: Patterns and mechanisms. Transl Oncol. 2021; 14(1): 100899, doi: 10.1016/j.tranon.2020.100899, indexed in Pubmed: 33080522.
17. Friedl P. Prespecification and plasticity: shifting mechanisms of cell migration. Curr Opin Cell Biol. 2004; 16(1): 14–23, doi: 10.1016/j.ceb.2003.11.001, indexed in Pubmed: 15037300.
18. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009; 119(6): 1420–1428, doi: 10.1172/JCI39104, indexed in Pubmed: 19487818.

19. Wang H, Guo S, Kim SJ, et al. Cisplatin prevents breast cancer metastasis through blocking early EMT and retards cancer growth together with paclitaxel. Theranostics. 2021; 11(5): 2442–2459, doi: 10.7150/thno.46460, indexed in Pubmed: 33500735.
20. Xu Bo, Jiang C, Han H, et al. Icaritin inhibits the invasion and epithelial-to-mesenchymal transition of glioblastoma cells by targeting EMMPRIN via PTEN/AKt/HIF-1α signalling. Clin Exp Pharmacol Physiol. 2015; 42(12): 1296–1307, doi: 10.1111/1440-1681.12488, indexed in Pubmed: 26356761.
21. Vara-Ciruelos D, Russell FM, Hardie DG. The strange case of AMPK and cancer: Dr Jekyll or Mr Hyde? . Open Biol. 2019; 9(7): 190099, doi: 10.1098/rsob.190099, indexed in Pubmed: 31288625.
22. Konieczny P, Adamus T, Sułkowski M, et al. Impact of AMPK on cervical carcinoma progression and metastasis. Cell Death Dis. 2023; 14(1): 43, doi: 10.1038/s41419-023-05583-9, indexed in Pubmed: 36658117.
23. Fatkhutdinov N, Sproesser K, Krepler C, et al. Targeting RRM2 and Mutant BRAF Is a Novel Combinatorial Strategy for Melanoma. Mol Cancer Res. 2016; 14(9): 767–775, doi: 10.1158/1541-7786.MCR-16-0099, indexed in Pubmed: 27297629.
24. Wang L, Meng L, Wang Xw, et al. Expression of RRM1 and RRM2 as a novel prognostic marker in advanced non-small cell lung cancer receiving chemotherapy. Tumour Biol. 2014; 35(3): 1899–1906, doi: 10.1007/s13277-013-1255-4, indexed in Pubmed: 24155212.

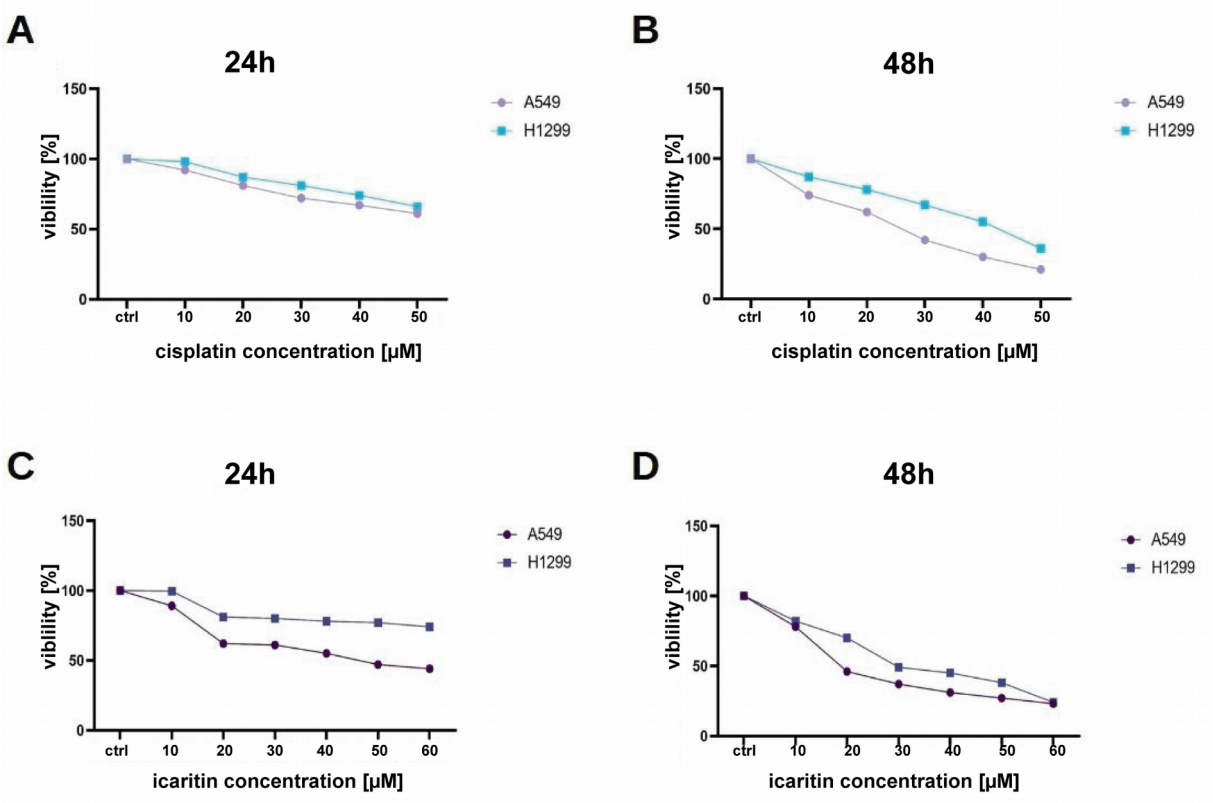


Figure 1. The effect of cisplatin and icaritin on the viability of A549 **(A, B)**, and H1299 cell line **(C, D)**

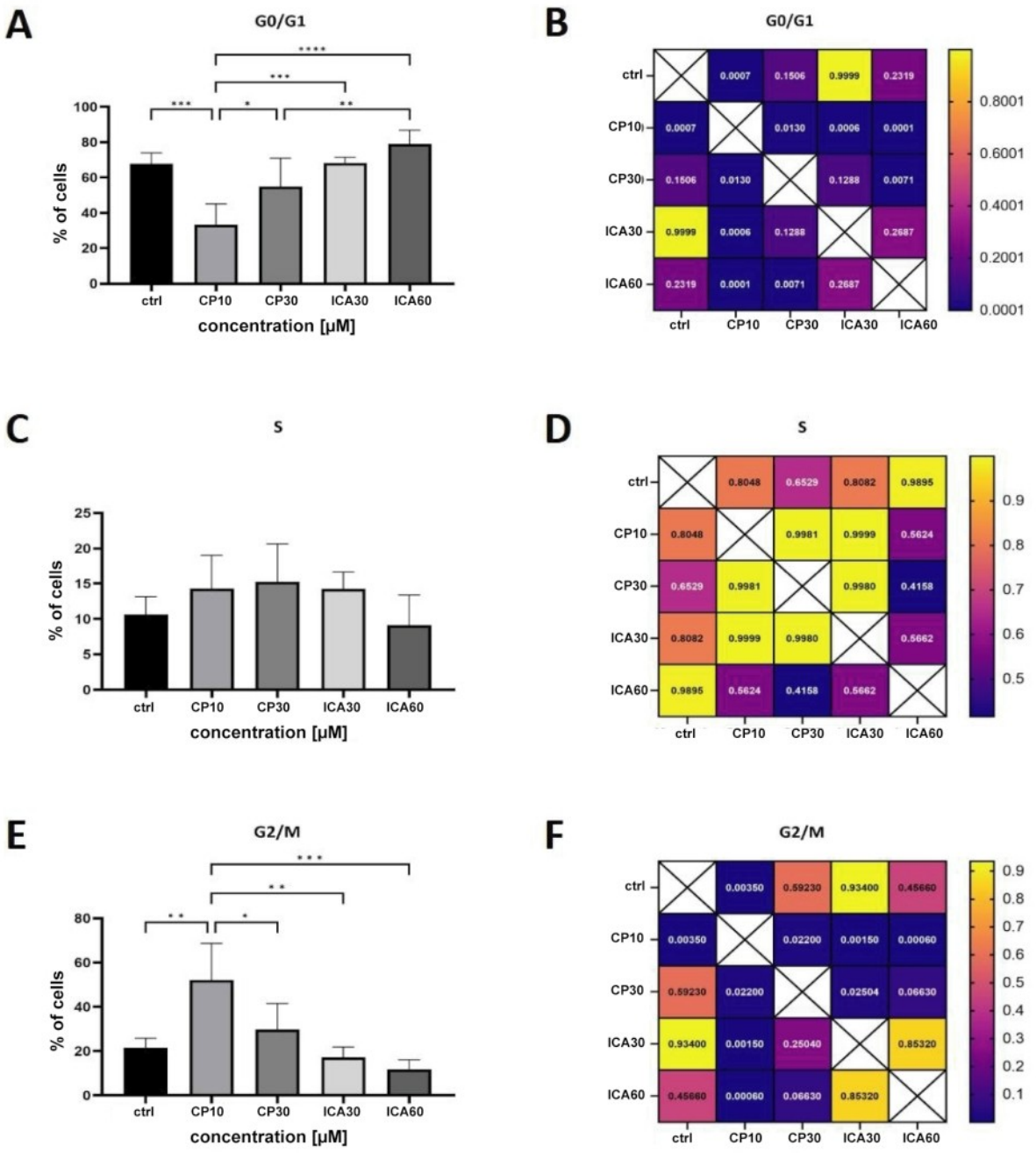


Figure 2. The effect of cisplatin and icaritin on cell cycle distribution in A549 cell line **(A, C, E)**. The heatmap shows the p-value between study groups. Asterisks denote statistically significant results ($p < 0.05$)

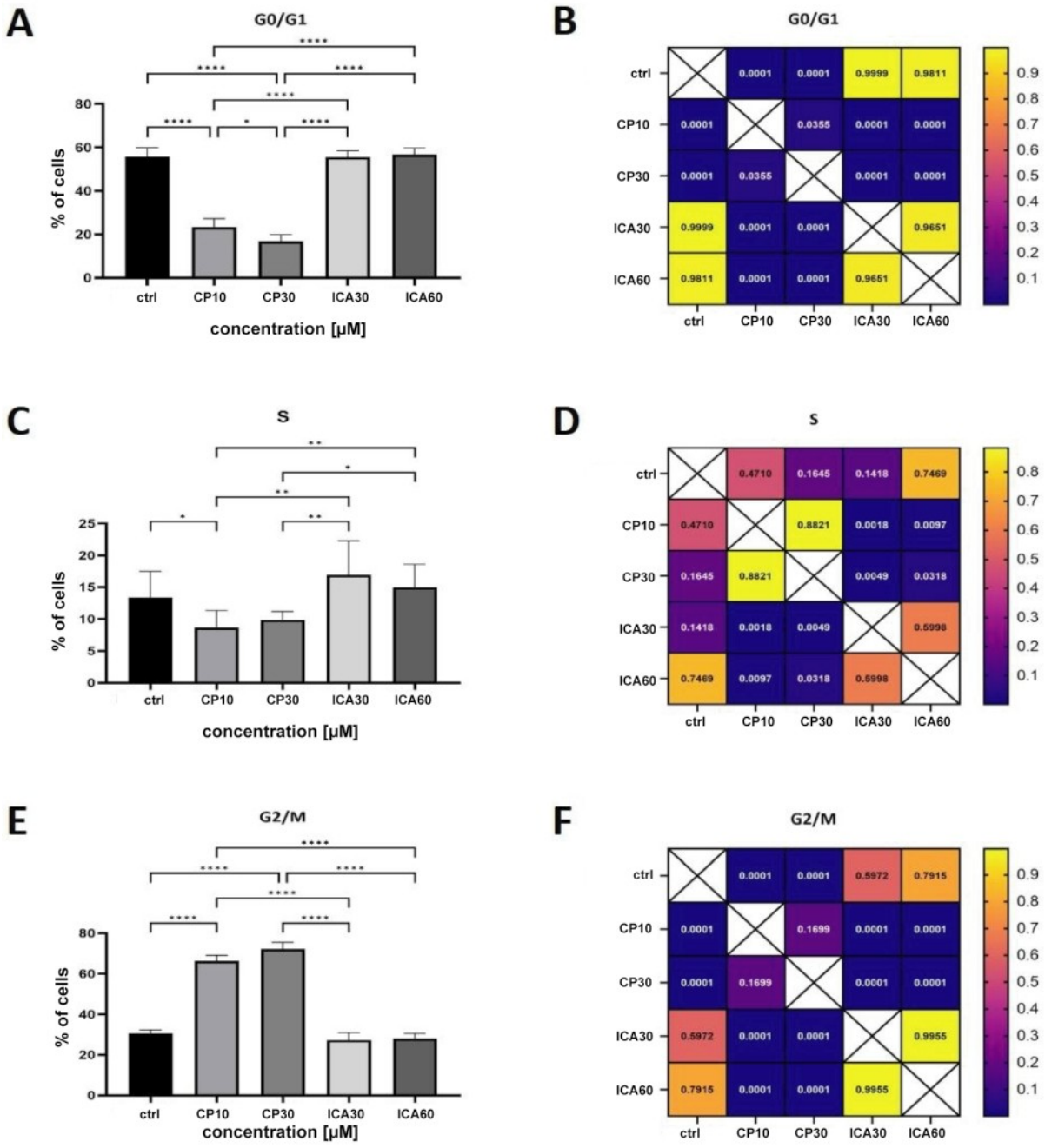


Figure 3. The effect of cisplatin and icaritin on cell cycle distribution in H1299 cell line **(A, C, E)**. The heatmap shows the p-value between study groups. Asterisks denote statistically significant results ($p < 0.05$)

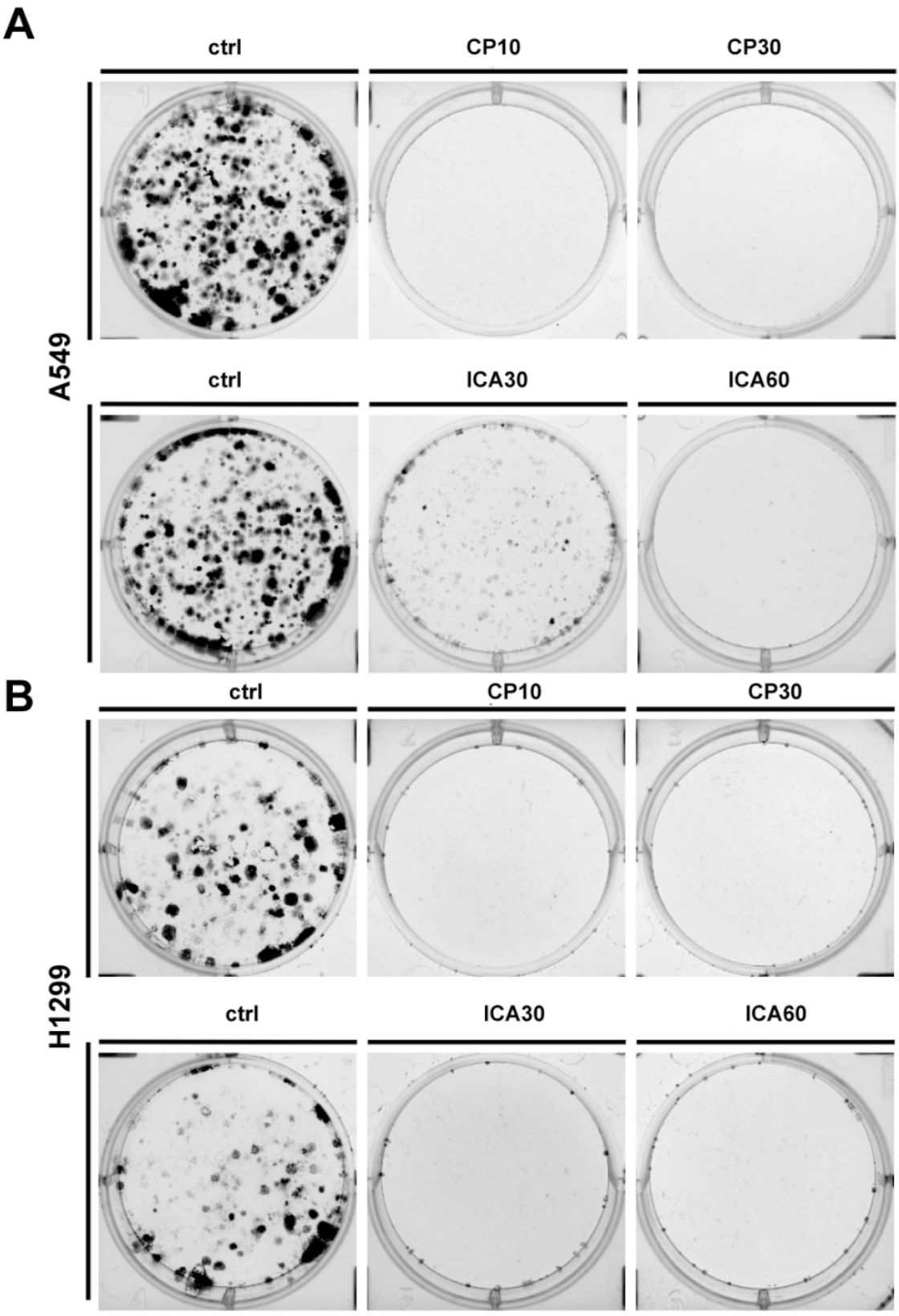


Figure 4. Colony formation assay shows a significant anti-proliferative effect of cisplatin and icaritin in the A549 **(A)** and H1299 **(B)** cell lines

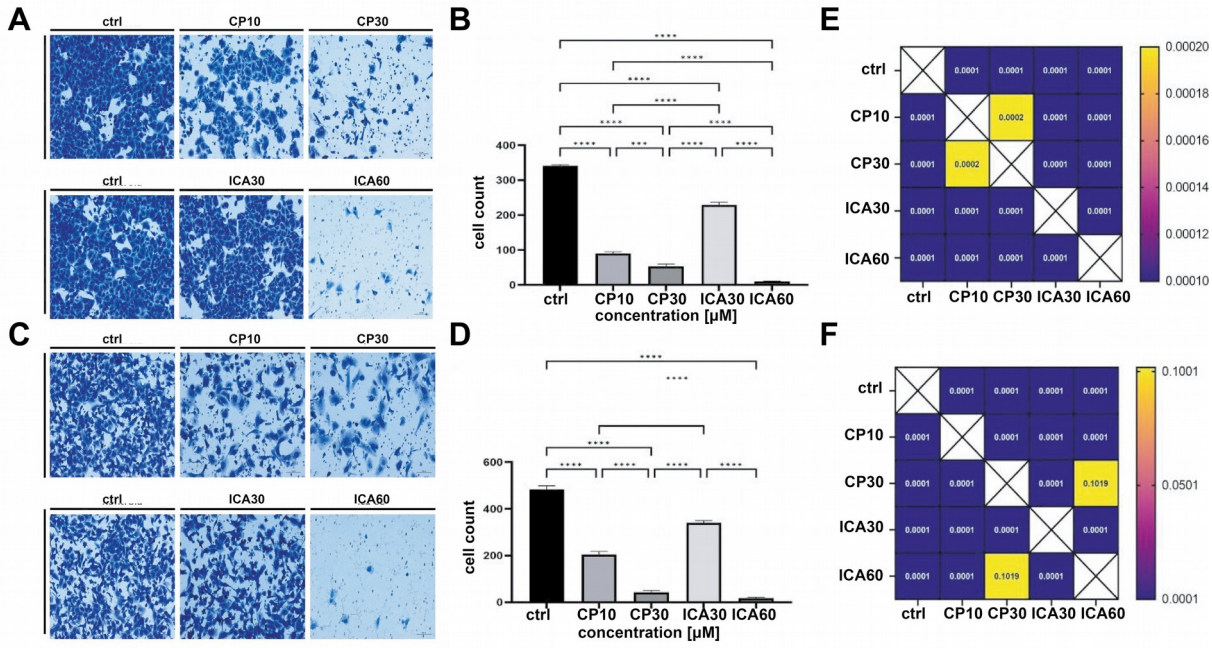


Figure 5. Cisplatin and icaritin reduce the migratory potential of A549 **(A, B)** and H1299 cells **(C, D)**. Heatmap shows p-value between A549 study groups **(E)** and H1299 groups **(F)**

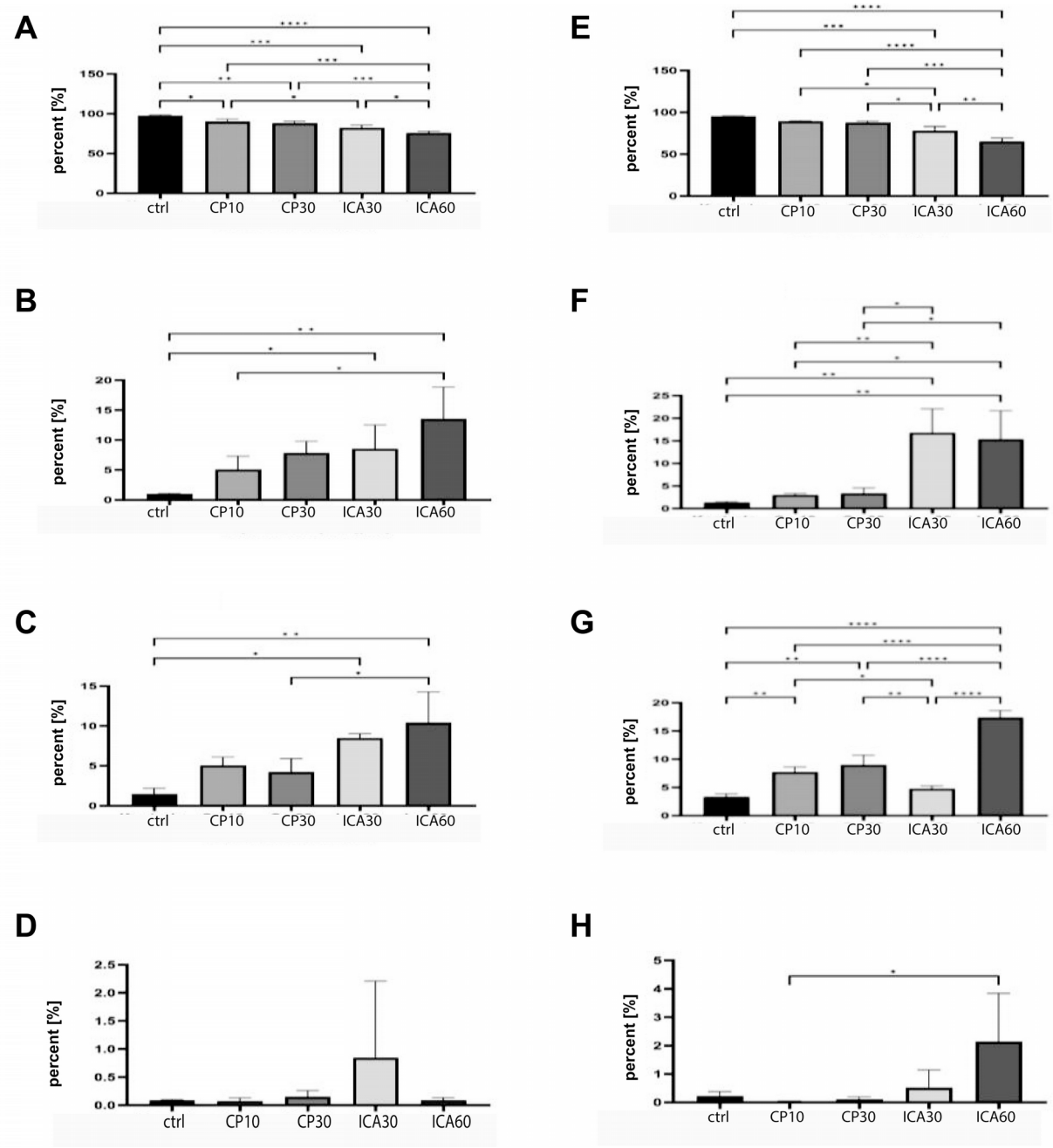


Figure 6. Apoptosis in A549 and H1299 cell lines after the CP and ICA treatment. A549 live **(A)** early apoptotic **(B)**, late apoptotic **(C)** and necrotic cells **(D)**. H1299 live **(E)**, early apoptotic **(F)** late apoptotic **(G)** and necrotic cells **(H)**. Asterisks denote statistically significant results ($p < 0.05$)

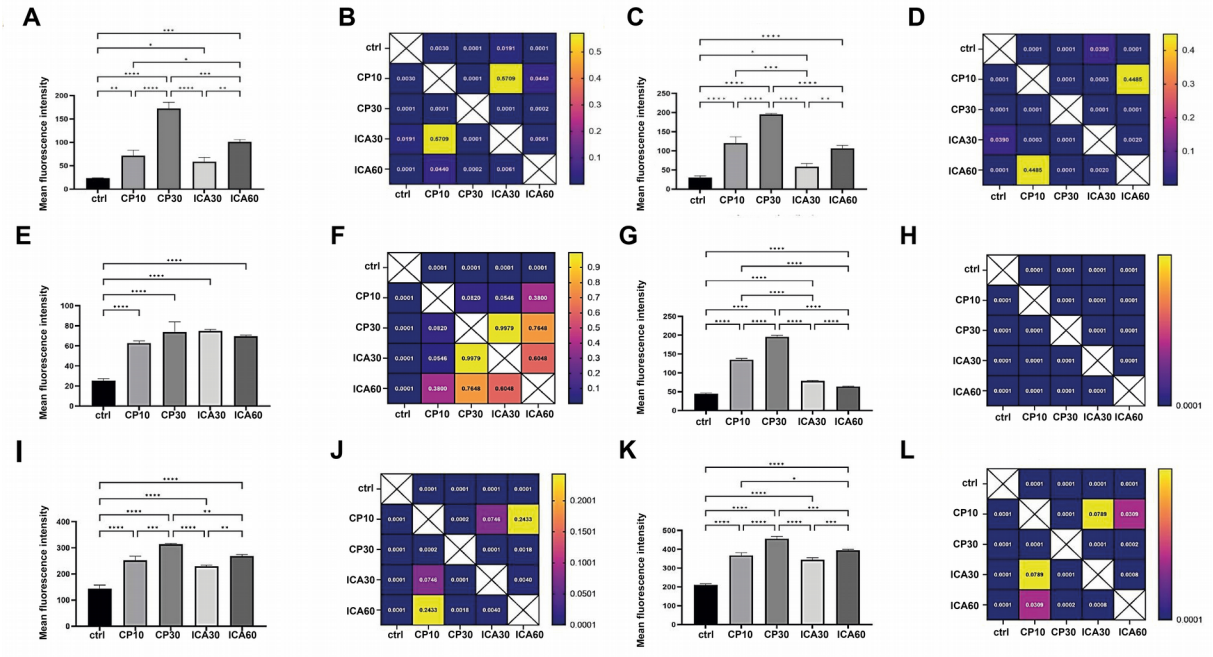
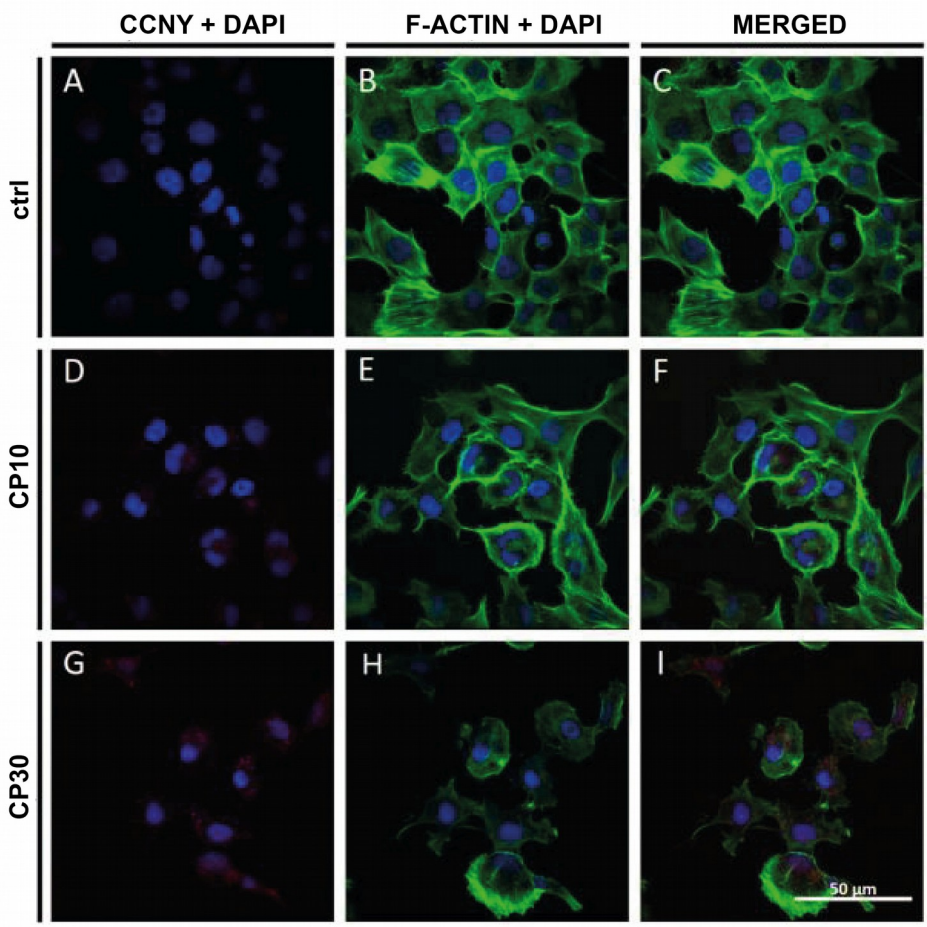


Figure 7. Expression of cyclin Y in A549 **(A)** and H1299 cell lines **(C)**. Expression of RRM2 in A549 **(E)** and H1299 **(G)** cell lines. Expression of β-catenin in A549 **(I)** and H1299 cell lines **(K)**. The heatmap shows the p-value between study groups. Asterisks denote statistically significant results ($p < 0.05$)


CCNY + DAPI
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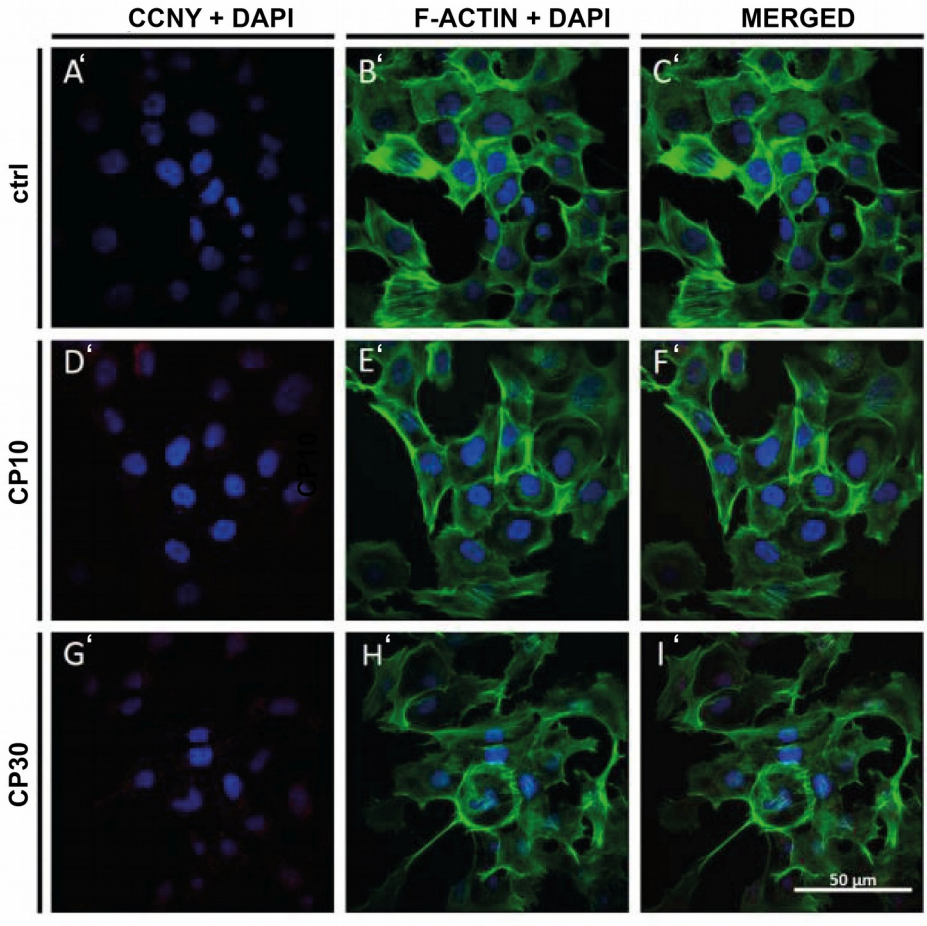
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Figure 8. Expression and localization of cyclin Y in A549 **(A–I)** and H1299 cell lines (A'–I') after the CP and ICA treatment