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Enhancing mitotane efficacy in adrenocortical carcinoma by calcineurin inhibition with cyclosporine A

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ABSTRACT

Aims: The aim of this study is to determine the effect of calcineurin (CaN) in adrenocortical cancer (ACC) cells, which is a rare but aggressive type of cancer resistant to mitotane therapy. The intracellular calcium signaling pathway is one of the most important mechanisms for cells. The effect of intracellular calcium concentration [(Ca^{2+}i)] on the function of cancer cells is also known. CaN, activated by the binding of calmodulin and Ca^{2+}, is critical in this pathway.

Methods: H295 adrenocortical cancer cells were treated with mitotane, cyclosporine A (CsA), and a combination of both. Cell viability, apoptosis, cell cycle, and gene expression levels of apoptosis-related genes (BCL2, BAX, TP53) were analyzed. Western blotting was used to measure CaN protein levels, and wound healing assays assessed cell migration.

Results: CsA significantly suppressed CaN protein levels in a dose-dependent manner, reducing cell viability and increasing apoptosis in H295 cells. Mitotane alone also suppressed CaN protein, but the combination of mitotane and CsA had a synergistic effect, further decreasing cell viability and increasing apoptosis. The combination treatment led to significant suppression of the BCL2 gene and upregulation of TP53. Cell cycle analysis showed increased arrest in the G0/G1 phase with combination treatment.

Conclusion: Suppression of CaN by CsA enhances the cytotoxic effects of mitotane on ACC cells, suggesting a potential therapeutic strategy to improve ACC treatment outcomes. This study highlights the importance of targeting intracellular calcium signaling pathways to overcome resistance and enhance the efficacy of existing cancer therapies.

Keywords: Adrenocortical carcinoma, calcineurin, cyclosporine A, intracellular calcium signaling, mitotane

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare epithelial neoplasm originating from the adrenal cortex with a high tendency to local invasion and distant metastasis. The annual incidence of ACC is 0.7 to 2 cases per million population. About 10-15% of ACCs are discovered incidentally in radiographic imaging. ACC has a poor prognosis, with overall five-year survival ranging from 60 to 80% in patients with ACC stage I to 13% in patients with stage IV disease.[1] Complete remission can be achieved in localized ACC, but the appearance of metastases or recurrences during the follow-up period significantly worsens the prognosis. Complete remission is extremely rare in patients diagnosed with ACC at the stage of regional invasion or distant metastasis. Current treatment regimens for ACC are limited, and radical surgical resection is the only curative option. Unfortunately, most ACC patients present with locally advanced or metastatic disease that is not amenable to surgical resection.[2] Mitotane is used as soon as possible after tumor removal, which is recommended for most patients, especially with stage III and IV cancers or with a high Ki-67 proliferation index.[3]

Mitotane is an adrenolytic drug, a synthetic derivative of the insecticide dichlorodiphenyltrichloroethane (DDT). Its toxic effect is observed in the bone marrow, liver, skin, and gastrointestinal tract.[4] Response to mitotane treatment is seen only in approximately 30-50% of ACC patients.[5] Response to treatment is observed in only 25% of ACC patients undergoing chemotherapy.[6] Although mitotane is an effective drug in the treatment of ACC, it must be administered in a controlled manner. Otherwise, its toxic effect will be more than its therapeutic effect. Not every ACC patient can give the same response to the same dose of mitotane. At the same time, it has been shown in recent studies that ACC cells develop resistance to mitotane.[7,8]

Calcineurin (CaN) is a serine/threonine phosphatase activated by the binding of calmodulin and Ca^{2+}, plays a crucial role in the intracellular calcium signaling pathway.[9] It is known to be expressed in the adrenal cortex.[10] CaN is the substrate of transcription factors, proteins involved in the cell cycle, and apoptosis.[9] When Ca^{2+} concentration

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increases in the cytoplasm, CaN becomes active. In cases where CaN is active, cell proliferation increases.[9,11] One of the best-known inhibitors of CaN is cyclosporine A (CsA).[12] CsA, a molecule of fungal origin (*Tolypocladium inflatum*), was discovered in 1970 to develop a new antifungal therapy.[13,14] CsA is an immunosuppressive drug. While CsA is primarily used in transplant patients to prevent rejection, its potential role in cancer therapy, particularly in combination with other anticancer agents, is of significant interest.[12,15]

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METHODS

Since the study was performed cell line and these results obtained from cell line, ethics committee approval is not need. In this cell line study, informed consent is not need. All procedures were carried out in accordance with the ethical rules and the principles.

Cell Lines and Culture Conditions

H295 human adrenocortical cancer cell line was purchased from the American Type Culture Collection (CRL-2128-ATCC). After H295 cells were cultured, it was incubated in a humidified incubator containing 5% CO2 at 37°C. The culture medium DMEM-F12 was Gibco. It was supplemented with 10% FBS, penicillin (1x10^5 U/L), and L-glutamine (2 mmol/L). Cells were harvested with trypsin (0.05%), and EDTA (0.02%) and resuspended in the medium.

Chemicals and Drugs

Mitotane and CsA chemicals were treated to H295 cells. It was ordered from sigma alderich mitotane stock solution was prepared with ethyl alcohol at a concentration of 10^{-2} M and stored at -20°C. It was freshly prepared from a stock solution of mitotane at different concentrations applied to H295 cells. CsA was ordered from Sigma Alderich. CsA stock solution was prepared with methanol at a concentration of 10^{-3} M and stored at -20°C.

Cell Proliferation Assay

The cell viability was measured using methyl thiazole diphenyl tetrazolium (MTT) salt, frequently used in cell viability tests based on the measurement of metabolic activity. Since metabolic activity continues in living cells, the MTT molecule can turn into formazan and a color change is observed, while this change does not occur in dead cells. 100 µl of the H295 cell solution (5000 cell/well) was seeded in a 96 well plate. Chemicals were treated to the cells for 24 hours. After 24 and 72 hours of incubation of cells with mitotane, CsA, and mitotane+CsA complex, cells were treated with MTT solution. At the end of the incubation period, absorbance values were measured by multiplate reader.

Apoptosis Assay

Apoptosis measurement was performed with Annexin V to determine whether the cells underwent the apoptotic process 24 and 72 hours after mitotane, CsA, and mitotane+CsA complex treatments to H295 cells. Phosphatidylserines found in the outer membranes of apoptotic cells were measured. Muse Annexin V & Dead Cell reagent (Luminex MCH100105) was added to cell suspensions. Samples were measured on the Muse analyzer (Merck).

Cell Cycle Assay

Muse Cell Cycle kit was used for easy and fast quantitative measurement of the percentage of cells in the G0 / G1, S, and G2 / M phases of the cell cycle of cells. Cells were fixed 24 and 72 hours after the mitotane, CsA, and mitotane+CsA complex treatment. After adding Muse cell cycle solution, it was measured with Muse analyzer.

RT-qPCR Assay

The expressions of Bcl2, Bax, and TP53 genes and GAPDH as the housekeeping gene in H295 cells were evaluated by quantitative RT-PCR. From H295 cells, total RNA was isolated using a commercially available kit (high pure RNA tissue kit; Roche). cDNA was synthesized using a commercially available kit (first strand cDNA synthesis kit; Roche). SYBR Green was used as a reporter dye. The expression of these genes was evaluated by real-time PCR method using the Roche LightCycler® 480 system (Germany). The protocol for real-time PCR was as the following: initial incubation at 95°C for 5 min., 45 cycles of 95°C for 10^{sec}, 60°C for 10^{sec}, and 72°C for 10^{sec}.

The Ct values of genes were calculated by the Roche Light Cycler® 480 system. Expression values were determined by the $2^{-\Delta\Delta Ct}$ method. The $2^{-\Delta\Delta Ct}$ method was calculated according to the housekeeping gene. Gene expression levels were evaluated in the drug-free control group and compared with the medicated control group. Expression levels of genes were determined according to the fold change.

Wound Healing Assay

H295 cells were seeded in 12-well plates. When cells density reached 85-90% in the wells, a scratch was created in the monolayer in each well using the tip of a sterile 100-µl pipette, the cells were gently washed with medium to remove detached cells, and fresh medium was added and mitotane, CsA, and mitotane+CsA complex was added to cells in the wells. The scratched areas were imaged at 0, 24, and 72 hours using phase-contrast microscopy. The distance traveled by cells at the leading edge of the wound at each time point was measured by ImageJ software.

Western Blotting

The total protein solution that is 30 ug diluted with sample buffer was denatured at 95°C for 5 min. After denaturation, the protein samples were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane with protein was incubated with a blocking buffer (0.1% tween 20-PBS/3% nonfat dry milk). It was incubated overnight at 4°C with the primary antibody.

After washing in 0.1% tween 20 for 1 hour, membranes were incubated with secondary antibody for 1 hour at room

temperature, followed by washing in 0.1% Tween 20 for 1 hour. Immunodetection was performed using the Chemidoc MP imaging system (Biorad). The optical density of the appropriately sized bands was measured using the image J (Fiji-Win64) software.

Statistical Analysis

GraphPad Prism 8 (GraphPad software, San Diego, CA) was used for statistical analysis. Experiments were repeated at least three times (n=3). IC50 values (fifty percent growth-inhibitory concentrations) were calculated using nonlinear regression curve-fitting program. The comparative statistical evaluation among groups was first performed by the one-way ANOVA test. Paired groups were tested with one simple t-test. The results were considered statistically significant when p-values were <0.05. (*p<0.05, **p<0.01, ***p<0.001).

RESULTS

CsA Suppresses CaN Protein and Reduces Proliferation of ACC Cells

In our study, we focused on CsA being the best-known CaN inhibitor as well as being a potent immunosuppressive. Initally, it was determined whether CsA inhibited the CaN protein in H295 cells (Figure 1A, B). According to CaN protein expression analysis in H295 cells treated with CsA after 24 hours of incubation, CsA suppressed CaN protein significantly compared to the control group (p<0.001) (Figure 1C). After 72 hours, CsA continues to suppress the CaN protein. The level of CaN protein decreased significantly compared to the control group (p<0.001) (Figure 1D).

We treated H295 cells with varying concentrations of CsA (10^{-4} to 10^{-8} M) and measured cell viability after 24 and 72 hours. CsA at 10^{-4} M reduced cell viability to 46.06% after 24 hours (Figure 1E) and 17.80% after 72 hours (p<0.001) (Figure 1F). IC50 values for CsA were10^{-4} M at 24 hours and $5x10^{-5}$ M at 72 hours (Figure 1G).

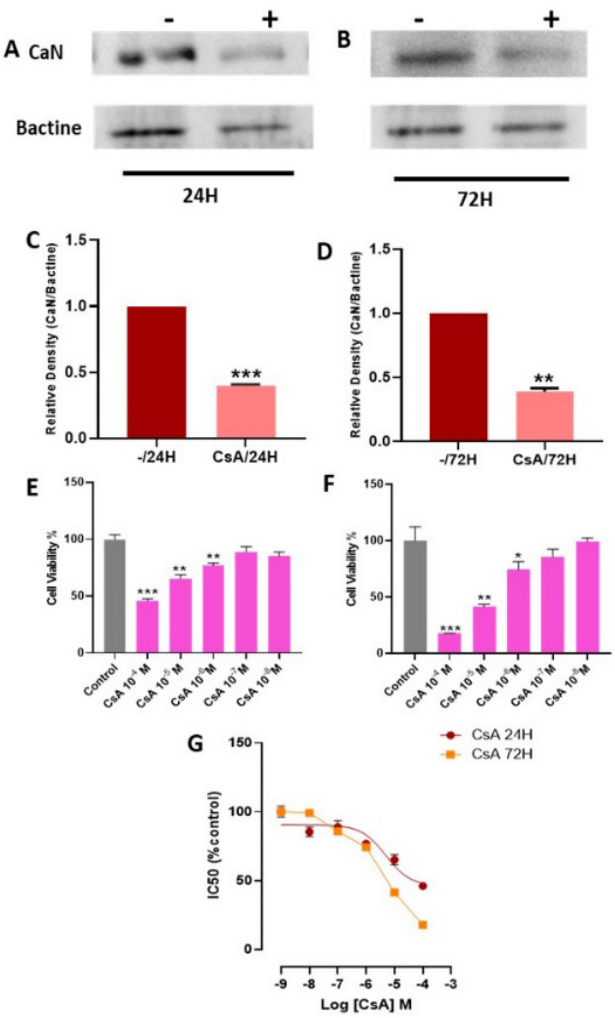

Figure 1. Band and relative density graph showing the effect of CsA on CaN protein and effects of CsA on H295 cell viability A) Protein bands of CsA treated cells (+) after 24 hours incubation time. B) and Protein bands of CsA treated cells (+) after 72 hours incubation time. C) The relative density graph shows the band densities after 24 hours incubation time. D) The relative density graph shows the band densities after 72 hours incubation time. E) Cell viability after treatment for 24H with CsA, F) for 72H with CsA. G) dose response curve of H295 cells with CsA. *p<0.05, **p<0.01, ***p<0.001

Mitotane and Mitotane+CsA Complex Affect CaN Protein and Cell Viability

The effect of mitotane on CaN protein in H295 cells was unknown. The effect of mitotane and mitotane+CsA complex on CaN protein in H295 cells changes after 24 and 72 hours (Figure 2A, B). After 24 hours, only mitotane significantly suppressed CaN protein compared to the control group (p<0.001). However, when combined with CsA, CaN protein levels initially increased after 24 hours (p<0.001) but significantly decreased after 72 hours (p<0.001) (Figure 2C, D).

Mitotane significantly decreased cell viability in a dose-dependent manner. At high concentrations (10^{-4}, 10^{-5}, and 10^{-6} M), mitotane reduced cell viability to 14.10%, 49.17%, and 62.81% respectively after 24 hours (p<0.01) (Figure 2E). After 72 hours, cell viability was further reduced to 11.57% and 22.20% at concentrations of 10^{-4} and 10^{-5} M respectively (p<0.01) (Figure 2F). IC50 values for mitotane were 10^{-5} M at 24 hours and 10^{-6} M at 72 hours. When H295 cells were treated with the mitotane+CsA complex, cell viability decreased to 36.78% after 24 hours (p<0.001) and to 35.20% after 72 hours (Figure 2G, H).

CsA and Mitotane+CsA Complex Induce Apoptosis in H295 Cells

When mitotane is treated alone, 17.79% of H295 cells are in early apoptosis after a 24 hour incubation period. While 10.98% of the cells were in the late apoptosis process, 4.82% of the cells were measured as dead. After 72 hours, these rates changed and the dead cell rate increased to 26.54%. While 3.25% of cells are in early apoptosis, 4.87% are in late apoptosis (Figure 3A).

H295 cells treated with CsA alone were 5.22% dead after 24 hours. Cells are in the early and late apoptosis process at a rate of 15.72% and 12.14%, respectively. After 72 hours, the rate of dead cells increases to 34.01%. On the other hand, early and late apoptosis rates are 2.06% and 3.28%, respectively. 24 hours after the mitotane+CsA complex is applied to H295 cells, cells are in the early and late apoptosis process at a rate of 18.00% and 15.48%, respectively. In contrast, the cells were dead at a rate of 6.96%. After 72 hours, 52.34% of the cells die, 0.56% of the cells are early and 10.94% are late in the apoptosis process (Figure 3B).

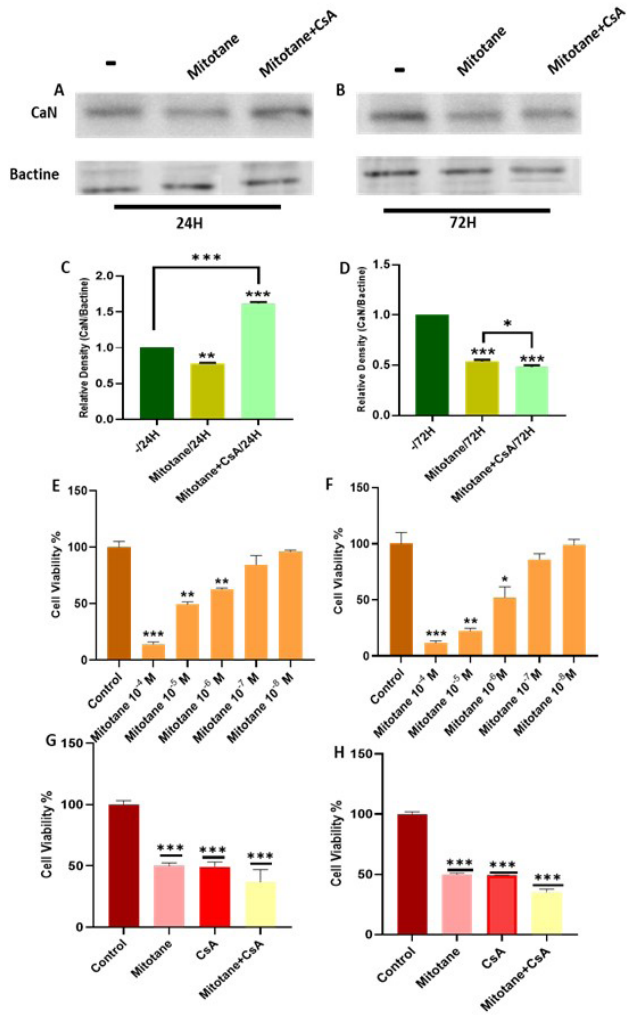

Figure 2. CaN protein band and relative density graph and effects of Mitotane and Mitotane+CsA complex on H295 cell viability. A, B) CaN protein bands for24 and 72 hours after Mitotane and Mitotane+CsA complex treatment. C, D) Relative density graphs (CaN/Bactine) for 24 and 72 hours after Mitotane and Mitotane+CsA complex treatment. After measuring band intensities, protein amounts were calculated with reference to B-actin. It was normalized to 1 relative to the control group (-) and statistical analyzes were performed according to the control group. E) Cell viability after treatment for 24 hours with Mitotane, F) for 72 hours with Mitotane. G) Cell viability with Mitotane+CsA for 24 hours. H) Cell viability with Mitotane+CsA for 72 hours ***p<0.001, ** p<0.01, * p<0.05

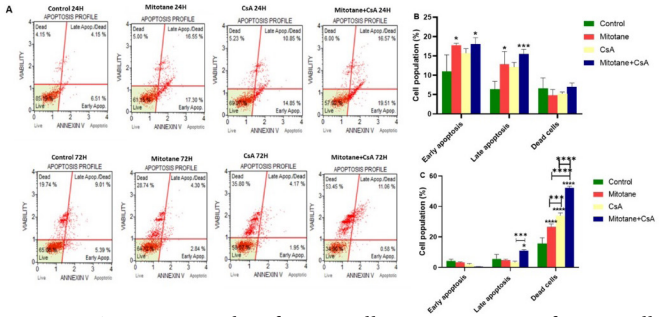

Figure 3. Apoptosis graphs of H295 cells. Measurements of H295 cells treated with Mitotane, CsA, and Mitotane+CsA complex after 24 and 72 hours by Annexin V A) Apoptosis profile of H295 cells treated with Mitotane, CsA, and Mitotane+CsA complex. B) Apoptosis graph of H295 cells treated with Mitotane, CsA, and Mitotane+CsA complex for 24 hours C) for 72 hours.*** p<0.001, ** p<0.01* p<0.05

Mitotane+CsA complex suppressed the BCL2 gene significantly compared to the control group 24 hours after being treated to H295 cells (p<0.05) (Figure 4A). Likewise, after 24 hours, the BAX gene was also significantly suppressed compared to the control group (p<0.05) (Figure 4C). 72 hours after administration of the mitotane+CsA complex, the BCL2 gene was significantly suppressed compared to the control (p<0.01) (Figure 4B). Although no significant difference was calculated between the BAX gene and the control, the BAX gene increased 1.1-fold compared to the control (Figure 4D). However, the TP53 gene was upregulated 1.23-fold compared to the control and there was a significant difference with the control after 72 hours (p<0.01) (Figure 4F).

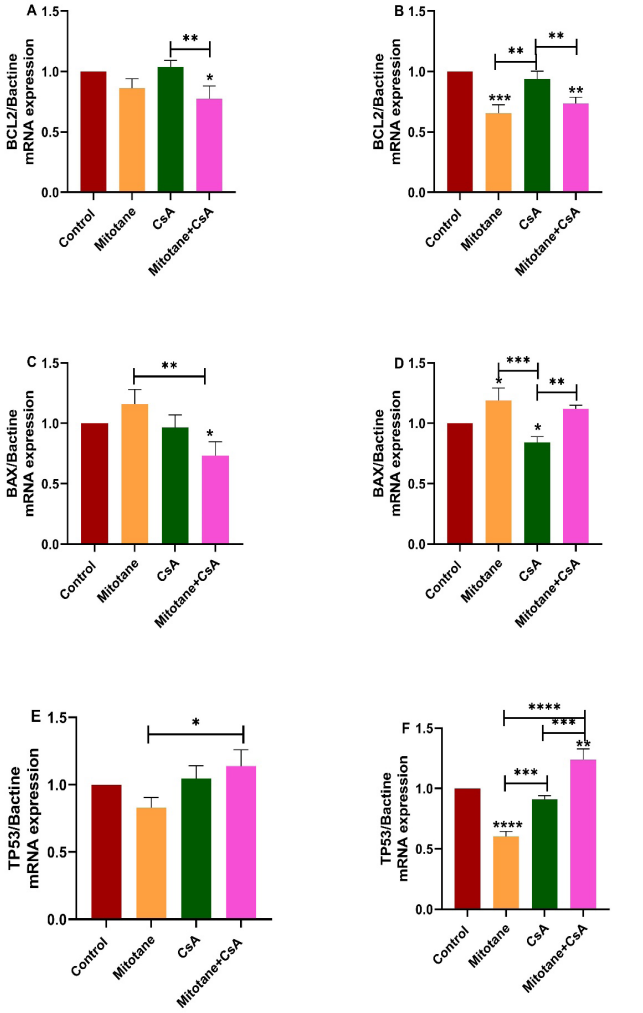

Figure 4. Fold change graph of apoptosis genes BCL2, BAX, and TP53. A) Expression levels of apoptosis gene BCL2 for 24 hours. B) for 72 hours. C) Expression levels of apoptosis gene BAX for 24 hours. D) for 72 hours and E) Expression levels of apoptosis gene TP53 for 24 hours F) 72 hours. Expression levels of apoptosis gene BCL2, BAX and TP53 were determined according to time in H295 cells treated with Mitotane, CsA, and CsA+Mitotane complex. ***p<0.001, **p<0.01 and *p<0.05

CsA and Mitotane+CsA Complex Arrested H295 Cells in G0/G1, S, and G2/M Phases

Cell cycle analysis showed that after 24 hours, 52.95% of mitotane-treated cells, 49.2% of CsA-treated cells, and 52.05% of mitotane+CsA-treated cells were arrested in the G0/G1 phase, compared to 38.35% in the control group (Figure 5A). After 72 hours, the arrest rates were 58.2%, 49.1%, and 58.05% respectively. In the S phase, 31.7% of mitotane-treated cells, 34.65% of CsA-treated cells, and 30.35% of mitotane+CsA-treated cells were arrested after 24 hours, compared to 8.85% in the control group (Figure 5B). After 72 hours, the arrest rates were 31.85%, 38.05%, and 31.75% respectively (Figure 5C).

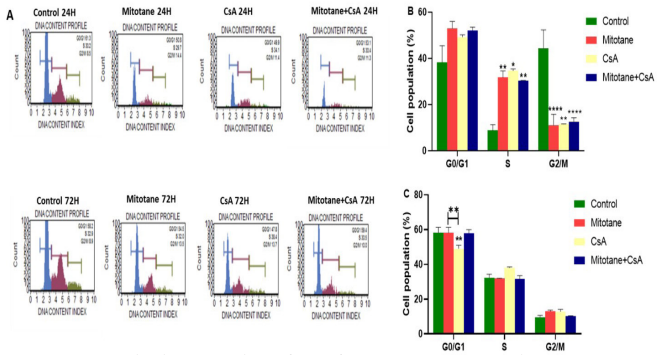

Figure 5. Graph showing the effect of mitotane, CsA, and mitotane+CsA complex on the cell cycle of H295 cells. A) DNA content profile of H295 cells after chemicals are treated. B) Graph according to cell cycle phases of H295 cells for 24 hours and C) for 72 hours

CsA and Mitotane+CsA Complex Inhibited Migration of H295 Cells

Wound healing assays showed that there was no significant difference in cell migration between CsA-treated cells and the control group after 24 and 72 hours. Similarly, the mitotane+CsA complex did not significantly affect cell migration compared to the control group (Figure 6).

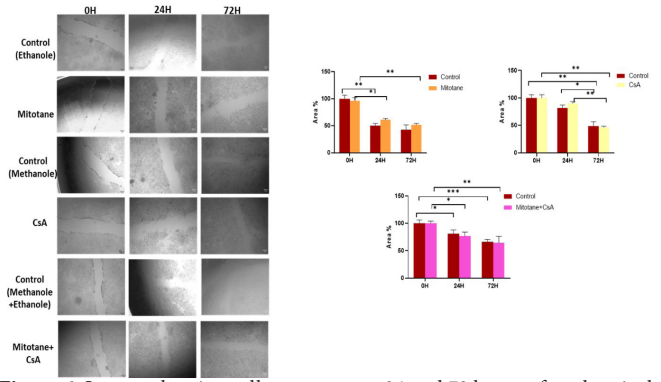

Figure 6. Images showing cell movement at 24 and 72 hours after chemicals were treated to H295 cells and graphs of gap area (%) according to time. Mitotane, CsA, and Mitotane+CsA complex were treated 24 hours after the cells were seeded and the gap between the cells was imaged after 0, 24, and 72 hours. Then, this gap area was measured with the ImageJ software. Graphs were drawn according to time. ***p<0.001, **p<0.01, *p<0.05

DISCUSSION

This study aimed to enhance the effect of mitotane on ACC by targeting the intracellular calcium signaling pathway, specifically focusing on the role of CaN and its inhibition by CsA. As an immunosuppressive drug, CsA inhibits CaN, a crucial enzyme in the intracellular Ca^{2+} signaling pathway known to influence tumorigenic potential. In our study, it was shown that CaN protein was expressed in ACC cells. Activation of CaN is known to increase tumorigenic potential Specifically, CsA decreased CaN protein levels by approximately 60% at both 24 and 72 hours post-treatment in H295 cells. This effect contrasts with our previous findings in breast cancer cells, where CaN suppression by CsA occurred only after 72 hours.[16] The immunosuppressive properties of CsA, alongside its ability to inhibit CaN, suggest potential dual benefits in treating ACC, a cancer characterized by excess glucocorticoid production, which can impair immunotherapy efficacy.[17] It was noted in a study that adrenocortical steroid production is under the control of Ca^{2+} signaling.[18] Molecularly, aldosterone-producing adenomas are characterized by somatic mutations in several genes involved in the regulation of intracellular calcium concentration.[19]

An important finding of this study is the impact of mitotane on CaN in ACC cells. While the direct relationship between mitotane and CaN is not well-established, mitotane likely influences CaN activity indirectly through its effects on cortisol levels and steroid hormone signaling.[20] Mitotane's known disruption of mitochondrial-associated membranes, which play crucial roles in apoptosis, calcium homeostasis, and steroid synthesis, supports this hypothesis.[21]

It surprised that after 24 hours of application of mitotane+CsA complex to H295 cells, CaN protein was expressed 1.61-fold compared to control, and after 72 hours, this complex suppressed CaN protein by approximately 50%. To understand the reason for this, the effects of CsA and mitotane+CsA complex on the viability of H295 cells were investigated. The effect of CsA on the viability of H295 cells was shown to vary depending on dose and time. CsA reduces the viability of H295 cells at high doses. It was found in a previous study that CsA reduced the viability of breast cancer cells.[16]

The mechanism by which CsA inhibits CaN involves binding within the cell, preventing activation by the Ca^{2+}/CaM complex.[22] Unlike other cytotoxic immunosuppressants, CsA selectively inhibits the proliferative activation of immune effector cells without killing them, interfering with IL-2 synthesis necessary for T lymphocyte activation and differentiation.[14] In this study, while H295 cells treated with CsA were in a high rate of early and late apoptosis at the end of 24 hours, it was observed that the cells largely went into necrosis after 72 hours. Some studies have reported that patients undergoing organ transplantation have a high risk of cancer because they use immunosuppressive drugs such as CsA.[23] But in other studies, 0.1 to 10 µM CsA applied to pituitary cells in-vitro decreased the viability of the cells. CsA dose-dependently induced apoptosis of GH3 pituitary cells.[24] CsA treatment to A549 lung cancer cells decreased the viability of these cells and increased the percentage of apoptosis depending on the dose. CsA is thought to affect cell apoptosis via caspase 9 and 3.[25] It has also been reported that CsA reduces viability in U937 cells.[26]

Mitotane+CsA complex decreased cell viability in ACC cells. At the same time, this complex increased the apoptosis rate of the cells. When we look at the effects on apoptosis genes, the antiapoptotic gene BCL-2 level did not change in H295 cells treated with CsA. CsA did not affect the pro-apoptotic BAX gene, either. However, in a study with pituitary cells, CsA increased BCL-2, BAX, and p53 protein levels in GH3 cells.[24] In our study, CsA did not change the TP53 gene expression in H295 ACC cells. As it is known, although TP53 mutation plays an important role in the pathogenesis of ACC,[17] this gene is not used as a marker because it shows variability in most ACC patients. The reason for this is that while the TP53 mutation is seen in pediatric patients, it has been reported that this mutation decreases with age.[27] Mitotane inhibited the TP53 gene in H295 cells. Mitotane inhibited the BCL-2 gene in H295 cells and increased the level of the BAX gene. Changes in these genes are as expected, as mitotane exerts

its cellular effect on mitochondria.[8] Mitotane+CsA complex showed different results on genes depending on time. There is another confusing pathway in the effect of CsA and mitotane cells on the apoptosis pathway. When CsA and mitotane inhibited the CaN protein, they also affected the CaN-induced apoptosis pathway. CaN is also known to interact with several receptors and ion channels and help regulate their activity.[9] In our previous studies, it was reported that CaN was indirectly associated with the plasma membrane calcium pump (PMCA) in breast cancer cells.[16] There is no known direct interaction between CsA and mitotane. Although these drugs have different mechanisms of action, they can both have significant side effects and interact with other drugs. For example, CsA can interact with drugs metabolized by the cytochrome P450 enzyme system and affect the levels of these drugs in the body. Similarly, mitotane can cause significant hormonal disorders, including adrenal insufficiency and hypothyroidism.

Roy et al.[26] reported that lower doses of CsA (10μM) resulted in the arrest of the cell population in G0/G1 and a decrease in cells in S and G2/M in their study with U937 cells. Our study showed no significant difference in the rates of cell arrested in the G0/G1, S, and G2/M phases after CsA treatment compared to the control. When evaluated according to time, the arrest rates of cells in the S and G2/M phases decreased a little more. When the mitotane+CsA complex was treated to H295 cells, most of the cells were arrested in the G0/G1 phase compared to the cell groups that were treated with only mitotane and CsA. There was no significant difference in the effect of CsA on the migration of H295 cells compared to the control. Compared to the effect of mitotane on cell motility, CsA slows down the migration of cells. The mitotane+CsA complex did not significantly change the migration of cells compared to the control group.

The effect of CsA varies depending on the dose. If high-dose CsA inhibits the activation of T cells, evidence indicates that low-dose CsA can induce autoimmunity and immune hyperactivity as well as proinflammatory cytokines.[15] It can be thought that low-dose CsA stimulates the immune response in specific conditions.[14] In mouse models, low-dose CsA has been shown to induce proinflammatory cytokines, such as IL-12, IFN-γ, and TNF-α.[28] A phase I/II study of 44 patients with advanced non-small cell lung carcinoma (NSCLC) compared low-dose CsA (1-2 mg/kg per day) with high-dose CsA (3-6 mg/kg). Low-dose CsA is thought to be therapeutic in cancer.[14]

CONCLUSION

This study demonstrates that CaN plays a significant role in the intracellular Ca2+ signaling pathway in ACC cells and that its inhibition by CsA can enhance the cytotoxic effects of mitotane. These findings suggest that targeting CaN may provide a promising therapeutic strategy to improve ACC treatment outcomes.

ETHICAL DECLARATIONS

Ethics Committee Approval

Since the study was performed cell line, ethics committee approval is not need.

Informed Consent

Since the study was performed cell line, informed consent is not need.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

REFERENCES

1. Else T, Kim AC, Sabolch A, et al. Adrenocortical carcinoma. *Endocr Rev.* 2014;35(2):282-326. doi:10.1210/er.2013-1029
2. Creemers SG, van Koetsveld PM, de Herder WW, et al. MDR1 inhibition increases sensitivity to doxorubicin and etoposide in adrenocortical cancer. *Endocr Relat Cancer.* 2019. doi:10.1530/ERC-18-0500
3. Karwacka I, Obołończyk Ł, Kaniuka-Jakubowska S, Sworczak K. The role of immunotherapy in the treatment of adrenocortical carcinoma. biomedicines. 2021;9(2):1-12. doi:10.3390/BIOMEDICINES9020098
4. Fassnacht M, Dekkers OM, Else T, et al. European Society of Endocrinology Clinical Practice Guidelines on the management of adrenocortical carcinoma in adults, in collaboration with the European Network for the Study of Adrenal Tumors. *Eur J Endocrinol.* 2018;179(4):G1-G46. doi:10.1530/EJE-18-0608
5. Creemers SG, Hofland LJ, Korpershoek E, et al. Future directions in the diagnosis and medical treatment of adrenocortical carcinoma. *Endocr Relat Cancer.* 2016;23(1):R43-R69. doi:10.1530/ERC-15-0452
6. Kerkhofs TMA, Ettaieb MHT, Hermsen IGC, Haak HR. Developing treatment for adrenocortical carcinoma. *Endocr Relat Cancer.* 2015;22(6):R325-R338. doi:10.1530/ERC-15-0318
7. Poli G, Guasti D, Rapizzi E, et al. Morphofunctional effects of mitotane on mitochondria in human adrenocortical cancer cells. *Endocr Relat Cancer.* 2013;20(4):537-550. doi:10.1530/ERC-13-0150
8. Corso CR, Acco A, Bach C, Bonatto SJR, de Figueiredo BC, de Souza LM. Pharmacological profile and effects of mitotane in adrenocortical carcinoma. *Br J Clin Pharmacol.* 2021;87(7):2698-2710. doi:10.1111/BCP.14721
9. Creamer TP. Calcineurin. *Cell Commun Signal.* 2020;28;18(1):137. doi: 10.1186/s12964-020-00636-4
10. Berber M, Leng S, Beuschlein F, Breault DT, Loffing J, Ribas DP. Calcineurin-NFATc4 pathway is activated upon K+-stimulation of adrenal aldosterone production. *J Endocr Soc.* 2021;5(Suppl 1):A805. doi:10.1210/JENDSO/BVAB048.1638
11. Chen L, Song M, Yao C. Calcineurin in development and disease. *Genes Dis.* 2022;9(4):915-927. doi:10.1016/j.gendis.2021.03.002

12. Tedesco D, Haragsim L. Cyclosporine: a review. *J Transplant*. 2012;2012:1-7. doi:10.1155/2012/230386

13. Adithan A, John Peter JS, Hossain MA, et al. Biological effects of cyclosporin A on CD3-CD161+ and CD3+CD161+ lymphocytes. *Mol Cell Biochem*. 2019;458(1-2):159-169. doi: 10.1007/s11010-019-03539-2

14. Flores C, Fouquet G, Moura IC, Maciel TT, Hermine O. Lessons to learn from low-dose cyclosporin-A: a new approach for unexpected clinical applications. *Front Immunol*. 2019;10:588. doi:10.3389/FIMMU.2019.00588

15. Devaux CA, Melenotte C, Piercecchi-Marti M-D, Delteil C, Raoult D. Cyclosporin A: a repurposable drug in the treatment of COVID-19? *Front Med*. 2021;8:663708. doi: 10.3389/fmed.2021.663708

16. Caner A, Onal MG, Bitgen N, Sezer G. The effect of cyclosporine A on proteins controlling intracellular calcium concentration in breast cancer cells. *J Membr Biol*. 2021:1-7. doi:10.1007/S00232-021-00201-1

17. Cosentini D, Grisanti S, Volta AD, et al. Immunotherapy failure in adrenocortical cancer: where next? *Endocr Connect*. 2018;7(12):E5. doi:10.1530/EC-18-0398

18. Spät A, Hunyady L, Szanda G. Signaling interactions in the adrenal cortex. *Front Endocrinol (Lausanne)*. 2016;7:17. doi:10.3389/fendo.2016.00017

19. Xing Y, Lerario AM, Rainey W, Hammer GD. Development of adrenal cortex zonation. *Endocrinol Metab Clin North Am*. 2015;44(2):243-274. doi:10.1016/j.ecl.2015.02.001

20. Hescot S, Amazit L, Lhomme M, et al. Identifying mitotane-induced mitochondria-associated membranes dysfunctions: metabolomic and lipidomic approaches. *Oncotarget*. 2017;8(66):109924-109940. doi:10.18632/ONCOTARGET.18968

21. Boulate G, Amazit L, Naman A, et al. Potentiation of mitotane action by rosuvastatin: New insights for adrenocortical carcinoma management. *Int J Oncol*. 2019;54(6):2149-2156. doi:10.3892/IJO.2019.4770/HTML

22. Matsuda S, Koyasu S. Mechanisms of action of cyclosporine. *Immunopharmacology*. 2000;47(2-3):119-125. doi:10.1016/S0162-3109(00)00192-2

23. Vial T, Descotes J. Immunosuppressive drugs and cancer. *Toxicology*. 2003;185(3):229-240.

24. Kim HS, Choi S Il, Jeung EB, Yoo YM. Cyclosporine A induces apoptotic and autophagic cell death in rat pituitary GH3 cells. *PLoS One*. 2014;9(10):108981. doi:10.1371/JOURNAL.PONE.0108981

25. Sato M, Tsujino I, Fukunaga M, et al. Cyclosporine A induces apoptosis of human lung adenocarcinoma cells via caspase-dependent pathway. *Anticancer Res*. 2011;31(6):2129-2134.

26. Roy MK, Takenaka M, Kobori M, Nakahara K, Isobe S, Tsushida T. Apoptosis, necrosis and cell proliferation-inhibition by cyclosporine A in U937 cells (a human monocytic cell line). *Pharmacol Res*. 2006;53(3):293-302. doi:10.1016/J.PHRS.2005.12.007

27. Pereira SS, Monteiro MP, Antonini SR, Pignatelli D. Apoptosis regulation in adrenocortical carcinoma. *Endocr Connect*. 2019;8(5):R91-R104. doi:10.1530/ec-19-0114

28. López-Flores R, Bojalil R, Benítez JC, et al. Consecutive low doses of cyclosporine A induce pro-inflammatory cytokines and accelerate allograft skin rejection. *Molecules*. 2011;16(5):3969-3984. doi:10.3390/molecules16053969